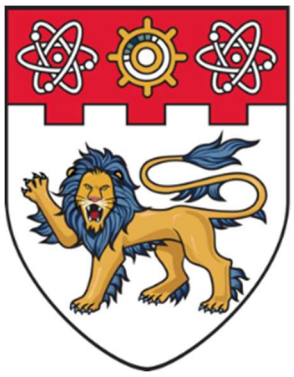

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

DISCOVERY AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI

MARTIN MUTHEE GAKUUBI

SCHOOL OF BIOLOGICAL SCIENCES

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Discovery and characterization of bioactive compounds from endophytic fungi

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A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2023

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

4 Jan 2023

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Martin Muthee Gakuubi

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4 Jan 2023

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Date

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Prof. Zhao-Xun Liang

4 Jan 2023

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Date

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Dr. Siew Bee Ng

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals and 2 papers accepted at conferences in which I am listed as an author.

Chapter 1 is published as:
Gakuubi M.M., Munusamy, M., Liang Z-X. and Ng S.B. Fungal endophytes: a promising frontier for discovery of novel bioactive compounds. *Journal of Fungi* **7**, 786 (2021). DOI: 10.3390/jof7100786

The contributions of the co-authors are as follows:

- Dr. Siew Bee Ng and I conceptualized the study. I corrected the primary data and prepared the initial draft manuscript.
- Prof. Zhao-Xun Liang and Dr. Madhaiyan Munusamy suggested the materials area and contributed to the editing of the draft manuscript.

Chapter 2, 3 and 4 are published as:

Gakuubi M.M., Ching K.C., Munusamy M., Wibowo M., Liang Z-X., Kanagasundaram Y. and Ng S.B. Enhancing the discovery of bioactive secondary metabolites from fungal endophytes using chemical elicitation and variation of fermentation media. *Frontier in Microbiology* **13**, 898976 (2022). DOI: 10.3389/fmicb.2022.898976.

The contributions of the co-authors are as follows:

- Dr. Siew Bee Ng and I conceptualized and planned the study with input from Dr. Madhaiyan Munusamy.
- I performed the biological studies, data analysis and wrote the draft manuscripts.
- Dr. Kuan Chieh Ching, Dr. Mario Wibowo and Dr. Yoganathan Kanagasundaram performed the chemical analysis of bioactive extracts, compound isolation, and structure elucidation and contributed to the editing of the draft manuscripts.
- Dr. Siew Bee Ng and Prof. Zhao-Xun Liang provided research direction, supervision and contributed to the editing of the draft manuscripts.

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- I performed the knockout experiments and other biological studies, data analysis and wrote the draft manuscript.
- Dr. Madhaiyan Munusamy performed phylogenetic studies and analysis.
- Dr. Guang-Lei Ma and Mr. Lim Chun Teck performed bioinformatics analyses.
- Dr. Kuan Chieh Ching, Dr. Mario Wibowo and Dr. Yoganathan Kanagasundaram performed the chemical analysis of bioactive extracts, compound isolation, and structure elucidation contributed to the editing of the draft manuscript.
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Table of Content

Abstract

Fungal endophytes represent a huge resource for the discovery of molecules with greater chemical novelty and interesting bioactivities. Compounds of great therapeutic value such as antibiotics, anticancer, antiviral, antidiabetic and immunosuppressive compounds and those with other industrial and biotechnological applications have been isolated from these largely untapped microbes. However, several factors limit the exploitation of the full biosynthetic potential of these microbial factories. These include, low productivity and titers from native producers, technical barriers associated with endophytic fungi isolation, growth and secondary metabolites (SMs) extraction and the current analytical technology detection threshold. An even greater limitation is the fact that many of the fungal SMs biosynthetic gene clusters (BGCs) are never expressed under conventional laboratory growth conditions. Furthermore, there is also a lack of a versatile genetic engineering system that can be employed across the vast majority of non-model filamentous fungi, making it challenging to fully unravel the biosynthetic capabilities of such fungal strains. The main objective of this study was to evaluate the diversity and bioactive potential of fungal endophytes from A*STAR's Natural Product Library (NPL) and previously isolated from different habitats of Singapore. We employed bioassay-guided approach to uncover the secondary metabolite synthesizing potential of the study fungal isolates. Furthermore, we assessed the influence of chemical elicitation using two compounds; 5-azacytidine and suberoylanilide hydroxamic acid (SAHA) and variation of fermentation media on the biosynthesis of bioactive fungal SMs. A total of 222 fungal strains were identified on the basis of sequence analysis of ITS2 region of the rDNA gene, with majority (99%; 219 strains) belonging to the phylum *Ascomycota* while two strains belong to the phylum *Basidiomycota*, and only one strain was from *Mucoromycota* phylum. A total of 146 fungal isolates were selected for metabolites extraction following phylogenetic analysis. Chemical elicitation and variation of fermentation media resulted in the discovery of

more strains exhibiting inhibitory activities against a panel of microbial pathogens and cancer cell lines. Bioassay-guided isolation and structure elucidation of active constituents from three prioritized fungal strains led to the isolation of palmarumycin C_8 and a new analogue palmarumycin CP_{30} from *Lophiotrema* sp. F6932. Three new polyketides designated as muyocopronol A - C were isolated from *Muyocopron laterale* F5912 while a novel 26-membered macrolide designated as tropicicolide was isolated from *Colletotrichum tropicicola* F10154. Tropicicolide displayed the strongest antifungal activity against *Aspergillus fumigatus* with an IC_{50} value of 1.8 µg/mL but with a weaker activity against cthe *Candida albicans* presenting an IC_{50} of 7.1 µg/mL. Palmarumycin C_8 revealed the best antiproliferative activity with IC_{50} values of 1.1, 2.1 and 10.7 µg/mL against MIA PaCa-2, PANC-1 and A549 cells, respectively. Biosynthesis of palmarumycin compounds has previously been proposed to involve phenolic oxidative dimerization of 1,8-dihydroxynaphthalene (DHN), the same precursor that is involved in melanin biosynthesis in many filamentous fungi. Thus, we sought to identify and validate the BGC involved in biosynthesis of this group of compounds from *Lophiotrema* sp. F6932. Whole-genome sequencing analysis resulted in the identification of a putative type 1 iterative PKS (iPKS) predicated to be involved in the biosynthesis of palmarumycins. To verify the involvement of palmarumycin (PAL) gene cluster in the biosynthesis of palmarumycins, we employed ribonucleoprotein (RNP)-mediated CRISPR-Cas9 to induce targeted deletion of the ketosynthase (KS) domain of PAL. Double-strand breaks (DSBs) upstream and downstream of the KS domain were followed by homology-directed repair (HDR) with a hygromycin resistance cassette flanked by a 50 bp of homology on both sides of the DSBs. The resultant deletion mutants displayed completely different phenotypes compared to the wild-type strain; they had different colony morphology and were no longer able to produce palmarumycins or melanin, thus confirming the involvement of iPKS in the biosynthesis of palmarumycins and melanin in *Lophiotrema* sp. F6932.

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List of Abbreviations

BGC	Biosynthetic gene clusters
BLAST	Basic local alignment search tool
CFU	Colony forming unit
CRISPR	Clustered regularly interspaced short palindromic repeats
DHN	1,8-dihydroxynaphthalene
DIN	DNA integrity number
DNMT	DNA methyltransferases
DOPA	3,4-dihydroxyphenylalanine
EIC	Extracted ion chromatogram
FAC	Final assay concentration
HDCA	Histone deacetylase
HPLC	High-performance liquid chromatography
HRESIMS	High resolution electrospray ionization mass spectrometry
KS	Ketosynthase
LC-MS	Liquid chromatography - mass spectrometry
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MRSA	Methicillin-resistant *Staphylococcus aureus*
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetases
OSMAC	One strain-many compound

OTU	Operational taxonomic units
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PKS	Polyketide synthases
RNP	Ribonucleoprotein
SAHA	Suberoylanilide hydroxamic acid
SBHA	Suberoyl bishydroxamic acid
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
WT	Wild-type

Chapter 1: Background

1.1: Introduction

Fungi, bacteria, and plants produce an array of specialized low-molecular weight and structurally diverse chemical entities known as secondary metabolites (SMs) also referred to as natural products (Baral et al., 2018). These molecules are not essential for the normal growth and development of the producing organism, and their production involves polymerization of primary metabolites (Keller, 2019). Though not necessary for the normal growth and development, secondary metabolites are thought to have a multitude of ecological functions such acting as signal molecules, virulence factors, and for protection against abiotic stress and defense against predators and competitors (Bills and Gloer, 2016). Human exploitation of natural products as a source of medicine date back several millennia, with the earliest recorded used of natural products in human health care in ancient Mesopotamia dating back from 2900 to 2600 BCE (Borchardt 2002). While both plants and microbes have a long history as sources of beneficial natural products, microorganisms have played a significant role in the evolution of modern drug discovery thanks to their remarkable diversity, ease of growth and amenability to genetic manipulation.

During the early days of microbial-based drug discovery, soil microbiota especially actinomycetes was the principal source of bioactive compounds (Von Bubnoff et al., 2016). However, the increase in the rate of rediscovery of known compounds from soil-based microorganisms necessitated a focus on other groups of microorganisms as sources of novel bioactive compounds. Among the most promising groups of microbes are those from the less studies habitat and those from specialized ecological niches such as marine microorganisms, microbes from extreme environments and plant-associated microbes represented mainly by endophytic, mycorrhizal and

rhizosphere fungi and bacteria (Gunatilaka, 2006; Sayed et al., 2020). Interesting molecules with great therapeutic value such as anticancer agents, antibiotics, antiviral, antidiabetic and immunosuppressive compounds have already been discovered from microbes sourced from these largely untapped habitats (Gakuubi et al., 2021; Gouda et al., 2016)

1.2: Endophytic fungi

Endophytic fungi refers to a heterogeneous group of fungi that are found in association with plants. The term endophytes (*endo* = inside; *phytón* = plant) was coined by Anton de Bary in 1866 to denote fungi that live inside their host plant tissues as opposed to 'epiphytes' a term assigned to fungi found on the surface of plants (Dutta et al., 2014). Fungal endophytes were originally defined as fungal strains that colonize internal plant tissues without causing apparent disease to their host. Over the years, the term endophyte has undergone redefinitions as more data continue to be generated from research in endophytic fungal biology. Petrini (1991) defined endophytes as "all organisms inhabiting plant organs that at some time in their life cycle can colonize internal plant tissues without causing apparent harm to their host". The above definition however, has numerous limitations. First, it can be quite difficult in certain instances to make a clear distinction between endophytes, mycorrhizae and even phytopathogenic fungi (Hardoim et al., 2015). For example, some fungal strains are pathogenic to certain plants while having neural or even beneficial qualities towards other host plants. Certain strains of fungi have also been known to exist as latent pathogens within their host plant tissues only for their pathogenicity to be activated once certain environmental conditions become favourable (Kloepper et al., 2013), or can live as conditional pathogens, only causes diseases to the host plant as it ages or when under stress (Kusari et al., 2014). Besides, there is a grey area regarding plant pathogenic fungi that have lost their virulence.

Should they be considered as endophytes or pathogens? Moreover, definition of fungal endophytes on the basis of pathogenicity rules out unculturable fungi whose phytopathogenicity can be difficult to ascertain (Hardoim et al., 2015). More recently, Hardoim et al. (2015) defined endophytes as microbes that colonize plants internal tissues notwithstanding the outcome of the association. The lack of a precise definition of fungal endophyte points at the heterogeneity and complex life histories of many members of endophytic fungi community (Rodriguez et al., 2009). In the context of the current study therefore, fungal endophytes refer to fungal stains that are generally isolated from plants.

It is estimated that there are more than 1 million species of endophytic fungi associated with plants growing in essentially all climatic and ecological zones (Strobel and Daisy, 2003; Chowdhary et al., 2012). Fungal endophytes have been isolated from virtually all of the plant types and plant tissues, and a single plant tissues such as a leaf, root or stem can harbor a great diversity of endophytic fungal strains (Abhijeet Singh, 2014). While the diversity of fungal endophytes varies greatly from place to place, majority of the fungal endophytes belongs to the phylum Ascomycota with occasional isolation of members of Basidiomycetes and Zygomycetes (Sun & Guo, 2012). Fungal endophytes are thought to play fundamental role in host plants' ecology and physiology such as acting as growth regulators, suppressing pathogens, increasing host plants' tolerance to various biotic and abiotic stresses and acting as mediators of plant-insect and plant soil interactions (Rim et al., 2021; Chen et al., 2020).

Because fungal endophytes represent a polyphyletic group whose members are taxonomically and morphologically diverse, their classification can be quite challenging. Endophytic fungi have been

primarily divided into two groups namely; the clavicipitaceous (C-endophytes) and non-clavicipitaceous endophytes (NC-endophytes) (Bamisile et al., 2018; Rodriguez et al., 2009; Uzma et al., 2018). The C-endophytes are mostly associated with grasses and belong mainly to the family Clavicipitaceae while the NC-endophytes are not found in grasses but in association with vascular and non-vascular plants with majority of the members occurring in the phyla Ascomycota and Basidiomycota. Other schemes of endophytic fungi classification based mostly on fungi ecology, diversity and functions has recently emerged. These include classification of fungal endophytes on the basis of host range, mode of transmission and infection, reproduction strategies, host's sites of colonization, and the position of the fungi within the continuum of interactions with their hosts (Bamisile et al., 2018; Rodriguez et al., 2009). On the basis of host plants, fungal endophytes are classified on the basis of specific categories of plants. For example, medicinal plants fungal endophytes (Kaul et al., 2012), mangroves fungal endophytes (Demers et al., 2018), coniferous trees fungal endophytes (Kim et al., 2013) and fungal endophytes of grasses (Tanaka et al., 2012). Regarding the modes of transmission, two main categories of fungal endophytes are recognized, namely the vertically and horizontally transmitted fungal endophytes. The former consist of fungal strains that are transmitted between host generations mostly through seeds, while the latter consist of fungi that are transmitted between different individuals of a population through spores or other vegetative propagules (Daley et al., 2017). Fungal endophytes have similarly been classified on the basis of their preferential colonization of specific host plant tissues. This is because studies have shown that most fungal endophytes rather than colonizing their host plant systemically have preferential tissues colonization with. Thus there are root endophytic fungi which are mostly

isolated from host plants' roots (Chadha et al., 2014) and foliar fungal endophytes which are found in hosts' stems and leaves (Jia et al., 2020).

1.3: Host plant selection and prioritization

Almost all of the higher plant species in natural ecosystems harbours one to several hundred species of endophytic fungi (Meshram & Gupta, 2019). Such a high number of possible sources of fungal endophytes present a challenge in selecting which plant hosts one has to priorities in endophytic fungal isolation. The selection of the plant sources for the isolation of fungal endophytes will largely depend on the nature and objective of the study among other factors. In the case of a study whose primary focus is on the discovery of new fungal natural products, the process of identifying the host plants for fungal isolation is very crucial because if well carried out, it can increase the likelihood of selecting host plants that might harbour uncharacterized fungi thus enhancing the prospect for the discovery of novel molecules. Though no formalized guidelines exist on the selection of plant sources for the isolation of fungal endophytes, a four-point criteria proposed as a rationale for selection of promising plants for the isolation of fungal endophytes is a good starting point (Strobel and Daisy, 2003). This approach proposes the selection of (i) plant from distinctive ecological niche, unique biology and survival strategies, (ii) plants from biodiversity hotspots, (iii) plants that are endemic to specific regions and, (iv) medicinal plants and plants of ethnobotanical importance, (iv) (Strobel and Daisy, 2003). The first two points focusses of plant that have unique ecological features, inimitable biology and survival strategies and those from the global biodiversity hotspots such as tropical forests. Such characteristics may have allowed these plants to attracted a unique assortment of fungal endophytes with capacity for production of compounds with interesting chemistry and biological activities (Demers et al., 2018;

Shearer et al., 2007). Targeting plants from endemic plants is another promising avenue for the discovery of novel fungal SMs. Having occupied a distinctive geographical area for a long time and being subjected to unique biotic and abiotic stresses, endemic plants may be host to a unique assortment of fungal endophytes with capacity for biosynthesis of molecules with fascinating bioactivities (Tan et al., 2018). The last point focuses on medicinal plants and is based on a hypothesis that the medicinal activity attributed to such plants may have something to do with their associated fungal endophytes. This viewpoint has gained traction in recent years following the discovery of fungal strains that can produce similar molecules as their host plants (Venieraki et al., 2017). A well-known case is that of anticancer agent paclitaxel which was originally discovered from the barks of *Taxus brevifolia* albeit at lower concentrations. This was followed by the discovery of paclitaxel-producing endophytic fungi *Taxomyces andreanae* from *T. brevifolia,* and more paclitaxel-producing endophytic fungi from other *Taxus* and non-*Taxus* plant species over the years (Roopa et al., 2015; Xiong et al., 2013).

1.4: Isolation of fungal endophytes

The next step after the identification of plant sources for fungal isolation and collection and sampling of plant materials is the isolation of fungal endophytes from the sampled plant materials. An important procedure during this step is the complete removal of surface or epiphytic microbiota. The standard protocol for surface sterilization involves washing of the plant materials thoroughly under running water followed by sterilization using chemical sterilizers such as ethanol, sodium hypochlorite or hydrogen peroxide. This is then followed by rinsing with sterile water before inoculation of the explants on suitable growth media (Sahu et al., 2022). A number of studies have shown that the procedures of surface sterilization of plant tissues have an influence

on the rate of recovery of culturable endophytic fungi (Verma et al., 2011). Indeed, the success of surface sterilization is influenced by numerous factors such as the type of chemical sterilants used (Masumoto and Degawa, 2019) and their concentration (Yu et al., 2022).

The next important consideration in the process of endophytic fungi isolation is the selection of culture media for fungal isolation. Numerous culture media protocols exist for endophytic fungal isolation with the most commonly used media being potato dextrose agar (PDA) (Dos Reis et al., 2022). Other growth media that have been used in the isolation of fungal endophytes include Czapek medium (Chand et al. 2020), Malt Extract Agar (Khalil et al., 2021), Hagem Minimal Medium (Khan et al. 2016), Tryptone Bovine Extract Agar, Tryptone Soybean Agar, and Luria–Bertani medium (Gong et al. 2019). For simple studies focusing on the diversity of commonly cultivatable fungal endophytes, the use of a single medium could suffice (Ibrahim et al. 2021). However, to enhance the rate of recovery of fungal endophytes, use of two or more growth media is often required. Moreover, supplementing the growth media with plant materials from host plants has also been shown to improve the rate of recovery of fungi (Mourad et al., 2018). It is also recommended to use one or a combination of broad-spectrum antibiotics such as chloramphenicol, streptomycin or tetracycline to prevent the growth of endophytic bacteria and other contaminants (Dos Reis et al., 2022).

1.5: Identification of endophytic fungi

Being a polyphyletic group consisting of morphologically, ecologically and phylogenetically diverse strains, a crucial step in the study of fungal endophytes is the correct identification of individual isolates. The classical procedures for fungal identification relies on the use of

morphological and cultural characteristics of fungi. This involves an assessment of the various phenotypic characters such as such as spore-producing bodies and colony morphologies (Raja et al., 2017). However, although the uses of morphology in the identification of fungi is still widely used today and has greatly contributed to the understanding of the evolution of different morphological characters in fungi, this approach faces numerous challenges especially in lower taxonomic levels (i.e. species). Morphological identification of fungi can lead to incorrect labelling of isolates due to factors such as convergent evolution, hybridization and cryptic speciation (Raja et al., 2017). Moreover, a good number of fungi including many endophytic fungal strains do not sporulate in culture (Ko Ko et al., 2011). Indeed, it is estimated that up to 50% of fungal endophytes do not sporulate in culture and therefore lacks the phenotypic characters that can be used in their identification (Dos Reis et al., 2022).

Due to the aforementioned shortcomings of morphological identification of fungi, molecular-based methods have emerged as an alternative to morphological characterization of fungi. These approaches typically involve the isolation of genomic DNA followed by PCR amplification of conserved DNA sequences, sequencing of the resultant PCR amplicons and sequence analysis and comparison with appropriate sequences deposited in databases (Thi et al., 2019). The nuclear ribosomal internal transcribed spacer (ITS) is currently accepted as the principal molecular marker for fungal identification due to its ease of amplification and wide coverage within the kingdom (Fernandes et al., 2015; Raja et al., 2017; Schoch et al., 2012). The strengths and shortcoming of using the ITS region in fungal identification have been dealt with in details in section 2.4. In cases where the use of ITS region is insufficient and much better discrimination among taxa is required, the use of secondary barcodes is recommended. Among the secondary barcode regions that have

been used in fungal identification include two other nuclear ribosomal genes, i.e. 18S nuclear small subunit ribosomal DNA (nrSSU) and nuclear large subunit (nrLSU), and protein-coding loci such as the largest (*RPB1*) and second largest (*RPB2*) subunits of RNA polymerase II, beta-tubulin (*tub2/BenA*) and translation elongation factor 1-alpha (*TEF1-α*) (Dos Reis et al., 2022; Gakuubi et al., 2022b).

1.6: Bioactive secondary metabolites from fungal endophytes

Fungi are renowned for their capacity for biosynthesis of a wide range of bioactive secondary metabolites. The discovery penicillin, the first natural antibiotic from *Penicillium notatum* by Alexander Fleming in 1928 heralded a new era in natural products research and positioned fungi as one of the most important source of therapeutic agents. Fungi continue to be a useful source of bioactive molecules with the discovery of novel fungal SMs increasing significantly in the last two decades compared to the rate of discovery in other groups of microbes such as actinomycetes, filamentous bacteria (Bills and Gloer, 2016). Among the different groupings of fungi, endophytic fungi have received much attention recently as potential source of novel bioactive secondary metabolites. Their ability to synthesis a great diversity of bioactive natural products has been attributed to the close biological relationship and interactions that these microbes have established over the years with their host plants (Hashem et al., 2023). Numerous hypotheses have been advanced regarding the close association of microbial endophytes and their host plants and the ability of endophytes to produce bioactive metabolites. One of the widely cited of these hypotheses is that the ability for biosynthesis of SMs in endophyte is tied to their co-evolution with their host plants where endophytes may have integrated some genetic information from the host plants (Gouda et al., 2016). It is postulated that this enabled endophytes to adapt to the host plant

environment in addition to allowing them to offer beneficial services to their host such as protection against pathogens, insects and even herbivore (Strobel, 2003). It has also been suggested that the mutual benefit that exist between microbial endophytes and their host has been established over the years resulting in a 'balanced antagonism' in which the microbes' virulence factors balances with the hosts' defenses. If this balance is offset and the fungal virulence factor are overcome by the host defenses, then endophytes will be destroyed. Conversely, if the endophytes virulent factors overpower the host defense mechanisms, a plant-pathogen relation arises leading to a plant disease and possible death of the host plant (Schulz et al., 1999). Recent studies however have revealed that endophytism is not a mere balance between host defenses and endophytes' virulence factors but rather, consists of a complex and multifaceted interactions that varies widely between different endophyte-host associations and no single mechanism can be used to explain them sufficiently (Khare et al., 2018).

Fungal endophytes therefore represent a vast resource for discovery of natural products with a broad range of applications. Bioactive compounds belonging to a wide range of chemical classes such as alkaloids, phenolic acids, flavonoids, steroids, quinones, terpenoids and saponins and among other have been isolated from these microbial factories (Fadiji & Babalola, 2020; Gouda et al., 2016). Fungal secondary metabolites can be group into numerous categories on the basis of their biosynthetic origin with each group assigned on the basis of the backbone or core enzymes that are involved in their production (Brakhage, 2013; Keller, 2019). Therefore, polyketides are produced from acyl-CoA by the multimodular enzyme complex polyketide synthases (PKSs), non-ribosomal peptides are produced by non-ribosomal peptide synthetases (NRPSs) from amino acids, while terpenes are generated from terpene synthases/ cyclases (TPs/TCs). Furthermore, certain

SMs are hybrids that are produced by two synthases and/or synthetases as the NRPS-PKS hybrids secondary metabolites (Keller, 2019)

The non-pathogenic nature of majority of the fungal endophytes makes most of the SMs derived from them more suited for human usage as most are non-toxic to mammalian cells (Ancheeva et al., 2019). Some of the biologically active compounds that have been isolated from fungal endophytes include antibiotics, anticancer, antifungal, immunosuppressive and antiviral compounds (Figure 1.1). A number of review articles have been published focusing on the major classes of bioactive compounds sourced from fungal endophytes including; antibiotics (Deshmukh et al., 2014; Martinez-Klimova et al., 2017), antifungal compounds (Deshmukh et al., 2018) antimycobacterial compounds (Alvin et al., 2014), anticancer/cytotoxic compounds (Chen et al., 2014; Kharwar et al., 2011; Uzma et al., 2018), antioxidants (Toghueo & Boyom, 2019) and valuable enzymes (Corrêa et al., 2014; Khan et al., 2017).

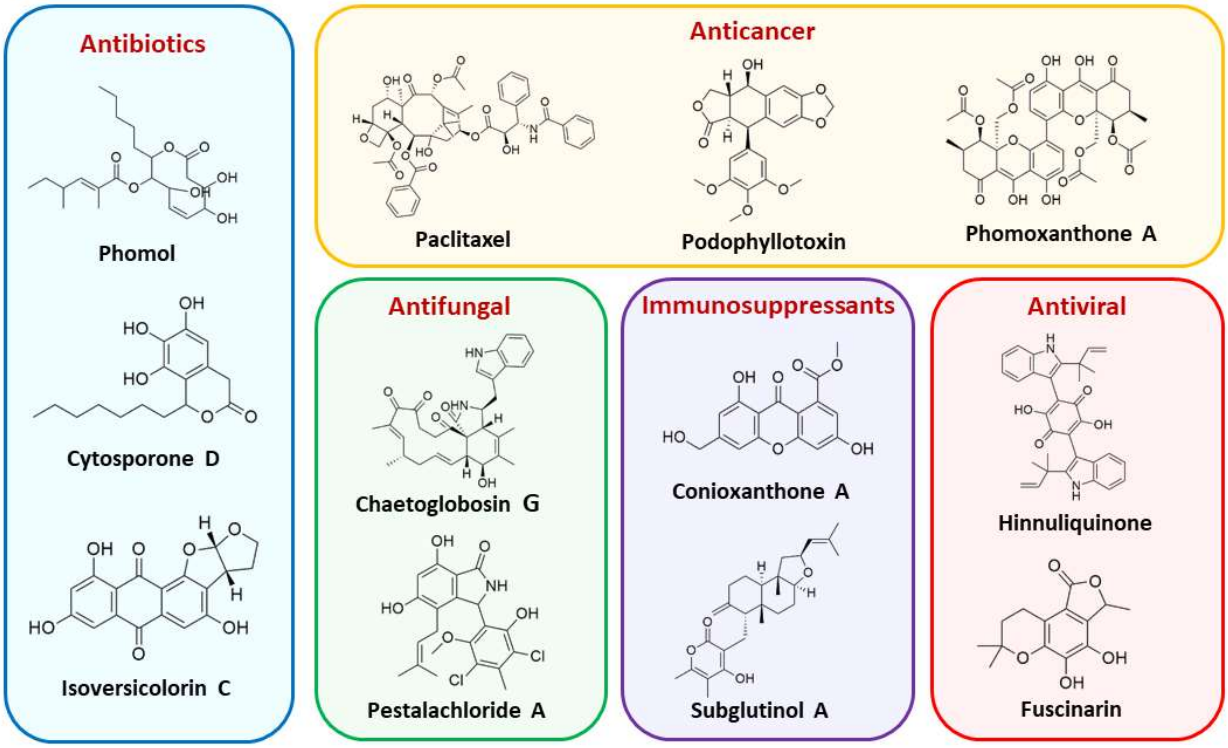


Figure 1:1 Some bioactive compounds that have been isolated from fungal endophytes grouped according to their bioactivities (Gakuubi et al., 2021).

1.7: Enhancement of secondary metabolite biosynthesis in fungal endophytes.

Despite their potential for biosynthesis of an array of bioactive secondary metabolites, only a small fraction of the chemical diversity of fungal endophytes is accessible through the conventional laboratory growth systems. The reason behind this is that majority of the fungal SMs gene clusters in fungi remain silent under standard laboratory growth conditions (Fisch et al., 2009; Schüller et al., 2020). Fungal genes that encode the enzymes needed for the biosynthesis of the core structure of the compound together with the tailoring enzymes that modifies the core structure, and those responsible for the transport of SMs are typically clustered on a single gene locus forming contiguous gene assembles that are referred to as biosynthetic gene clusters (BGCs) (Deepika et

al., 2016; Keller et al., 2005; Khaldi et al., 2010; Kjærbølling et al., 2019). This design of fungal SMs biosynthetic genes allows for the development of bioinformatics algorithms that are capable of predicting the core enzymes involved in the biosynthesis of various classes of secondary metabolites. Moreover, it allows for the prediction of the biosynthetic capacity of individual fungal strains (Keller, 2019).

Due to the fact that the fact that the bulk of biosynthetic potential of majority of fungi cannot be uncovered under standard laboratory growth conditions, there is a need therefore to come up with appropriate techniques that can be used to induce the activation of silent biosynthetic pathways to enhance the discovery of new natural products in fungal endophytes. Figure 1.2 shows some of the approaches that have been employed to induce the activation of silent or weakly expressed gene clusters in fungal endophytes.

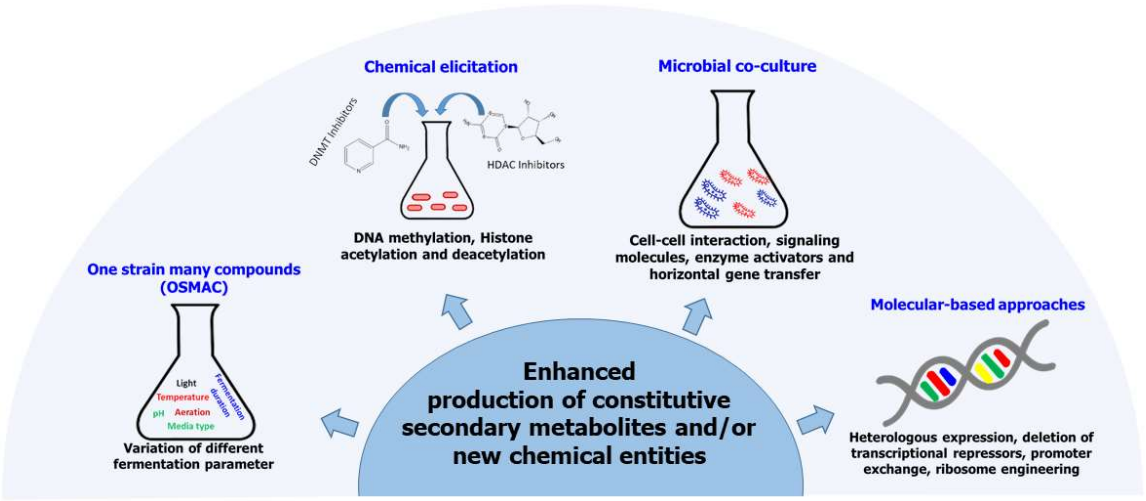


Figure 1:2 Some approaches that have been employed to enhance secondary metabolites biosynthesis in fungal endophytes (Gakuubi et al., 2021).

1.7.1: One strain many compounds approach

The one strain-many compound (OSMAC) approach entails a systematic manipulation of the various growth parameters such as the media type and composition, aeration, culture vessel size and use of selected enzyme inhibitors (Bode et al., 2002). Such changes have been found to induce changes not only in the amount of fungal secondary metabolites produced, but have in some certain cases led to the discovery of new chemical entities altogether (Ahsan et al., 2017; Reen et al., 2015). While manipulation of media composition has traditionally been used as a tool for optimized production of already known metabolites, this technique has recently found widespread use as a mean of awakening silent or poorly expressed BGCs in fungi. On aspect of growth media manipulation involves the supplementing of growth media with various salts. Addition of salts such as $MgSO_4$, $NaNO_3$, $CuSO_4$ and NaCl have been shown to completely alter the biosynthetic profile of fungal endophytes resulting in enhanced biosynthesis of constitutive secondary metabolites and in many cases production of novel secondary metabolites that are not produced in control cultures (Ariantari et al., 2019; Mafezoli et al., 2018; Supratman et al., 2019). For example, addition of sodium chloride (NaCl) salt in the media resulted in enhance production of geodin 1 by a marine-derived fungus *Aspergillus* sp. (Said and Ahmad, 2022).

Even the source of growth media components has been found to have an influence on fungal SMs biosynthetic potential. In one study, growth of endophytic fungus *Dothideomycete* sp. CRI7 in PDB prepared from fresh potato tubers and one prepared from commercial potato powder resulted in considerable changes to the SM profile of the fungus (Hewage et al., 2014). Interestingly, variations the fungus SM biosynthetic profile were observed when the strain was grown in Czapek malt media prepared from commercial malt extracts sourced from different countries. The source

of water for media preparation too has been shown to have an influence on SM biosynthetic capacity of some fungal strains. For instance, the use of either tap or distilled water in preparation of culture media was shown to result in production of different types and quantities of fungal secondary metabolites, with such variations attributed to the presence of certain trace ions in tap water (Paranagama et al., 2007). Other cultivation parameter that have been shown to influence fungi SMs biosynthesis includes the volume of culture media and/or culture vessel (Daletos et al., 2017), incubation temperature and salinity (Venkatachalam et al., 2019), oxygen concentration and level of aeration (Barberel and Walker 2000), pH (Tudor et al., 2013), light type and/or intensity (Kim et al., 2014).

Since its conceptualization, OSMAC approach has become a mainstream in microbial natural product research. Figure 1.3 shows that between 2002 – 2006, only 2 journal articles containing the phrase “one strain many compounds” or “OSMAC” within the title, abstract or as a keyword were published compared to 86 such papers that have been published between 2016 - 2020.

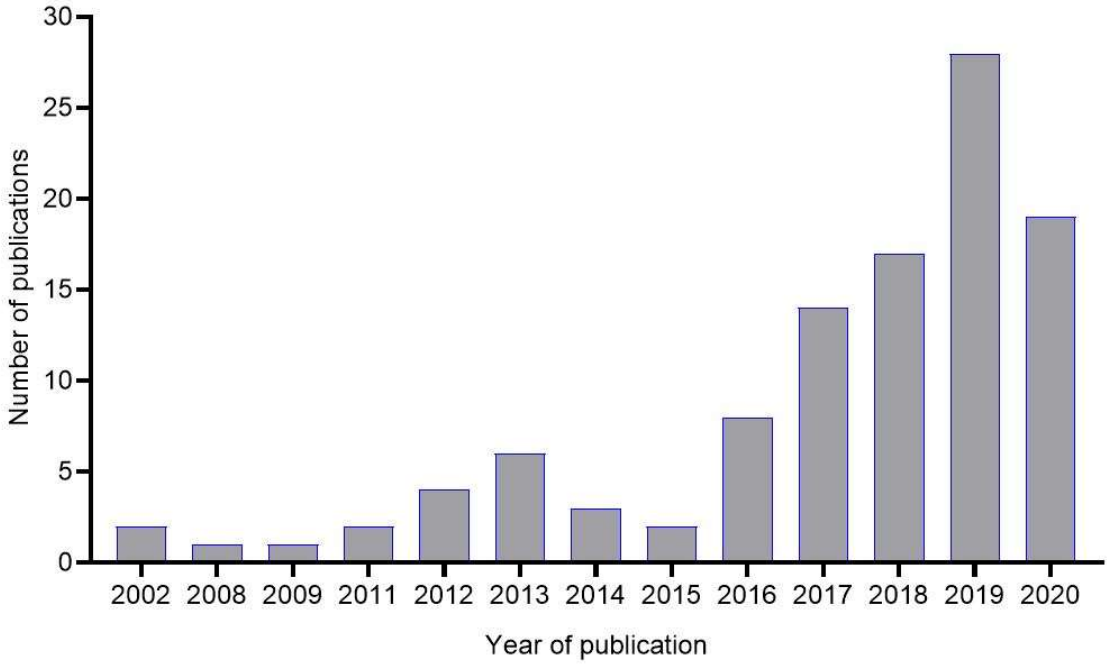


Figure 1:3 Number of journal articles published between 2002-2020 containing the phrase "one strain many compounds", "one strain-many compounds" or "OSMAC" within the title, abstract or as a keyword (Web of Science, January 14, 2021) (Gakuubi et al., 2021).

1.7.2: Microbial co-culture

Microbial co-culture is another strategy that has been found to be effective in enhancing the biosynthesis of secondary metabolites among fungal endophytes. In contrast to the mainly axenic conventional laboratory cultivation systems, endophytic fungi in their natural environment exist in complex communities consisting of members of their own and those of other populations. Members of different microbes within these microbial communities inevitably interact with other members as they compete for nutrients and space. Moreover, by utilizing specific resources and releasing specific chemicals into their surroundings, microorganisms modify their environment, which affect their growth and that of other resident microbes (Gakuubi et al., 2021). Thus, these natural microbial communities are characterized by complex intra-species and inter-species

interactions that fall within the continuum of mutualism and antagonism (Reen et al., 2015; Stroe et al., 2020). Microbial co-culture strategy entails the growth of two or more microbial strains together with the aim of mimicking the naturally occurring conditions of the strains of interest together with the myriad of interactions that occurs when these microbes coexist naturally (Bertrand et al., 2014). Stimulation of biosynthesis of new natural products by fungi through co-culture may involve one of the numerous mechanisms. Among the known mechanisms of elicitation of secondary metabolites biosynthesis through microbial co-cultures include physical cell to cell interaction, production of diffusible signaling molecules, production of enzyme that activate secondary metabolite precursors from the producing microbe and horizontal gene transfer (Abdelmohsen et al., 2015; Moody, 2014).

The two common co-culture schemes that have been employed for the induction of cryptic BGCs in fungi endophytes are fungal-bacterial and fungal-fungal co-culture. Induction of secondary metabolite biosynthesis through fungal-fungal co-culture may involve the growth of two fungal endophytes together or the growth of fungal endophyte of interest with other fungal strains including common fungal pathogens (Li et al., 2017; Wang, et al., 2013). For fungal-bacteria co-culture studies, various members of the Genus *Streptomyces* and *Bacillus* are among the most widely studied inducing bacteria (Ebrahim et al., 2016; Ola et al., 2013; Stroe et al., 2020). While the mechanisms involved in some fungal-fungal and fungal-bacterial elicitation processes have been elucidated, in other cases, the elicitation processes have remain poorly understood due to their complexity.

Co-cultivation is increasingly emerging as a powerful tool for unlocking the chemical diversity of fungal endophytes by enhancing the expression of silent or weakly expressed biosynthetic pathways. Co-culture has proven to be a particularly popular approach owing to its simplicity and low cost when compared with other more complex approaches such as the use of various gene manipulation techniques. However, when working with many strains, the method requires large screening of diverse strains combinations before coming up with the most promising combinations, a task that can be quite burdensome and time consuming. Furthermore, co-culture approach is only applicable to cultivable microorganisms and even for these microbes, there is a need to select the growth media that will support the growth of all the microbes involved.

1.7.3: Chemical elicitation

Fungi like all other living organisms are ingrained with an intricate network of multi-level regulatory systems that governs their gene expression. Such control mechanisms though essential for the normal growth and development of fungi often place some restrictions on secondary metabolism. Many fungal SMs are subject to complex regulatory systems consisting of signaling cascade and pathway-specific regulators and is dependent upon a complex network of cellular, chemical and genetic determinants (Rojas-Aedo et al., 2018). This tight regulation occurs through a set of cluster-specific regulators as well as globally acting regulators. The latter are usually encoded for by genes that are not associated with any specific gene cluster and are mediated by a wide range signals or triggers (Brakhage, 2013; García-Estrada et al., 2018).

Epigenetic modification is achieved in two ways: molecular epigenetic modification and chemical epigenetic modification. The former involves deletion or overexpression of epigenetic-related

genes while the latter entails the use of one or more of the chemical inhibitors of epigenetic modification such as DNA methyltransferase (DNMT) inhibitors, histone deacetylase (HDAC) inhibitors, and histone acetyltransferase (HAT) inhibitors (Xue et al., 2023). DNA in eukaryotes is wound tightly into a complex known as chromatin. Nucleosome, which is the basic unit of chromatin, is formed when DNA wound around a histone core consisting of two molecules of each of the four histone proteins H2A, H2B, H3 and H4 (Armeev et al., 2019). Chromatin is quite dynamic and under the influence of various signals can switch between two types of domains namely; the 'open' and transcriptionally active euchromatin and the 'closed' transcriptionally inactive heterochromatin (Collemare & Seidl, 2019). Figure 1.4 shows an illustration of the two different states of chromatin. Histones and their associated posttranslational modifications are the main components of epigenetic machineries that partition the genome into euchromatin or heterochromatin (DiPiazza et al., 2021). Secondary metabolite BGC may be found within the transcriptionally inactive heterochromatin. However, in response to various environmental cues, chromatin can transit to the transcriptionally active euchromatin conformation thus allowing transcription of the BGCs associated with the secondary metabolite and thus allowing the production of the associated SMs.

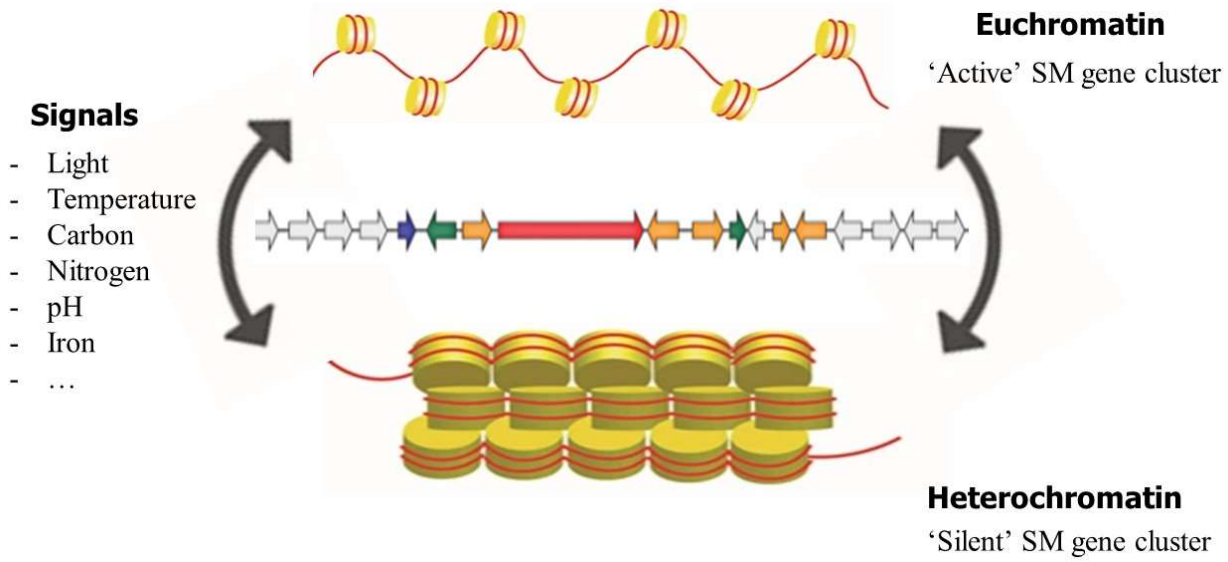


Figure 1:4 Chromatin structure showing the two states; the transcriptionally active euchromatin and the transcriptionally inactive heterochromatin (Illustration adapted from Collemare & Seidl, 2019).

Chemical epigenetic modification the most common of the two epigenetic modification approaches entails the growth of fungal strains in small chemical molecules known as chemical epigenetic modifiers the most common being histone deacetylases (HDAC) inhibitors and DNA methyltransferases (DNMT) inhibitors (Akone et al., 2016; Baral et al., 2018). Addition of such compounds in the growth cultures at micromolar or even nanomolar concentrations has been found to suppress or activate the associated enzymes resulting in the reengineering of SMs biosynthetic pathways in fungi (Magotra et al., 2017; Yang et al., 2014; Gakuubi 2022).

Some of the chemical inhibitors of epigenetic modification that have been used to enhance the activation of silent gene clusters in fungi include 5-azacytidine, 5-aza-2′-deoxycytidine (decitabine) and hydralazine hydrochloride which are DNMT inhibitors. Some HDAC inhibitors

on the other hand include suberoylanilide hydroxamic acid (vorinostat or SAHA), Suberoyl bishydroxamic acid (SBHA), trichostatin A, trapoxin B, sodium butyrate, valproic acid and nicotinamide (Figure 1.5).

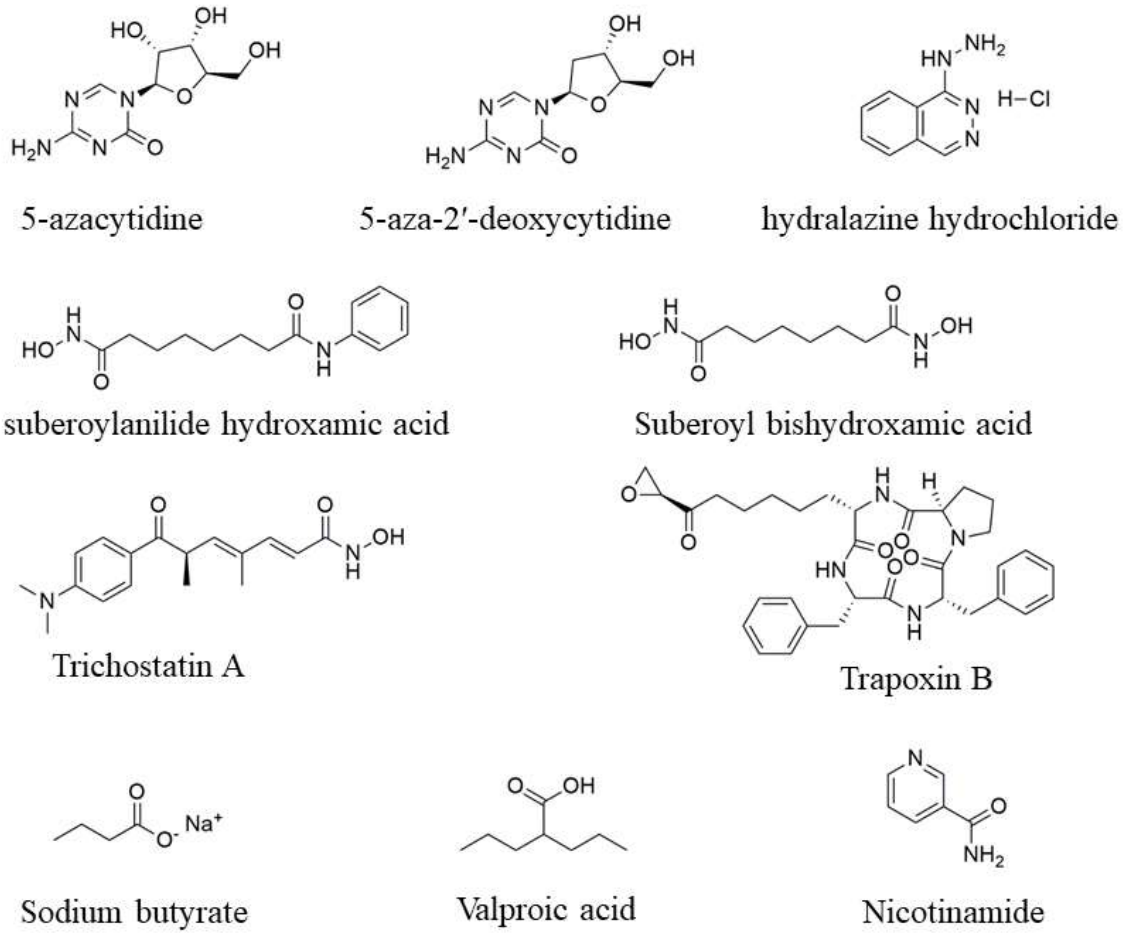


Figure 1:5 Some of the chemical elicitors that have been used in fungal natural products research (Gakuubi et al., 2021).

1.7.4: Molecular-based approaches

The one strain-many compound approach, microbial co-culture and chemical elicitation are all untargeted approaches that aims at activation of putative cryptic biosynthetic pathways whose

identity or number is mostly unknown beforehand. Because of their untargeted nature, these approaches do not permit the association of a newly identified secondary metabolite with any particular gene clusters. Genetic or metabolic engineering of target strains represents a more precise avenue that can be exploited to awaken cryptic BGCs. This approach is BGC-specific and relies largely on genome mining. It involves first the 'mining' of whole genome of the target strains for the typical secondary metabolites gene clusters using appropriate bioinformatics tools (Brakhage et al., 2008; Wiemann & Keller, 2014). Based on the nature of the identified putative cryptic BGCs, appropriate genetic manipulations of the strain of interest are performed using various synthetic and molecular biology tools to induce the expression of the silent biosynthetic pathways.

If the silent putative BGCs are found within a native host that is both cultivatable and amenable to genetic engineering, various strategies for triggering their expression can be performed directly on the native fungal strain (Kjærbølling et al., 2019; Soldatou et al., 2019). (Kjærbølling et al., 2019; Soldatou et al., 2019). Overexpression of global regulatory factors is one method that has been widely used to activate cryptic fungal SMs gene clusters (Calvo & Cary, 2015). Global transcription factors which regulate genes that are not involved in secondary metabolism are coded for by genes outside the cluster region and regulate fungal response to various environmental stimuli such as nutrients, light, PH and stress (Fox & Howlett, 2008; Hautbergue et al., 2018; Zeilinger et al., 2015).

In addition to the perturbations of the global regulators of fungal secondary metabolism, manipulation of pathway-specific regulators is an especially promising approach for activation of

silent SMs gene cluster considering that up to 50% of fungal BCGs have been found to harbours cluster-specific transcription factors (Keller, 2019). Moreover, this approach is more targeted in nature and although more labor intensive, it allows for the understanding of the role of each gene (Skellam, 2019). Cluster-specific regulators are involved in the inactivation or repression of biosynthesis of SMs associated with a particular BGC (Keller et al., 2005; Macheleidt et al., 2016).

In instances where the strain of interest is difficult to grow in the laboratory or cannot be easily manipulated genetically, heterologous expression strategies are employed to activate the identified silent BGCs. Selection of an appropriate host is an important consideration and will depend on a number of factors such as the level of genetic optimization required to express the genes, capability of the host to produce SMs i.e. availability of precursors and capability for post-translational modifications, toxicity of the compound and coping mechanisms among others (Kjærbølling et al., 2019). Three most commonly used categories of host for fungal heterologous expression are bacteria, yeast and filamentous fungi (Kjærbølling et al., 2019; Qiao et al., 2019). *Escherichia coli* and *Saccharomyces cerevisiae* are so far the most widely used bacteria and yeast host for fungal heterologous expression respectively while numerous filamentous fungal host exist, especially member of the genus *Aspergillus* such as *A. nidulan, A. oryzae*, *A. niger* and *A. fumigatus* (Qiao et al., 2019).

The advancements in DNA sequencing technologies and the drop in the cost associated with whole genome sequencing that has been witness in recent years has enhance the ability of natural product researchers to predict the biosynthetic capacity of fungal strains without having to culture them. This has in turn resulted in a paradigm shift towards genomic-driven discovery of microbial

secondary metabolites. However, this approach too has its limitations. For one, it still remains a challenge to link the predicted compounds to any possible inhibitory activities and hence, validation bioassays have to be performed to confirm with certainty the activity of predicted molecules (Wohlleben et al., 2016). Moreover, bioinformatics tools used in SMs gene clusters prediction rely on existing databases that are assembled based on data from previous studies and thus their inbuilt algorithms may hinder the discovery of more novel BGCs (Chen et al., 2019). Furthermore, being predictive by nature, bioinformatics tools are also prone to overprediction and underprediction when enlisting genes in particular BGCs hence the need for their progressive improvement and refinement to enhance their robustness and predictive power (Keller, 2019). The choice of any of the four strategies described herein for inducing the activation of silent or weakly expressed biosynthetic gene clusters in fungal endophytes will largely depend on the needs of an individual researcher, one's expertise and the nature of the fungal strains among other factors. None of the approaches can be considered superior to others as each has its strengths and limitations.

1.8: Aim and objectives

Fungi are renowned for their ability for biosynthesis of an array of bioactive secondary metabolites including beneficial molecules such as antibiotics and useful enzymes and harmful compounds such as mycotoxins. It has also been established that many of the fungal biosynthetic gene clusters (BGCs) that are involved in the production of SMs are 'silent' and thus never expressed under standard laboratory growth conditions. The main aim of this study therefore was to evaluate the diversity and bioactive potential of a target of at least 200 fungal isolates from the A*STAR's Natural Product Library (NPL) housed at the Singapore Institute of Food and Biotechnology

Innovation (SIFBI), Singapore. To achieve this, selected fungal strains were subjected to two different growth regimes namely; chemical epigenetic manipulation and variation of growth media. Fungal strains showing promising antimicrobial and cytotoxic activities were subjected to chemical analysis with the most promising strains progressed to compound isolation and structural elucidation. To achieve this aim, this study was guided by following three specific objectives:

i) Evaluate the diversity and bioactive potential of fungal endophytes from A*STAR's Natural Product Library (NPL).

ii) Assess the influence of chemical elicitation and diversification of fermentation media on the biosynthesis of bioactive fungal secondary metabolites.

iii) Elucidate/validate the biosynthetic pathway involved in the production of at least one isolated compound.

1.9: Thesis summary

This thesis is comprised of six chapters. Chapter one gives a comprehensive background and introduction to fungal endophytes and their importance as repositories of useful bioactive secondary metabolites. In this chapter, an attempt has been made at the definition and delimitation of fungal endophytes on the basis of the various definitions from literature and a working definition of fungal endophytes in the context of the current study is given. Furthermore, four approaches that are employed to induce the activation of silent or weakly expressed gene clusters or to enhance the production of SMs in fungal endophytes are described in details. This chapter additionally summaries the overall aim and the specific objectives of the study. Chapter two describes the diversity and characterization of fungal endophytes originally isolated from eight sites that correspond to four different habitats in Singapore. In this chapter, the molecular characterization

of fungal isolates through sequencing of the ITS2 region of rDNA gene is described. This chapter additionally delves into the phylogenetic analysis of the identified fungal isolates with the aim of identify those strains that would undergo fermentation for metabolites extraction. Following the selection of fungal strains that would undergo fermentation for metabolite extraction, chapter three explores the capacity of these strains to produce biologically active secondary metabolites. This chapter focusses on the use of two approaches namely; chemical elicitation and variation of fermentation media in the discovery of bioactive secondary metabolites. Among the areas covered under this chapter include small scale fermentation, processing of crude extracts, primary screening for antimicrobial and cytotoxic activities and dose-response testing for extracts showing promising growth inhibitory activities against a panel of four microbial pathogens and three cancer cell lines. Chapter four deals with isolation and identification of the bioactive secondary metabolites from selected endophytic fungi following a bioassay-guided approach. Here, chemical analysis using a wide range analytical methods such as HR-LC-MS/MS and NMR for the promising samples leading to isolation and structure elucidation of bioactive compounds from selected strains of interest is described. Chapter five describes the development of a CRISPR/Cas9-mediated system for the validation of the biosynthetic gene cluster (BGC) involved in the biosynthesis of palmarumycins in *Lophiotrema* sp. F6932, one of the three prioritized strains that were progressed to compound isolation and structure elucidation. Using a combination of bioinformatics, transformation and genetic engineering tools, this chapter details the establishment of a protocol for a PEG-mediated transformation of *Lophiotrema* sp. F6932 and ribonucleoprotein-mediated CRISPR-Cas9 induced deletion of the ketosynthase (KS) domain of the hypothesized palmarumycin pathway. This is followed by description of the chemical analysis of extracts from

the resultant knockout mutants and those of the wild-type strain for validation of the involvement of the gene cluster of interest in the biosynthesis of palmarumycins. Furthermore, the chapter proposes the biosynthetic routes to palmarumycin C_8, palmarumycin CP_{30} and melanin from the shared precursor DHN in *Lophiotrema* sp. F6932. Lastly, chapter six gives the conclusions arising from the major findings of the study and recommendations for future studies.

Chapter 2: Characterization of endophytic fungi

2.1: Introduction

Endophytic fungi are a diverse group of microorganisms that are found in association with plants. It has been estimated that most if not all plants in natural ecosystems are found in association with one or more fungal endophytes (Rodriguez et al., 2009). Indeed, fungal endophytes have been isolated from plants from nearly all of the known climatic and ecological zones from the hot deserts and cold Arctic and Antarctic regions to the temperate and tropical forests, the mangrove and coastal forest, grasslands and savannas among natural ecosystems (Kharwar et al., 2011). In this study, endophytic fungal isolates were obtained from A*STAR's Natural Product Library (NPL) housed at the Singapore Institute of Food and Biotechnology Innovation (SIFBI), Singapore. Aptly described as among the largest natural organism library (NOL) in the world, the library boasts a collection of more than 54,000 and 58,000 fungi and actinomycetes respectively in addition to thousands of eubacteria and plant specimens (Ng et al., 2018). Fungal strains selected for the current study were categorized into four groups on the basis of the habitats of their original sites of isolation. *Habitat 1* consist of strains isolated from the three water catchment areas of Upper Seletar, Upper Pierce and MacRitchie reservoirs. *Habitat 2* consist of strains isolated from Cluny road along Singapore Botanic Gardens. Strains from *Habitat 3* were isolated from two offshore islands of Pulau Ubin and St. John's while *Habitat 4* consist of strains isolated from Bukit Timah Nature Reserve and Kent Ridge Park.

2.2: Materials and methods

2.2.1: Revival of study fungal strains

Reference stock cultures which had been stored at -80 °C were revived by sub-culturing on either

malt extract agar (MEA, Oxoid, UK) or potato dextrose agar (PDA, Sigma, USA) plates followed by incubation at 24 °C for 5 days and up to 14 days for the fast-growing and slow-growing strains, respectively. For each strain, details such as strain's natural product library (NPL) ID code, preliminary preservation identity, isolation site/location, habitat of the plants and substrate from which the fungus was isolated among other details were noted. After the revived strains had grown sufficiently, plate photographs were taken and stock cultures for use during the course of the study prepared as follows: One milliliter of a mixture of malt extract broth (MEB; 30 g malt extract, 5 g mycological peptone/L; pH 5.4 ± 0.2) and 50% glycerol in 1:1 ratio was prepared and added into 1.5 mL cryogenic vials. For each fungal strain, mycelial plugs were prepared by cutting 5 mm diameter discs from the periphery of actively growing cultures grown on either MEA/PDA plates using sterile drinking straws. For each fungal strain, six such mycelial discs were aseptically added into the cryogenic vials which were then labeled and stored at -80 °C.

2.2.2: Extraction of fungal genomic DNA

Genomic DNA was isolated from mycelia harvested from freshly subcultured plates. Approximately 100 mg of mycelia from each fungus was scraped using sterile toothpick and grounded to powder in liquid nitrogen using a sterile mortar and pestle. When this method failed to produce quality genomic DNA from some fungal isolates, the following method was used to obtain better quality gDNA: Three mycelial agar discs (5 mm in diameter) from the periphery of an actively growing culture of the fungi grown on malt extract agar (MEA, Oxoid, UK) were inoculated in a 250 mL flask containing 15 mL MEB. The flasks were incubated at 24 °C in a shaking incubator at 200 rpm for 72 h for the fast-growing strains and up to 7 days for the slow-growing strains. The mycelial mass was harvested by filtering the growth culture through two

layers of miracloth followed by drying of the mycelia by thoroughly squeezing out the liquid media through additional layers of miracloth. The mycelia were then ground to powder in liquid nitrogen. In both cases, the genomic DNA was extracted from the ground mycelia using DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturers' instructions. The extracted DNA was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.3: Molecular identification fungal endophytes

For molecular characterization of the fungal isolates, PCR amplification of the internal transcribed spacer 2 (ITS2) region in rDNA was carried out using ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′) as the forward primer and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as the reverse primer (Turenne et al., 1999; White et al., 1990). The final PCR reaction mixture of 20 µL containing 1 µL gDNA, 1 µL of each of the primers at 10 µM, 2 µL of 2 mM premixed dNTPs, 2 µL of green buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 12.8 µL autoclaved Milli-Q water and 0.2 µL Dream Taq polymerase (Thermo Fisher Scientific, Vilnius, Lithuania). For the negative control, DNA was replaced with Milli-Q water. The PCR reactions were performed on a Veriti thermal cycler (Applied Biosystems, CA, USA) with the following conditions: initial denaturation at 95 °C for 5 mins, 40 cycles at 95 °C for 30 secs, annealing at 60 °C for 30 secs initial extension at 72 °C for 30 secs and a final extension 72 °C for 10 mins. The PCR products were electrophoresed on an agarose gels (1× TAE buffer, 1% agarose gel) stained with SYBR safe DNA gel stain (Thermo Fisher Scientific, Carlsbad, CA, US). Successful PCR amplification was confirmed by visualization of the gel on a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). Amplified PCR products were purified using MEGAquick-spin total fragment DNA purification kit (iNtRON Biotechnology, South Korea)

according to the manufacturer's instructions. The amplicons were sent for bi-directional sequencing at 1st Base, Axil Scientific Pte Ltd (Singapore) sequencing services (www.base-asia.com) using the aforementioned primer set.

2.2.4: Phylogenetic analysis of fungal strains

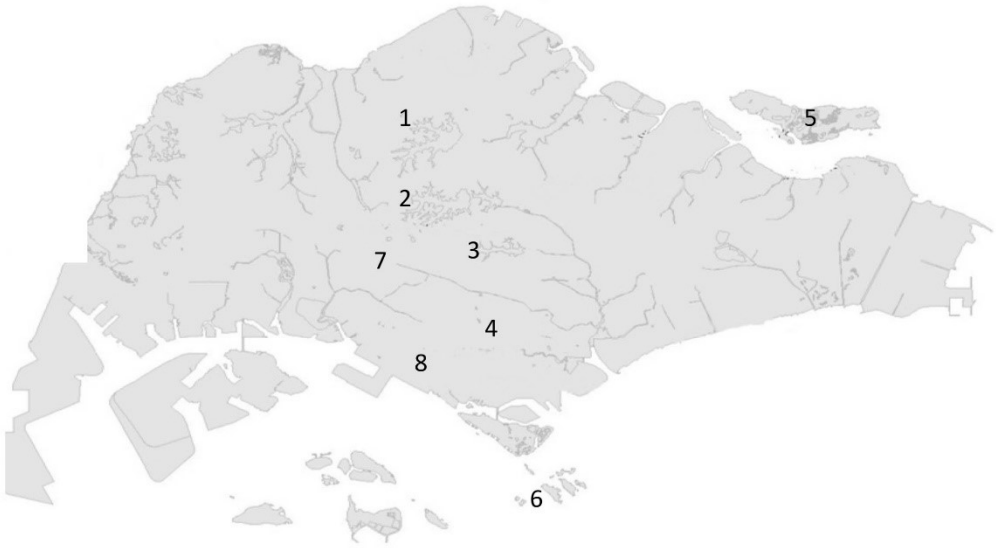
Generation of accurate consensus sequences for the ITS2 region from the forward (5′–3′) and reverse (3′–5′) sequence raw data for each fungal strain was performed using an Benchling alignment editor program (https://benchling.com). The alignments were trimmed, overhangs removed and gaps corrected. The resultant nucleotide sequences were uploaded onto the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the analysis of sequences similarity. The identity of each strain was assigned on the basis of maximum identity score. Phylogenetic analyses were performed using MEGA 7 software with DNA sequences aligned using ClustaIW program embedded within the software (Kumar et al., 2016). The evolutionary relationships between the strains were computed using the Neighbor-Joining and Maximum Likelihood methods. The resultant phylogenetic results were used for taxonomic analyses as well as selection of fungal strains that would undergo fermentation for metabolites extraction.

2.3: Results

2.3.1: Characterization of endophytic fungi

A total of 222 fungal strains were successfully revived and identified through molecular sequencing of the ITS2 region of rDNA gene. The sequences generated in this study were deposited to the National Center for Biotechnology Information (NCBI) database GenBank with accession numbers OM791857- OM792078. Appendix I gives a list of all the fungal strain

identified in each of the four study habitats, plant organ/tissue from where each of the strain was isolated from, the accession number of the closest relative in NCBI, classification/taxonomy summary among other details. The fungal strains in the current study had been preliminarily identified mainly up to the Genus level on the basis of cultural and morphological characteristic before preservation at A*STAR's Natural Product Library. Following successful revival, fungal strains were subjected to molecular identification, a form of DNA sequence-based identification system in which specific region in this case the rDNA internal transcribed spacer region (ITS-2) is used as a standard "barcode" locus for fungi identification (Badotti et al., 2017; Xu, 2016). Of the identified fungal strains, 23 had been isolated from the three water catchment areas of Upper Seletar Reservoir Park, Upper Pierce Reservoir and MacRitchie Reservoir (*Habitat* 1), 89 strains were from Cluny road along Singapore botanic garden (*Habitat* 2), 39 strains isolated from Pulau Ubin and St. John's islands represent *Habitat* 3 while 71 strains were isolated from Bukit Timah Nature Reserve and Kent Ridge Park designated as *Habitat* 4 (Figure 2.1). Among the 222 characterized fungal strains, 200 fungal strains had ≥97% identity matches to known strains based on the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). The remaining 22 fungal isolate fell below this threshold value with an identity match of between 80% - 96%. Strains with the lowest percentage similarity include F10161 with 80% similarity to *Myriangium* sp., followed by F11618 with 86% similarity to *Scorias spongiosa* (Appendix I).



Habitat 1	Habitat 2	Habitat 3	Habitat 4
Upper Seletar (1), Upper Pierce (2) and MacRitchie (3) Reservoirs	Cluny Road (4)	Pulau Ubin Island (5) and St. John's Island (6)	Bukit Timah Nature Reserve (7) and Kent Ridge Park (8)

Figure 2:1 A map of Singapore showing eight locations where the identified fungal endophytes were originally isolated from.

With regard to the plant tissue/substrate from which the fungal strains were isolated, majority (65%; 145 strains) of the strains had been isolated from the leaves followed by (19%; 41 strains) that had been isolated from plant stems. The fungal strains that were isolated from the flowers and fruits were 17 (8%) and 5 (2%) respectively. The remaining strains had been isolated from specialized plant organs such as the frond of palm trees and ferns (Figure 2.2).

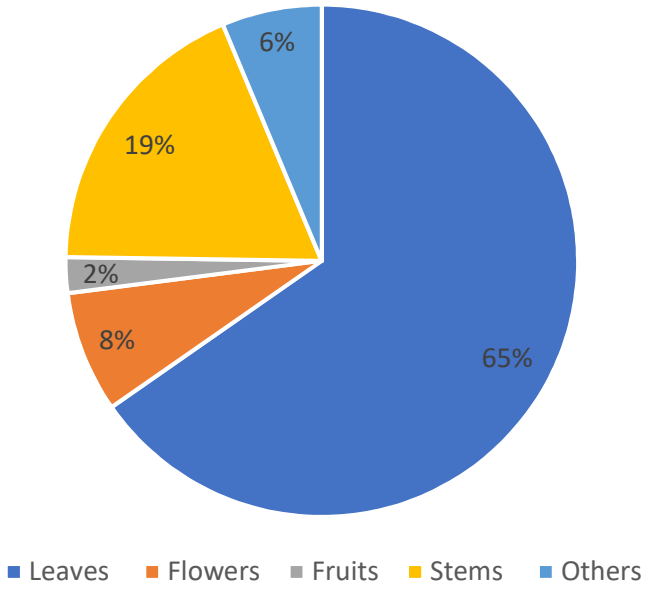


Figure 2:2 Percentage of endophytic fungal strains isolated from various plant organs/tissues

2.3.2: Diversity of endophytic fungi from different habitats

Majority of the identified fungal strains (99%; 219 strains) belongs to the phylum *Ascomycota*, while two strains (*Coprinopsis cinerea* and *Rhodofomitopsis feei*) belonged to the phylum *Basidiomycota* and only one strain (*Rhizomucor variabilis*) was from *Mucoromycota* phylum. Within the phylum *Ascomycota*, Class *Sordariomycetes* accounted for 79 % of the all the identified ascomycetes followed by *Dothideomycetes* which accounted for 14% of all the fungal reads belonging in this phylum. The identified fungal strains were distributed into 20 orders with *Glomerellales* and *Diaporthales* accounting for the highest number of strains (Figure 2.3A). Two fungal isolates belonging to the class *Dothideomycetes* lack a placement at the level of order and are designated as *incertae sedis* (Appendix I). At the Genus level, the fungal strains were distributed into 59 genera of which the dominating genera were *Colletotrichum* accounting for 27% of all the identified fungal isolates, followed by *Diaporthe* and anamorph *Phomopsis*

constituting 24% of the identified fungal strains. Others include *Aspergillus* (4%) and *Hypoxylon*, *Mycosphaerella*, *Phyllosticta* each accounting for 3% of all the fungal strains (Figure 2.3B). Furthermore, the majority of the identified genera (63%; 37 genera) were represented by a single fungal strain with the bulk of the identified fungal genera (73%; 43 genera) occurring in only one of any of the four studied habitats.

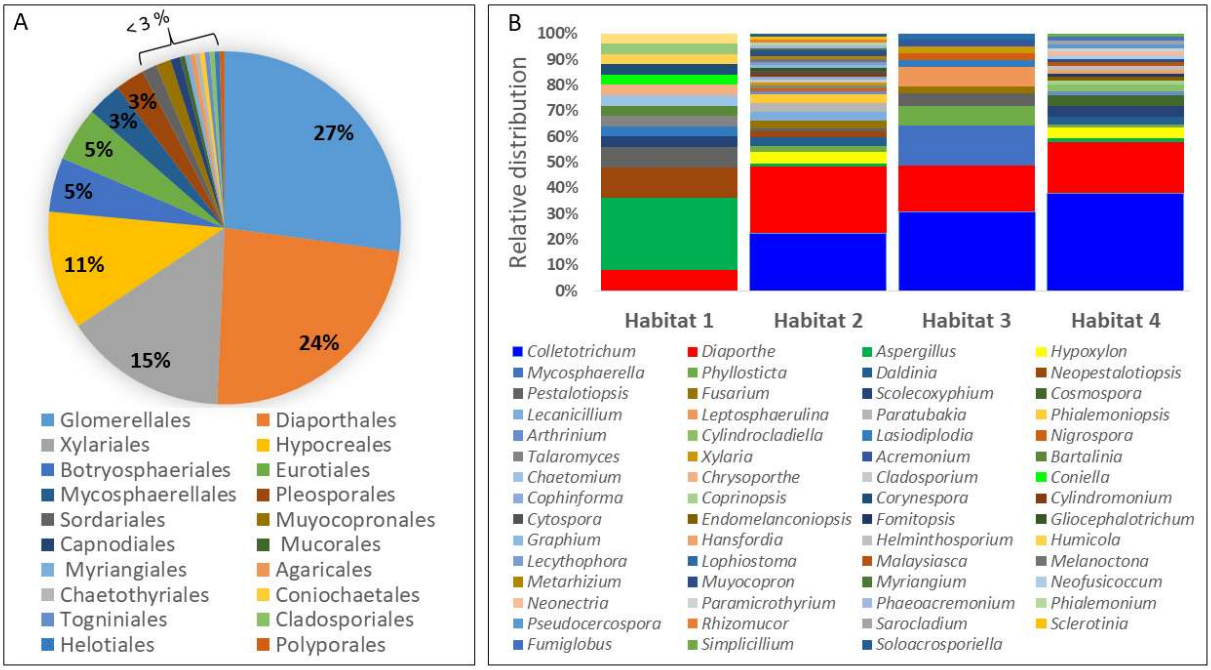


Figure 2:3 Diversity and distribution of identified endophytic fungi: (A) Percentage of fungal strains belonging to each of the 20 identified orders. (B) Relative distribution of fungal endophytes from different genera within the four study habitats (Gakuubi et al., 2022a).

Fungal endophytes isolated from the water catchment areas of Upper Seletar Reservoir Park, Upper Pierce Reservoir and MacRitchie Reservoir (*Habitat 1*) were distributed into 7 orders with 7 (30%) belonging to the order Eurotiales and 6 (26%) belonging to the order Xylariales. Cluny road (*Habitat 2*) had the highest number of identified strains of all the four study habitats. The 89

fungal endophytes that were isolated from this habitat were distributed into 17 orders with Diaporthales (30%; 27 strains), Glomerellales (23%; 20 strains) and Xylariales (16%; 14 strains) being the most dominant order in this habitat. For *Habitat* 3 namely Pulau Ubin and St. John's islands, and *Habitat* 4 that is Bukit Timah Nature Reserve and Kent Ridge Park, the most abundant fungal strains were those derived from the order Glomerellales representing 12 (30.8%) and 29 (40%) of the total fungal strains from the two habitats respectively. Figure 2.4 shows the morphological diversity of representative fungal isolates from each of the four study habitats while Figure 2.5 is a phylogenetic tree showing the relationship among all 222 fungal strains in this study.

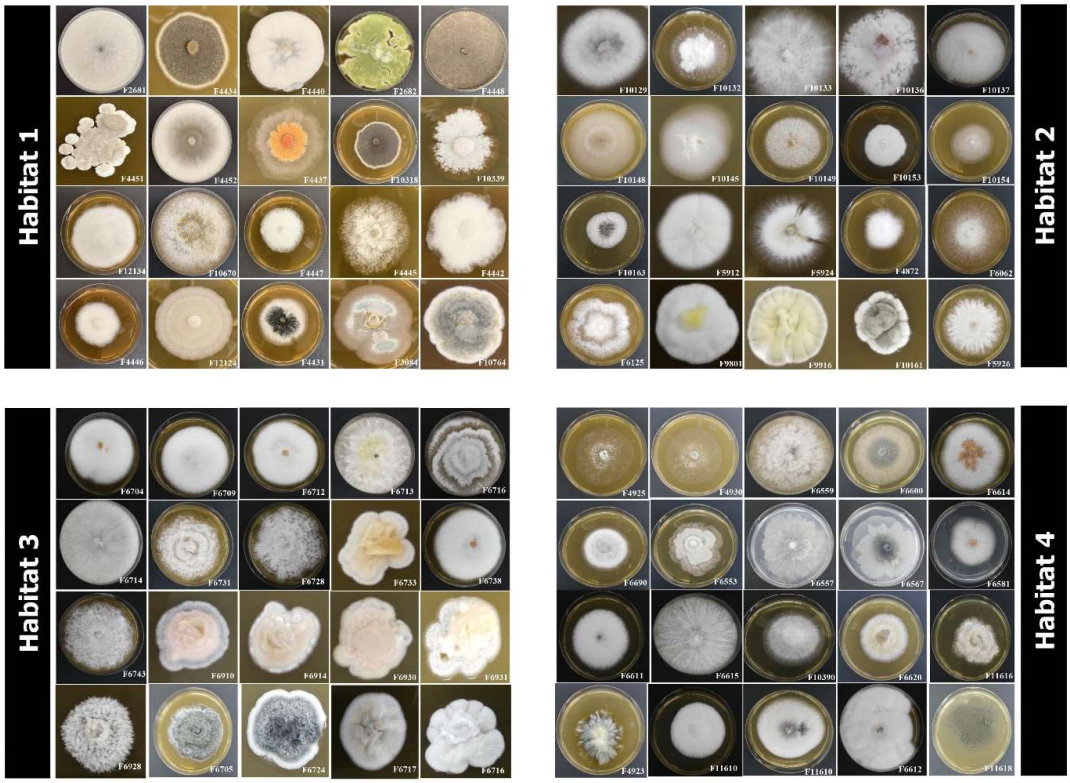


Figure 2:4 Morphological diversity of fungal endophytes. Shown here are representative plate pictures of fungal isolates from each of the four habitats in Singapore. *Habitat 1* (Upper Seletar Reservoir Park, Upper Pierce Reservoir and MacRitchie Reservoir), *Habitat 2* (Cluny road along

Singapore botanic garden), *Habitat 3* (Pulau Ubin and St. John's islands) and *Habitat 4* (Bukit Timah Nature Reserve and Kent Ridge Park.

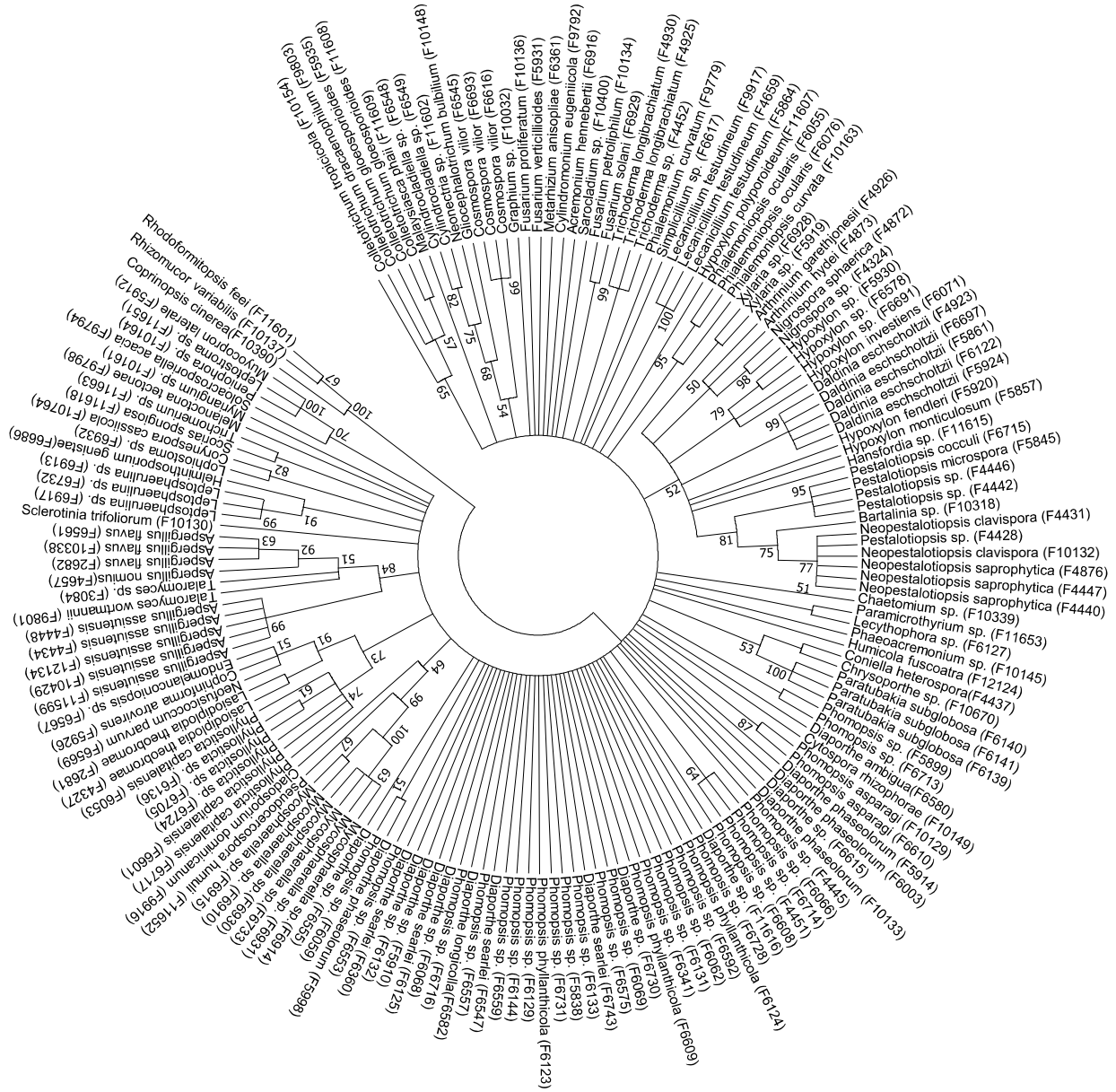


Figure 2:5 Phylogenetic tree of all the 222 fungal strains based on internal transcribed spacer (ITS2) rDNA gene sequences. The tree was constructed using the neighbor-joining method with MEGA 7 software (Bootstrap value = 500; values below 50% are not shown).

2.4: Discussion

Endophytic fungi constitutes a polyphyletic group of microbes that shows high degree of taxonomic and morphological diversity (Jia et al., 2016). Fungal endophytes have been isolated from essentially all of the different climatic and ecological zones (Chowdhary et al., 2012). Fungal endophytes have been isolated from virtually all plant types including trees, shrubs, herbaceous plants, grasses, mosses and ferns (Abhijeet Singh, 2014; Rodriguez et al., 2009). The number of endophytic fungi has been estimated to be in the range of one million on the basis of the 6:1 ratio of fungal: plant species (Sun & Guo, 2012). However, the actual figure could be much higher than this given the fact that only a small fraction of the approximately 5% of fungal species have been describe (Chávez et al., 2015). Furthermore, the validity of the 6:1 ratio has been questioned with some authors suggesting that the ratio could be up to five times higher in certain areas such as the tropical and subtropical regions (Gamboa et al., 2003).

Fungal endophytes whose unique ecological niche has been established through a co-evolution with their host plants over many years makes them a fascinating resource in the search for novel bioactive molecules (Aly et al., 2011; Meshram & Gupta, 2019). Recent advancement in genomics especially high-throughput sequencing has resulted in a renewal of interest in natural product-based drug discovery (Bachmann et al., 2015; Keller, 2019). This coupled with a decline in the hit rates of bioactive molecules from the traditional sources of microbes such as soil has led to an increased interest in microorganisms from other understudied and specialized niches as a potential source of novel bioactive molecules. Studies have revealed that the diversity of fungal endophytes is mainly a function of the host plants genotype and the environment with all its variables among other factors (Jia et al., 2016; You et al., 2017). Therefore, various environmental conditions such

as temperature, altitude and humidity in addition to a combination of geographical, ecological and edaphic factors influence the distribution range of various host plants which in turn determines the distribution and diversity of endophytic fungi (Bailey et al., 2005; Huang, 2020; Jia et al., 2016). Survey studies of endophytic fungi diversity have furthermore revealed that, even within the same locality, other factors such as sampling methods, season and methods employed in fungal isolation all have an influence on the number and diversity of fungal endophytes recovered within a given area (Li et al., 2016; Rather et al., 2018).

The fungal endophytes strains in the current study were originally isolated from eight sites within Singapore that roughly correspond to four different ecological habitats; the water catchment area, (Upper Seletar Reservoir Park, Upper Pierce Reservoir and MacRitchie Reservoir), urban setting (Cluny road), offshore islands (Pulau Ubin and St. John's islands) and nature park/reserves (Bukit Timah Nature Reserve and Kent Ridge Park). Singapore has a typical tropical climate that is characterized by abundant rainfall, high and uniform temperature and humidity throughout the year (Ai-Leen & Jin Song, 2000). Such environmental conditions have been cited as major predicator for fungal growth and thus have an influence on endophytic fungi community richness and diversity (Barnes et al., 2018; Rather et al., 2018). Despite its smaller land size, Singapore harbours an astonishing diversity of native and exotic plant species (Ho et al., 2019). However, very little published information exists regarding the diversity of fungal endophytes in the country and thus, the country's flora may host a diverse endophytic fungal community. In the current study, a total of 222 fungal strains from the four study habitats were characterized. The largest fraction of these strains (65%) had been isolated from the leaves followed by 41 (19%) that were obtained from stems. Plants leaves especially within the tropical ecosystems have been identified as the

hotspots of endophytic fungal diversity (Arnold & Lutzoni, 2007; Mahapatra & Banerjee, 2010). It is therefore not surprising that majority of the characterized fungal endophytes in this study had been originally isolated form plant leaves. In the current study however, generalizations regarding the plant tissues from which the fungal strains were isolated cannot be made since these strains were obtained from A*STAR's Natural Product Library (NPL) having been isolated previously from different sites within Singapore.

During the revival of fungal strains, it was observed that certain isolates that failed to grow on MEA plate were successfully revived upon growth on PDA plate. This selective growth of fungal isolates in specific media underpins the importance of diversification of growth media especially during the isolation step as a mean of enhancing the recovery rate of cultivable fungal endophytes. The retrieved fungal strains were subjected to molecular identification through sequencing analysis of the ITS2 region. The ITS region remains the mostly popular barcode region for fungal identification since its formal recognition as such by the International Fungal Barcoding Consortium and has gained widespread usage in fungal systematics and phylogenetic studies (Fernandes et al., 2015; Raja et al., 2017; Schoch et al., 2012). The entire ITS regions which is about 600 bp is made up of two variable spacer regions; ITS-1 and ITS-2, which are separated by a highly conserved 5.8s rRNA gene with the 5'-end of the ITS-1 bordering the conserved 18S rRNA gene and 3′ of the ITS-2 being flanked by another highly conserved 5.8S rRNA gene (Hill et al., 2000; Xu, 2016). After selection of the barcode region, proper selection of primers is another crucial step in successful identification of the microorganisms of interest where DNA barcoding approach is employed. The use of ITS86F/ITS4 primer set in the current study was supported by a number of studies that found it to be quite robust and to outperform other primer pairs in a number

of essential qualities such as high specificity for numerous fungal clades especially Ascomycetes, Basidiomycetes and Zygomycetes, *in silico* primer efficiency, PCR specificity and amplification efficiency, number of reads and number of species-level operational taxonomic units (OTUs) among other traits (De Beeck et al., 2014; Vancov & Keen, 2009). This has made ITS86F and ITS4 among the primer pair of choice for fungal diversity studies. However, although the ITS preforms quite well as a fungal barcoding marker, its use in accurate identification of fungal species is at time hindered by the presence of ambiguous sequences in genomic databases such as GenBank (Yamamoto & Bibby, 2014). Furthermore, the ITS region does not do well in discriminating highly speciose genera such as *Aspergillus, Fusarium, Penicillium, Trichoderma* and *Cladosporium* due to lack of barcode gaps or narrow barcode in their ITS region (Raja et al., 2017; Schoch et al., 2012). Recently, other barcode regions for use in fungal identification such as the translational elongation factor 1α (*TEF1-α*) and RNA polymerase II second largest subunit (RPB2) have been introduced to complement the use of ITS region and close the identification gap especially for pathogenic fungal species (Hoang et al., 2019).

In the current study, majority (99%; 219 strains) of the characterized fungal strains were from the phylum *Ascomycota* with just two strains belonging to phylum *Basidiomycota* and one strain from *Mucoromycota* phylum. These finding are in agreement with many studies that have reported ascomycetes are the most abundant representative of endophytic fungal communities with occasional isolation of basidiomycetes and zygomycetes (Hamzah et al., 2018; Lugtenberg et al., 2016; Sun & Guo, 2012). Indeed, the two strains belonging to the phylum *Basidiomycota* in the current study had been originally isolated from basidiomata and therefore are not endophytes. Within the ascomycetes, Sordariomycetes and Dothideomycetes were the most dominant classes

accounting for 79 % and 14% of fungal strains in this phylum. The remaining ascomycetes were mainly from Eurotiomycetes and Leotiomycetes classes. These finding are in agreement with a number of studies that have cited Sordariomycetes and Dothideomycetes as the dominant classes of fungal endophytes especially among the tropical and temperate plants (Arnold et al., 2000; Z. Wu et al., 2020). Furthermore, even within individual host plants such as *Acanthus ilicifolius, Zanthoxylum bungeanum* and *Rhizophora mucronata*, majority of the isolated endophytic fungal species were found to come from the Sordariomycetes and Dothideomycetes classes (Chi et al., 2019; Hamzah et al., 2018; Li et al., 2016).

The most abundant genus was *Colletotrichum,* which accounted for 27% of all the studied fungal strains followed by *Diaporthe* and its anamorph (asexual state) *Phomopsis* constituting 20% of the identified fungal strains. Significantly, in *Diaporthe*, only the *Phomopsis* which is the anamorph state was found in all the four study habitats. Members of *Colletotrichum* sp., were found in habitat 2, 3 and 4. Some of the most commonly occurring members of this genus were *Colletotrichum gloeosporioides*, *Colletotrichum asianum*, *Colletotrichum siamense*, and *Colletotrichum viniferum*. *Colletotrichum* is a cosmopolitan fungal genus whose members are adapted to a wide range lifestyles including as phytopathogens, endophytes and occasionally as saprobes (da Silva et al., 2020; Dean et al., 2012; Ma et al., 2018). The genus consists of 193 accepted species within 11 complexes with a further 3 more complexes having been recently proposed (Damm et al., 2019; Ma et al., 2018). Due to the ubiquitous nature of *Colletotrichum,* members of this genus have been widely studies for their secondary metabolites biosynthetic potential (García-Pajón & Collado, 2003; Kim & Shim, 2019). Diverse classes of bioactive molecules such as sterols, pyrones, phenolics, terpenes and fatty acids with wide-ranging inhibitory activities such as antimicrobial,

antioxidant, cytotoxic, antiviral and anti-inflammatory activities have been isolated from members of this genus (Kim & Shim, 2019). Other than being fungal endophytes, members of this genus are also recognized as important plant pathogens and the genus has been designated among the top ten most important plant pathogenic fungi (Dean et al., 2012).

Out of 59 fungal genera that were characterized in this study, majority of them (63%) were represented by only a single fungal isolate; a clear indication of great diversity of fungal endophytes in the studied habitats. The observed variations in fungal community composition within the four study habitats may be a function of the ecological variations as well as a reflection of the environmental structuring of Singapore's landscape. These aspects have been found to have an influence on the distribution and diversity of the host plants and subsequently endophytic fungi community composition (Fang et al., 2019; Zimmerman & Vitousek, 2012). Furthermore, of the 222 characterized fungal isolates, 22 isolates had <97% identity matches to the reference strains in NCBI. A cutoff value of 97% sequence similarity has been typically adopted in many fungal studies for delimiting fungal isolates into molecular operational taxonomic units (MOTUs) (An et al., 2020; Garnica et al., 2016). Therefore, some of the fungal isolates falling below this threshold value could be potentially new undescribed species. However, further studies including multilocus sequence analysis (MLSA) among other verification processes of the 22 fungal isolates may be required in order to rule out any errors that may have resulted in the observed low sequence similarity matches. This is because sequence-based identifications of fungal strains rely to a large extent on the accuracy of the reference sequences in public databases and thus, correct identification of either existing or new taxon will be compromised if the reference sequences are incomplete or erroneously labelled (Hofstetter et al., 2019; Lücking et al., 2020). It is worthy to

note that the targeted number of fungal strains in this study was at least 200 fungal isolates. Thus, the number of strains selected from each of the studied habitat was not predetermined nor is it an indication of the abundance of fungal isolates from each of the studies habitats. Rather, it is based on the number of fungal strains available within A*STAR's NPL from different study sites that had not been previously studied for their diversity and inhibitory activities and thus were considered more appropriate for inclusion in the current study. Because of this therefore, it would not be valid to make generalized conclusions on the comparative taxonomic diversity of fungal endophytes between the four studied habitats, but rather, the study gives a general representative picture of endophytic fungal community in the respective study habitats and Singapore in general. Furthermore, the number of selected fungal strain from each of the four study habitats varies greatly.

2.5: Conclusion

There has been a renewed interest in natural product-based drug discovery in the recent years. Endophytic microbes such as fungal endophytes represent an understudied component of the microbial diversity. This group of fungi holds an enormous potential for discovery of novel chemical entities of pharmaceutical importance owing to their unique ecological niche and inimitable biology. In this study, 222 fungal isolates from A*STAR's Natural Product Library were characterized on the basis of sequencing analysis of ITS region of the rDNA gene. The fungal strains had been originally isolated from 8 locations within Singapore representing four distinctive habitats namely; the water catchment areas of Upper Seletar Reservoir Park, Upper Pierce Reservoir and MacRitchie Reservoir (*Habitat 1*) Cluny road along Singapore botanic garden (*Habitat 2*) two offshore islands of Pulau Ubin and St. John's (*Habitat 3*) and Bukit Timah Nature

Reserve and Kent Ridge Park (*Habitat 4*). The bulk of the identified fungal endophytes belong to the phylum *Ascomycota* with only two and a single strain belonged to the *Basidiomycota* and *Mucoromycota* phyla respectively. Characterized fungal endophytes were distributed in 59 genera with the most dominating genera being *Colletotrichum* and *Diaporthe* and its anamorph *Phomopsis*. 21 strains (an equivalent of 9% of all the studied fungal strains) had < 97% sequence similarity in the ITS2 rDNA gene region when compared with the closest relative in the NCBI and thus some of these could potentially be new undescribed fungal endophytes strains and thus warrant further investigation. Following phylogenetic analysis of the identified fungal endophytes, 146 strains were selected for fermentation and isolation of secondary metabolites and thus forms the groundwork for the next phase of this study.

Chapter 3: Bioactivity screening of endophytic fungi

3.1: Introduction

The capacity for production of bioactive molecules, both beneficial (such as antibiotics e.g. penicillin) and detrimental (such as mycotoxins e.g. aflatoxin) by fungi has spurred a great deal of interest among natural product researchers (Hyde et al., 2019). During their co-evolution history with their host plants, fungal endophytes have developed mechanisms for biosynthesis of bioactive secondary metabolites (SMs) that are both similar or closely related to those produced by the host plants in addition to molecules that are unique to the fungi and not biosynthetically related to their respective host plants (El-Hawary et al., 2020). Fungal endophytes have been exploited for the discovery of biologically active molecules with a wide range of applications such as in the pharmaceutical, food, cosmetic and agrochemical industries (Gouda et al., 2016; Manganyi & Ateba, 2020). Among the compounds of pharmaceutical importance that have been isolated from fungal endophytes are antibiotics (Deshmukh et al., 2014; Martinez-Klimova et al., 2017), antifungal compounds (Deshmukh et al., 2018) antimycobacterial compounds (Alvin et al., 2014), anticancer/cytotoxic compounds (Chen et al., 2014; Kharwar et al., 2011; Uzma et al., 2018), antioxidants (Toghueo & Boyom, 2019) and enzymes with a wide range of commercial applications (Corrêa et al., 2014; Khan et al., 2017).

Following the isolation and selection of fungal endophytes of interest, the strains are fermented using appropriate methods and growth media for preparation of crude extracts. Since fungal endophytes constitute a heterogeneous and diverse group of microorganisms, the nutritional requirements needed for the production of secondary metabolites are complex and vary greatly among different strains (Supratman et al., 2019; VanderMolen et al., 2013). Besides the nutritional

requirements, other abiotic culture conditions that results in optimum production of secondary metabolites are specific to different fungal strains and need to be optimized. These include growth parameters such as static vs agitation growth, aeration and availability of oxygen, temperature, size and shape of culturing flask, light type, intensity and duration , growth duration among others growth factors (Ahsan et al., 2017; Reen et al., 2015). The term one strain-many compounds (OSMAC) was introduced to denote these different culture parameters whose manipulation can result in tremendous changes in the biosynthetic profile of a microbes resulting in changes not only in the amount of a specific metabolites produced, but in certain instances the production of new chemical entities altogether (Bode et al., 2002). In addition to the use of OSMAC approach, other growth strategies that have been adopted to enhance the biosynthesis of secondary metabolites in fungal endophytes includes microbial co-culture in which two or more microorganisms are grown together with the aim of mimicking the myriad of interactions that occurs when microbes coexist naturally (Daletos et al., 2017; Li et al., 2019). Chemical elicitation is another approach that has been widely used in enhancing the biosynthesis of SMs in fungal endophytes. This approach entails the growth of strains of interest in the presence of one or more of the chemical inhibitor of epigenetic modification such as histone deacetylases (HDAC) inhibitors and DNA methyltransferases (DNMT) inhibitors (González-Menéndez et al., 2019; Magotra et al., 2017). The addition of such compounds in growth cultures at micromolar or even nanomolar concentrations has been found to suppress or activate the associated enzymes, resulting in the reengineering of SMs biosynthetic pathways in fungi (Yang et al., 2014).

Isolation of fungal SMs is followed by assessment of their inhibitory activities using appropriate assays leading to the purification, and characterization of the active constituents within the active

samples. The techniques employed for screening of endophytic fungi and indeed all microbial secondary metabolites for their inhibitory activities have greatly evolved over the years fueled by the need for more speed and efficiency in the identification and characterization of bioactive compounds (Balouiri et al., 2016; Vasundhara et al., 2016). The 'Waksman platform' whereby streptomycetes were assessed for growth inhibitory activity through the detection of zone of inhibition on an overlay plate is considered among the most successful drug discovery platform of the early days of the 'golden era' of antibiotics (Balouiri et al., 2016; Lewis, 2013). This was followed by other methods such as agar disk-diffusion method, agar well diffusion, cross streak, poisoned food among other *in vitro* antimicrobial susceptibility testing methods that have been developed over time (Balouiri et al., 2016). In recent years, isolating and characterization of biologically active molecules has been significantly enhance through the development of high-throughput screening (HTS) techniques (Alvin et al., 2014; Henrich & Beutler, 2013). High-throughput screening platforms leverage on robotic and automation of many bioassay process in addition to the use of dedicated software for data acquisition and analysis allowing fast testing of a large number of samples. This coupled with advancement that has been witness in analytical techniques, mainly high-performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear magnetic resonance (NMR), has greatly boosted natural product-based drug discovery (Alvin et al., 2014; Mishra et al., 2008).

3.2: Materials and Methods

3.2.1: Small scale fermentation of endophytic fungi

For preliminary isolation and identification of bioactive metabolites, fermentation for extracts generation was done as follows: First, all the 146 fungal strains selected for extracts generation

following phylogenetic analysis were grown in 10 mL cultures in 50 mL conical glass flasks in two in-house liquid media (CF02LB and CF18LB) that have been formulated and optimized by the Natural Product Library group at SIFBI for fungal secondary metabolites production (Table 3.1). Thereafter, two approaches namely; chemical elicitation and variation of fermentation media were evaluated for their potential in enhancing the production of bioactive secondary metabolites from fungal endophytes. To this end, the 146 fungal strains were divided into two groups with the first group of 45 strains grown in the same two liquid media (CF02LB and CF18LB) in the presence of 50 µM 5-azacytidine (Sigma, Canada), a DNA methyltransferase inhibitor or 100 µM of suberoylanilide hydroxamic acid (SAHA, Cayman Chemical Company, USA), a histone deacetylases inhibitor (Figure 3.1). The objective here was to assess the effect of chemical epigenetic modification on the biosynthesis of active SMs from fungal endophytes. Stock solutions of the two chemical elicitors were prepared in 50% DMSO and then filter-sterilized through 0.2 µm filter. An appropriate volume of each of the chemical elicitor was then dispensed into each of the conical flask containing 10 mL of each of the two growth media using a micropipette such that each media flask contained an appropriate concentration of the chemical elicitor. After the addition of the elicitors, three mycelial discs (3 mm in diameter) were cut from the periphery of an actively growing fungal cultures grown on MEA/PDA plates and inoculated into each of the media flask.

Table 3:1 Composition of the five media used in the study

Components	Media (per L)				
	CF02LB	CF07LB	CF18LB	CF25ST	CF28ST
Glucose	20 g	15 g	20 g	–	–
Maltose	10 g	–	–	–	–
Cane molasses	–	20 g	–	–	–
Soluble starch	–	40 g	–	–	–
Pharmamedia	–	25 g	–	–	–
Peptone	–	–	5 g	–	–
Yeast extract	4 g	–	5 g	1 g	0.2 g
KH_2PO_4	–	–	0.5 g	0.5 g	0.1 g
$MgSO_4.\ 7H_2O$	–	–	1 g	–	0.1 g
$FeSO_4.\ 7H_2O$	–	–	–	–	0.01 g
$FeCl_3$	–	–	10 mg	–	–
$ZnSO_4.\ 7H_2O$	–	–	1.78 mg	–	0.01 g
$CaCl_2$	–	–	73.5 mg	–	–
$CaCO_3$	–	8 g	–	–	–
Sodium tartrate	–	–	–	0.5 g	0.1 g
Oatmeal	20 g	–	–	–	–
Brown rice	–	–	–	20 g / 40 mL	–
Cracked corn	–	–	–	–	5 g /10 mL
pH	7.5	Natural	5.5	Natural	Natural

The second group of 101 fungal isolates were grown in three additional media; one liquid media (CF07LB) and two solid media (CF25ST and CF28ST) (Figure 3.1). The objective of this experiment was to assess the media that would enhance the biosynthesis of active secondary

metabolites in fungal strains. This part of the study was modelled around the one strain-many compound (OSMAC) approach (Bode et al., 2002). The principles behind OSMAC is that by systematically manipulating media type and composition among other cultivation parameters, it is possible to completely alter the biosynthetic profile of a microbe thus making it possible to isolate as many bioactive SMs as possible from a single strain (Bode et al., 2002; Pan et al., 2019). All the culture flasks were appropriately labelled and incubated for 14 days in a shaking incubator (Infors HT Multitron Pro) at 24°C and 200 rpm for the liquid cultures and 21 days at 24°C in a static condition for the solid media cultures. The growth progress of the cultures was regularly monitoring. The selection of the number of strains that would be subjected to the second round of fermentation was based first on the overall workload that would entail the two experiments. The next important consideration was to have a representation of all the main taxonomic groups of fungal strains in the two experiments.

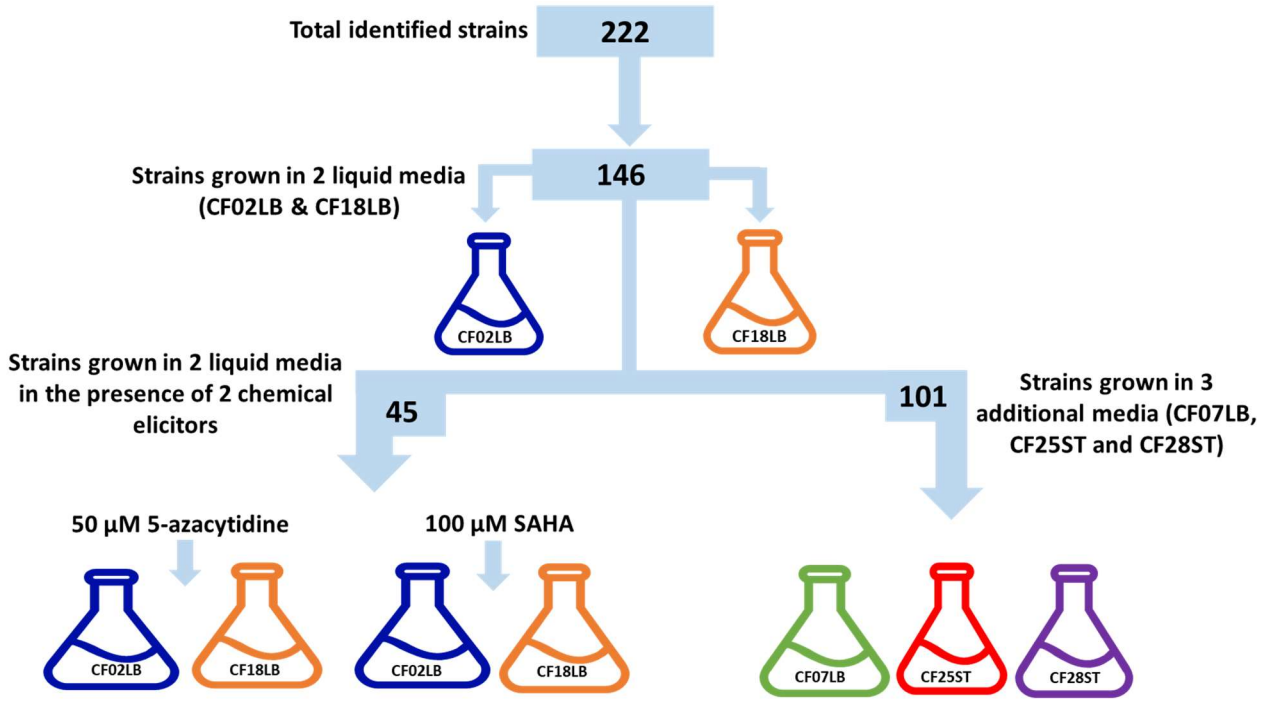


Figure 3:1 Experimental design for extracts generation for evaluation of the effects of chemical elicitation and variation of growth media on the inhibitory activity of fungal extracts.

3.2.2: Preparation of crude fungal secondary metabolites

Following two and three weeks of incubation for the liquid and solid cultures respectively, the culture flasks were frozen overnight at -80°C and then freeze-dried in a vacuum freeze-dryer for 4 days to expel all the moisture. This was followed by the addition of 20 mL of methanol and shaking overnight in an incubator at 24ºC and 200 rpm to extract crude extractables. The extracts were then filtered through a filter paper (Whatman No. 4) to separate the dissolved metabolites from the insoluble media components and mycelial residues. The extracts were dried using a centrifugal vacuum concentrator (Genevac Ltd, United Kingdom) to obtain dry crude extracts. The mass of extracts produced by each fungal strain was determined by subtracting the weight of the pre-weighed empty falcon tubes from that of the falcon tubes containing the dried extracts. The

dry extracts were then resuspended in an appropriate volume of methanol through sonication to obtain a final concentration of 5 mg/mL. The resuspended extracts were dispensed into micronic tubes (1 mL per tube) using an automated liquid handler (Tecan, Switzerland). The extracts were further dispensed into numerous copies of two sets in 96-well microtiter plates using a semi-automatic 96-fold pipettor (Analytik Jena, Germany). The two sets of microtiter plates contained 40 µL and 60 µL of the extracts per wells such that upon drying, each wells would contain 0.2 mg and 0.3 mg of the extracts respectively. The 0.2 and 0.3 mg extract samples in 96-well microtiter plates were to be used in primary screening assays while the 0.3 mg samples were meant for use in dose-response testing for confirmation of activity. Samples in micronic tubes were to be used in fingerprinting-based chemical dereplication for preliminary identification of bioactive compounds from the active samples. The remaining extracts were dispensed into labelled glass vials. All the extracts i.e. in microtiter plates, micronic tubes and glass vials were dried using a centrifugal vacuum concentrator and stored at 4^{o}C in a cold room until when next required.

3.2.3: Primary screening for antimicrobial activity

Screening for antimicrobial activity of fungal secondary metabolites was carried out against four human pathogens; two bacteria strains - *Staphylococcus aureus* (ATCC 25923) and *Klebsiella aerogenes* (ATCC 13048; formerly *Enterobacter aerogenes*) as representatives of the Gram positive and Gram negative bacteria respectively. Two fungal strains, *Candida albicans* (ATCC 10231) and *Aspergillus fumigatus* (ATCC 46645) served as representative of yeasts and mold human pathogens respectively. Primary screening of crude extracts was carried out at a single concentration of 200 µg/mL in duplicate in a 384-well assay following the guidelines of Clinical and Laboratory Standards Institute (CLSI, M07-A9, 2012) for the antibacterial assay, Clinical and

Laboratory Standards Institute(CLSI, M27-A2, 2002) for *C. albicans* and (CLSI, M38-A2, 2008) for *A. fumigatus*.

For the antibacterial assay, reference bacterial pathogens from glycerol stocks that had been stored at -80 ^{o}C were streaked onto Mueller Hinton agar (MHA) plates and incubated overnight at 37 ^{o}C. A new streak plate was prepared from the glycerol stock plate in MHA and incubated overnight at 37 ^{o}C. From this second streak plate, a bacterial suspension was prepared in 0.85% saline solution and its turbidity adjusted to 0.5 McFarland standard, approximately 1.5×10^{8} CFU/mL. The OD_{600} of the bacterial suspension was then measured and the bacterial inoculum adjusted accordingly to obtain OD_{600} reading in the range of 0.121 - 0.132. The bacterial suspension was then diluted 273× in cation-adjusted Mueller Hinton broth to obtain a final concentration of approximately 5.0×10^{5} CFU/mL. To prepare the 200µg/mL of the test compounds, 10µL of neat DMSO was dispensed into each well of the 96-well plate containing 0.2 mg dried extracts. The plates were then put on a plate shaker for 5 min followed by addition of 90 µL of sterile Milli-Q water into each well and further mixing on a plate shaker to give extract solutions at a concentration of 2mg/mL in 10% DMSO. 5µL of each of the extract sample solution were transferred from the 96-well plate and dispensed into a 384-well clear flat bottom plate (Greiner Bio-One, Austria) in duplicate. This was followed by the addition of 45 µL of 5.0×10^{5} CFU/mL of the bacterial suspension using Multidrop bulk reagent dispenser (Thermo Fisher Scientific). This ensured that the final concentration of DMSO, the solvent that was used to reconstitute the extracts in the assay was 1% (v/v). For the assay controls, 0% inhibition control (signal) wells contained 5µL of 10% DMSO and 45µL of 5.0×10^{5} CFU/mL bacterial suspension while the 100% inhibition control (background) contained 5µL of 10% DMSO and 45µL of MHB. Gentamicin was used as a standard inhibitor in bacterial

screens with a 16-point, 2-fold serial dilution prepared with a starting final assay concentration (FAC) of 25µg/mL. Drop plate method was additionally used for test inoculum validation.

The antifungal activity assay was similarly performed at a single concentration of 200µg/ml extracts in duplicate in a 384-well assay. For *C. albicans*, the reference strain from the glycerol stock that had been stored at -80 ºC was streaked on Sabouraud dextrose agar (SDA) plate and incubated at 24 ºC for 48 hours. A new streaked plate was prepared from this glycerol stock plate in SDA plate and incubated at 24 ºC for 48 hours. From this second streaked plate, an inoculum was prepared in 0.85% saline solution and its turbidity adjusted to 0.5 McFarland standard, approximately 2.5×10^6 CFU/mL. The OD_{600} measurement of the fungal suspension was taken and the concentration of the inoculum adjusted to obtain OD_{600} in the range of 0.22 - 0.23. The fungal suspension was then diluted 1000× in Sabouraud dextrose broth (SDB) to obtain a final concentration of approximately 2.5×10^3 CFU/mL. For *A. fumigatus*, a spore suspension (1×10^8 spore/mL) that had been stored at -80 ºC was thawed and diluted 4000× in SDB to obtain a final spore suspension of 2.5×10^4 spore/mL. The rest of the assay procedures were carried out in a similar manner to the antibacterial assay. Amphotericin B was used as the standard inhibitor for fungal screens with a 16-point, 2-fold serial dilution prepared with a starting final assay concentration of 20 µg/mL. Furthermore, drop plate and spread plates methods were additionally used for test inoculum validation for *C. albicans* and *A. fumigatus* respectively. The assay plates were incubated inside biotransport carriers. The bacterial assay plates where incubated at 37 ºC with the plate readings taken after 24 hours. The fungal assay plates were incubated at 24 ºC with plate readings for *C. albicans* and *A. fumigatus* taken after 48 and 72 hours respectively. The

growth inhibitory effects (% inhibition) of the crude extracts on the test microorganisms were evaluated by measuring the plates' OD_{600} using a microplate reader (Tecan Infinite M1000 Pro reader, Switzerland) and calculated using the following formula:

$$\% \text{ Inhibition} = 100 - \left[100 \text{ x } \frac{(\text{Raw value} - \text{Average Background})}{(\text{Average signal} - \text{Average background})} \right]$$

3.2.4: Primary screening of cytotoxic activity

Primary screening for cytotoxic activity of the fungal extracts was likewise done at a single concentration of 200 µg/mL in duplicate in a 384-well assay. Three adherent cancer cell lines that are widely used in basic research and drug discovery; the human pancreatic cancer cells PANC-1 and MIA PaCa-2 and human lung cancer cells A549 were used for cytotoxic screening of fungal extracts. The cell stocks in 1mL aliquots in Dulbecco's modified eagle medium (DMEM, Gibco, Life Technologies, Bleiswijk, the Netherlands) containing 50% heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, Paisley, UK) and 10% DMSO were stored in liquid nitrogen (-196˚C). The cells were retrieved from the frozen condition and thawed in a 37 ˚C water bath followed by centrifugation to remove the storage media and DMSO. The cell suspensions were then transferred into a flasks containing DMEM media enriched with 10% FBS and 1% penicillin-streptomycin solution (Gibco, Thermo Fisher Scientific). The cells were incubated at 37 ˚C with 5% CO_2 and 95% relative humidity for at least two passage in order to allow them to recover and achieve optimal growth conditions necessary for the cytotoxic screening.

Once the growing cells had attained confluence, they were twice washed with phosphate-buffered saline (Gibco, Life Technologies, Paisley, UK), and harvested by trypsinization. The cells were

then transferred into falcon tubes and centrifuged at 800 rpm for 5 minutes followed by the removal of the used media and trypsin. Fresh media was added to the cells and the cells suspension was homogenized using a syringe and needle to get rid of any cell clumps. In order to determine the cell density, viable cells were calculated by trypan blue exclusion method using a haemocytomter. The cells at a density of 3.3 $\times 10^4$ cells/mL for A549 and MIA PaCa-2 and 5.5 $\times 10^4$ cells/mL for PANC-1 were seeded in 384-well black clear bottom plates (Greiner Bio-One, Austria) with 45µl added into each well. The cells were incubated at 37˚C for 24 hours in 5% CO_2 and 95% relative humidity. After 24 hours, the crude extracts were added into the cells with 5µL added into each well using a semi-automatic pipettor. For the assay controls; 0% inhibition control (signal) wells contained 45µL cells and 5µL of 10% DMSO while the 100% inhibition control (background) wells contained 45µL of the media and 5µL of 10% DMSO. Puromycin, a protein synthesis inhibitor was used as the standard inhibitor in which a 16-point, 3-fold serial dilution was prepared with a starting final assay concentration of 184 µM. The plates were incubated at 37 ˚C for 72 hours in 5% CO_2 and 95% relative humidity. After incubation, 5µL of PrestoBlue (Invitrogen, Eugene, Oregon, USA), a resazurin based cell viability reagent was added in each well in darkness. The plates were covered with aluminum foil-coated plates covers, shaken gently over a plate shaker for 1 minute and re-incubated for 2 h for A549 and MIA PaCa-2 and 4 h for PANC-1. This was followed reading of plate's fluorescence at an excitation wavelength of 560nm and emission wavelength of 590nm.

3.2.5: Dose-response testing for confirmation of activity

Following primary screening of the antimicrobial and cytotoxic activity, the crude extracts showing promising growth inhibitory activity against any of the test microbial pathogens and cell

lines (average % inhibition ≥ 50) were subjected to dose-response testing for confirmation of activity. Furthermore, dose-response testing allowed for the generation of dose response curves which were used to determine the IC_{50} values in order to determine the samples that would be progressed to the chemical dereplication for putative identification of the active constituents. Dose-response testing was done in duplicate in an 8-point 2-fold serial dilution in a 384-well assay with a starting concentration 200µg/mL. The rest of the antimicrobial and cytotoxic assay and associated controls were carried out in a similar manner and conditions as is described in sections 3.2.3 and 3.2.4 respectively.

3.3: Results

3.3.1: Effects of chemical elicitors and growth media on extractable yields

The growth of fungal endophytes in media supplemented with the two chemical elicitors resulted in markedly increase in the weight of crude extracts for most fungal strains. Generally, it was observed that crude extracts were in some cases one to three orders of magnitude higher in cultures grown in the presence of the two chemical elicitors when compared with extracts derived from the same strains grown in the absence of the two chemical elicitors. For example, of the 45 fungal strains that were grown in two liquid media in the presence and absence of the chemical elicitors, 9 out of the 10 highest extractable yields were obtained from strains grown in the presence of the chemical elicitors. One-way ANOVA results revealed a significance difference in the yields of crude extracts from the fungal strains grown in the absence of an elicitor in comparison with those grown in the presence of the two elicitors (Figure 3.2A). With regard to the used of five different fermentation media, crude extracts from two solid media (CF25ST and CF28ST) were significantly higher than those derived from the liquid growth media (Figure 3.2B). Overall, fungal

strains grown in CF28ST media produced significantly higher amount of crude extracts compared to the four other media (Figure 3.2B).

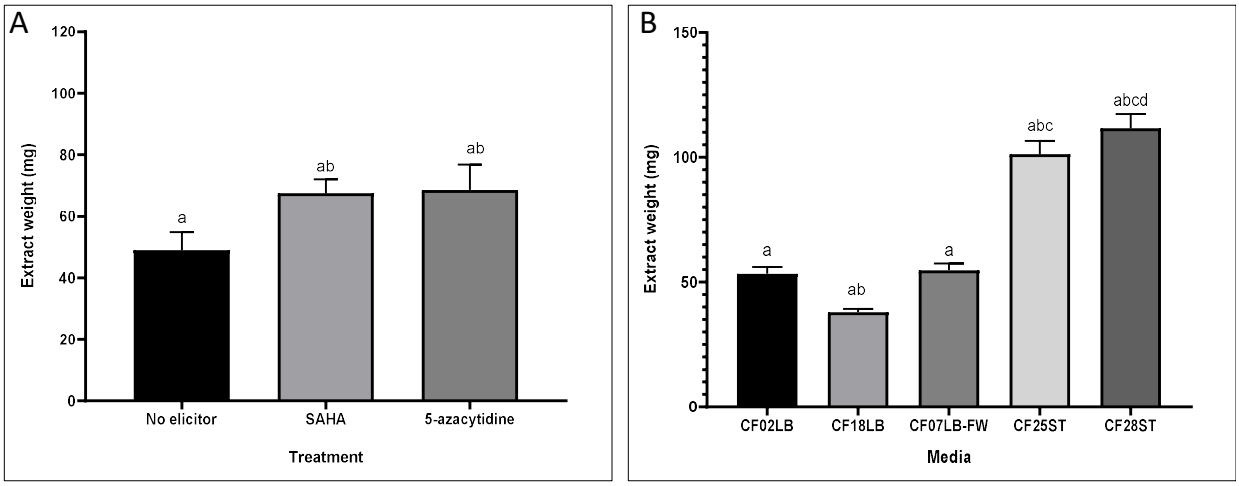


Figure 3:2 A comparison of mass of crude extracts from two fermentation experiments: (A) Fungal strains grown in the two liquid media in the presence and absence of the two chemical elicitors and (B) strains grown in three liquid media (CF02LB, CF18LB and CF07LB) and two solid media (CF25ST and CF28ST). Different lowercase letters (a–d) among the treatments indicate statistically significant differences between means by one-way ANOVA with Tukey–Kramer multiple comparison test ($p < 0.05$).

Other than the changes in the yield of extracts obtained from the same fungal strains under different growth conditions, a number of other observable changes were apparent during the growth and extracts generation from certain fungal strains. For example, there were morphological differences for strain F6932 when grown in three different media namely; malt extract agar (MEA), potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) (Figure 3.3A). For strain F4437, growth in CF02LB media in the presence and absence of the two chemical elicitors resulted in observable differences in the colour of the resultant crude extracts. There was an increase in the colour intensity in the extracts from F4437 when grown in the presence of 50 µM 5-azacytidine compared

with the extracts obtained when the fungus grown in the presence of 100 µM SAHA and in the absence of any of the two chemical elicitors (Figure 3.3B).

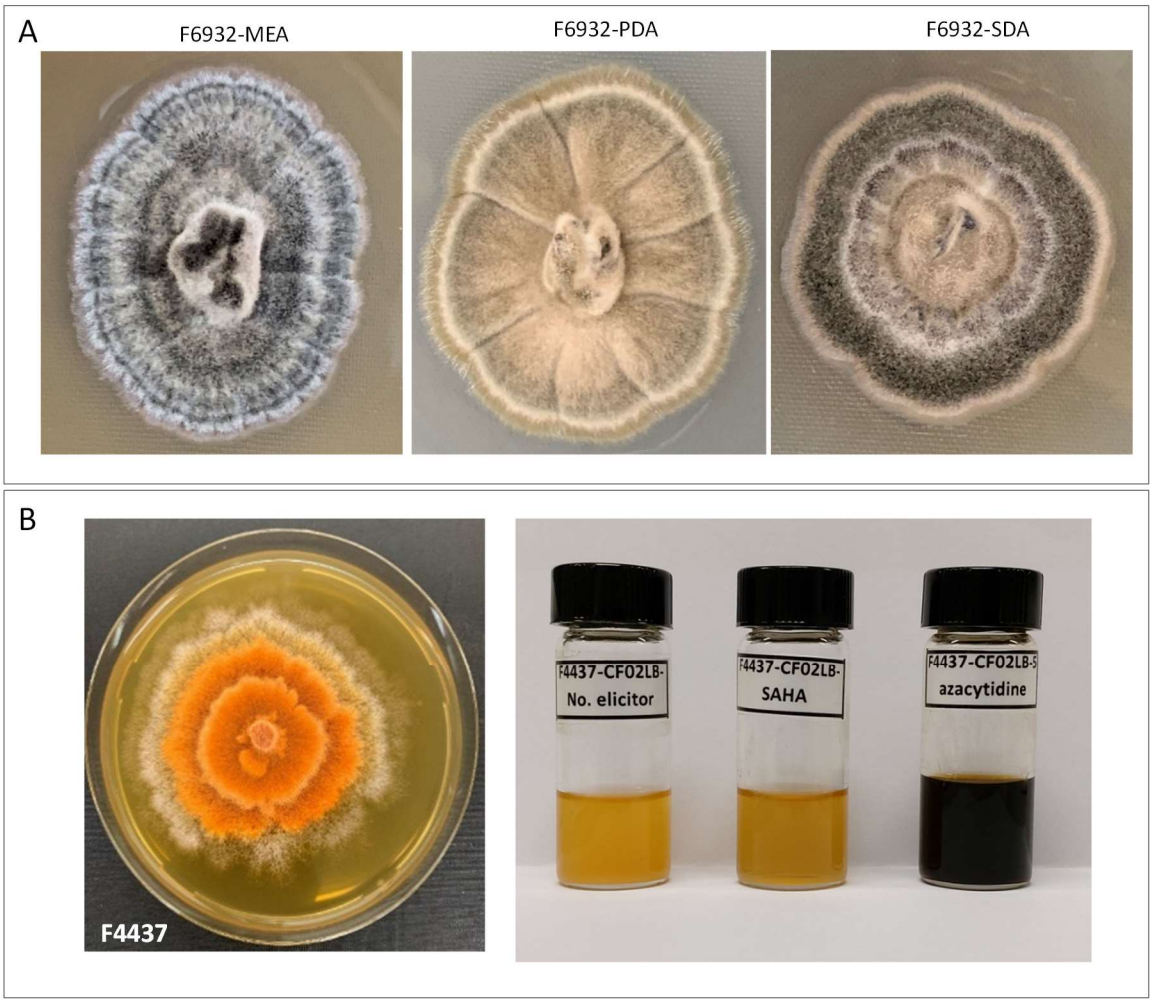


Figure 3:3 Observable changes in the colony morphology and crude extracts color from fungal strains under different growth regimes: (A) Morphological characteristics of *Lophiotrema* sp. F6932 grown in three common media MEA, PDA and SDA (B) Differences in the colour intensity of crude extracts of *Coniella heterospora* F4437 grown in CF02LB media in the presence and absence of chemical elicitors.

3.3.2: Antimicrobial and cytotoxic activity of fungal extracts

Fungal endophytes are renowned for their capacity to synthesize bioactive secondary metabolites. In this study, phylogenetic analysis of 222 fungal strains resulted in 146 fungal strains being selected for fermentation in two liquid media (CF02LB and CF18LB) for the assessment of bioactive metabolites production. Furthermore, in order to assess the effect of chemical elicitation and variation of growth media on the biosynthesis of bioactive secondary metabolites, the 146 strains were further divided into two groups. The first group of 45 strains was grown in the same two liquid media (CF02LB and CF18LB) in the presence of two chemical elicitors; 50 µM of 5-azacytidine and 100 µM of SAHA. The second group of 101 strains were grown in three additional media (CF07LB, CF25ST and CF28ST). The selection of the strains that would go into the second round of fermentation was based firstly on, the overall workload that would entail the two experiments and secondly, the representation of the main taxonomic groups of fungal strains in the two experiments.

For the 45 fungal strains that were grown in two liquid media in the presence and absence of the two chemical elicitors, a total of 8 strains revealed antimicrobial activity against at least one of the four studied microbial pathogens (average % growth inhibition ≥ 50). Significantly, four of these strains (F4434, F4437, F4448 and F6932), an equivalent of 50% of the active strains, revealed antimicrobial activity only when grown in the presence of chemical elicitors (Figure 3.4). For cytotoxic activity, out of the 45 strains that were grown in the presence and absence of the two chemical elicitors, 12 fungal strains exhibited cytotoxic activity against at least one of the three tested cell lines. Notably, chemical elicitation results revealed that five of these strains (F4437, F4446, F6731, F6932 and F10764) presented cytotoxic activities when grown exclusively in the

presence of chemical elicitors (Figure 3.4). One-way ANOVA was conducted to compare the activity of extracts derived from 13 fungal strains grown in CF02LB media in presence and absence of chemical elicitation. Comparisons were made between no elicitor, 5-azacytidine and SAHA groups for each of the test microbial pathogens and cancer cell lines (Appendix II). Interestingly, there were no significant difference in the inhibitory activities between the extracts from fungal strains grown in the presence of either of the two chemical elicitors and those from strains grown in the absence of chemical elicitation. For example, for *S. aureus,* there was no significant difference in the inhibitory activity at $p<.05$ level for the three treatment groups $F(2, 12) = 5.552$, $p = .076$. A summary of the Analysis of Variance results for the rest of the antimicrobial and cytotoxic screens is given on Appendix II.

#	Strain ID	Antimicrobial Screens								Cytotoxicity Screens					
		S.aureus		*K.aerogenes*		*C. albicans*		*A.fumigatus*		A549		MIA PaCa-2		PANC-1	
		-Eli	*Eli*	*-Eli*	*Eli*	*-Eli*	*Eli*	*-Eli*	*Eli*	*-Eli*	*Eli*	*-Eli*	*Eli*	*-Eli*	*Eli*
1	F2682									■		■	■	■	■
2	F4434		■				■			■	■	■	■	■	■
3	F4437		■								■		■		■
4	F4446												■		■
5	F4448		■				■			■	■	■	■	■	■
6	F4451	■	■					■	■			■	■		
7	F6724							■		■		■	■		
8	F6731										■		■		■
9	F6916	■	■												
10	F6932		■								■		■		■
11	F10318		■			■				■	■	■	■	■	■
12	F10764												■		
13	F12124											■	■	■	■

■ Active: Average % growth inhibition ≥ 50 *Eli* = Presence of chemical elicitor
Inactive: Average % growth inhibition < 50 - *Eli* = Absence of chemical elicitor

Figure 3:4 Antimicrobial and cytotoxic hits from 45 fungal strains grown in two media in the presence and absence of two chemical elicitors.

Elicitor-derived extracts from F4437, F6731 and F6932 exhibited cytotoxic activities against all the three cell lines evaluated while extracts from F4446 displayed cytotoxic activity against MIA PaCa-2 and PANC-1 cell lines, and extracts from F10764 exhibited inhibitory activity against MIA PaCa-2 cells only (Figure 3.4). The overall distribution of antimicrobial and cytotoxic hits from 45 fungal strains grown in the presence and absence of the two chemical elicitors are shown in Figure 3.5

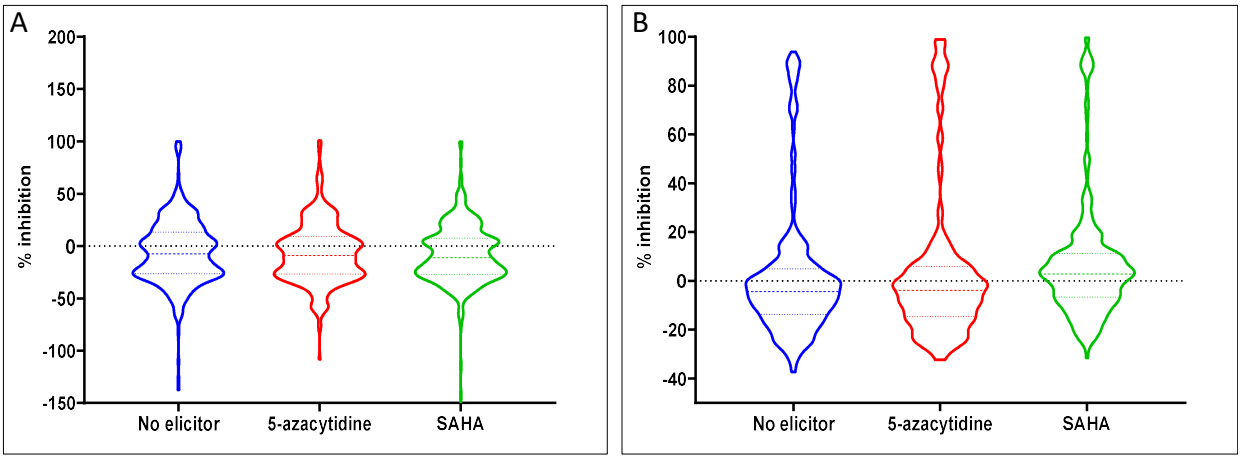


Figure 3:5 Distribution of antimicrobial hits (A) and cytotoxic hits (B) from 45 fungal strains grown in the presence and absence of the two chemical elicitors.

For the 101 fungal strains that were grown in five different media; two initial media (CF02LB and CF18LB) and three additional media (CF07LB, CF25ST and CF28ST), there were a total of 47 strains that exhibited inhibitory activity against at least one of the tested panel of microbial pathogens and cancer cell lines (Figure 3.6). Notably, 16 of these fungal strains, an equivalent of 34% of all the active strains, exhibited inhibitory activity only when grown in the three additional media. In other words, compared with the use of two initial growth media (CF02LB and CF18LB), the growth of fungal strains in three additional media (CF07LB, CF25ST and CF28ST) resulted in

16 additional hit strains (Figure 3.6). Among the three additional fermentation media, CF25ST had the highest number of active strains (28 strains) followed by CF28ST and CF07LB with 24 and 22 active strains respectively (Figure 3.6). One-way ANOVA was conducted to compare the cytotoxic activity of extracts derived from the 47 fungal strains grown five different types of media. (Appendix III). There was no significant difference in the inhibitory activities between the extracts from fungal strains grown in five different media against PANC-1 cells. However, there was a signifant difference in the inhibitory activity among the different types of fermentation media at $p < .05$ level for five groups $F (4, 46) = 4.42$, $p = .006$ for in A549 cells and $F (4, 46) = 3.26$, $p = .02$ for MIA PaCa-2 cells. For A549 screen, Tukey's post hoc test results revealed that extracts from CF07LB media had significantly lower inhibitory activity (M=11.44, SD=38.18) compared to those from CF02LB (M=28.44, SD=40.70). For MIA PaCa-2 screen, the post hoc test results revealed that extracts from CF18LB media had significantly lower inhibitory activity (M=25.68, SD=34.90) compared to those from CF02LB (M=41.86, SD=39.36). For MIA PaCa-2 screen (Appendix III).

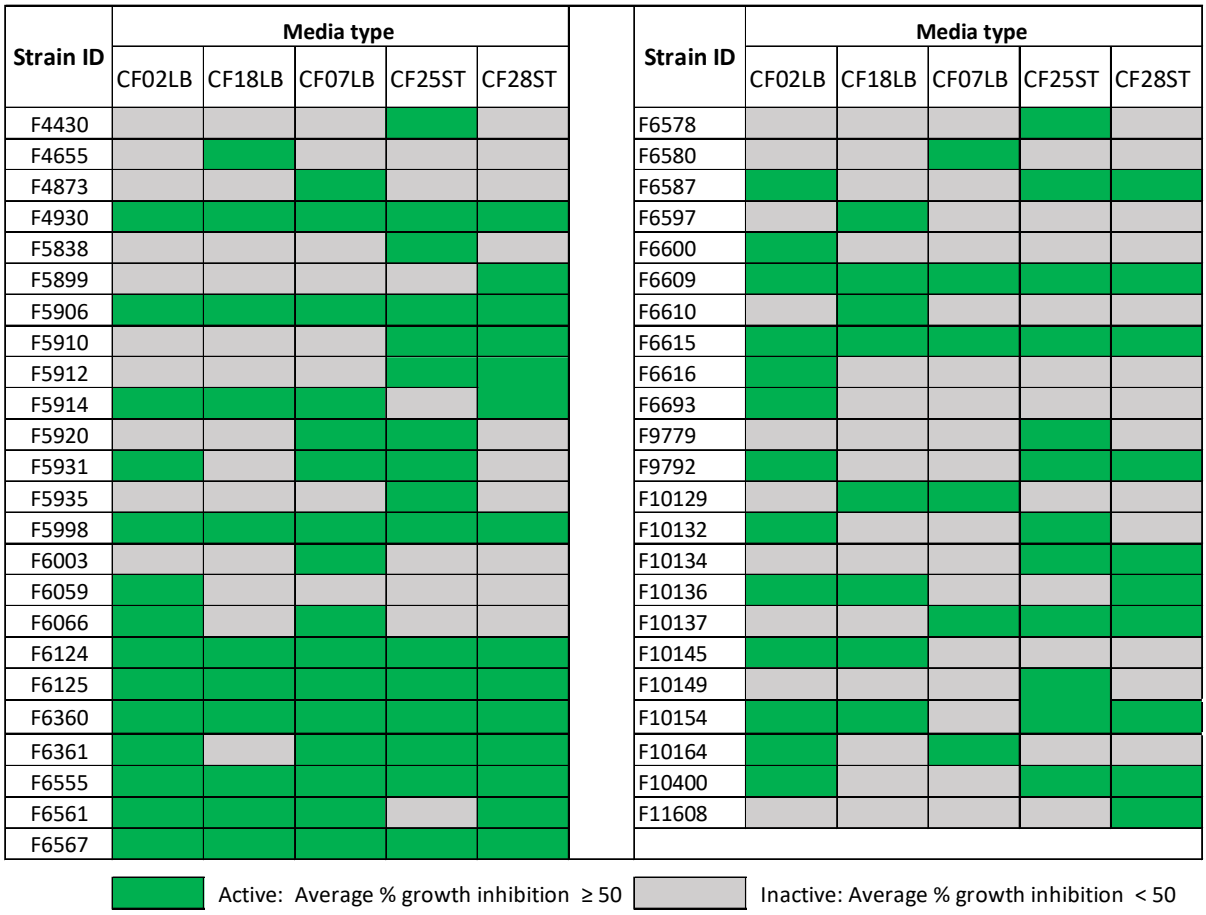


Figure 3:6 Hits from 101 fungal strains that were grown two initial media (CF02LB, CF18LB) and three additional media (CF07LB, CF25ST and CF28ST).

The overall distribution of antimicrobial and cytotoxic hits from the 101 fungal strains that were grown in five different media are represented by violin plots in Figure 3.7.

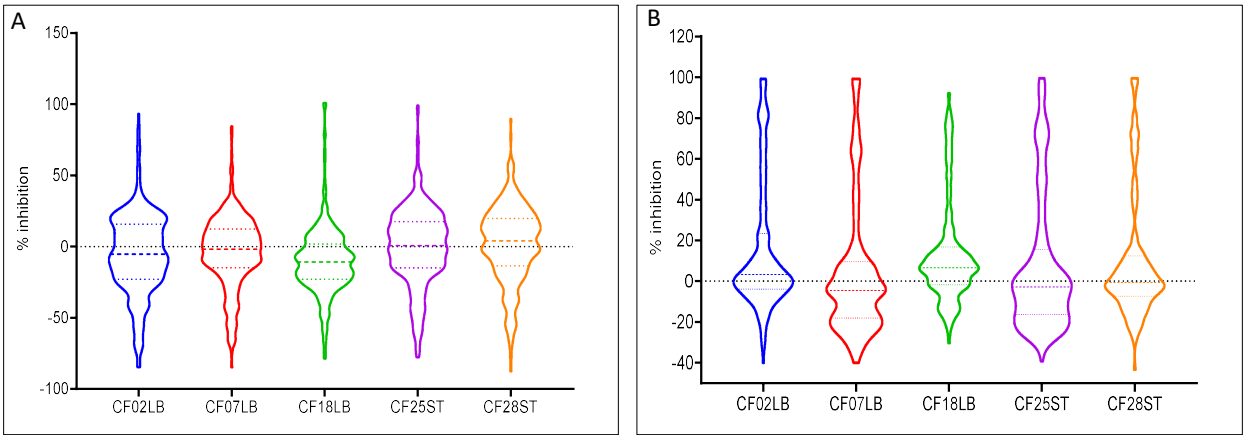


Figure 3:7 Distribution of antimicrobial hits (A) and cytotoxic hits (B) from 101 fungal strains grown in five different media.

Following dose-response testing for selected extracts that revealed promising activities during the primary screening, in a number of instances, there were observable differences in the expression of inhibitory activities by crude extracts derived from the same fungal strains when grown in the presence or absence of chemical elicitor or in different growth media. Figure 3.8 and Figure 3.9 shows some examples of differential expression of antimicrobial and cytotoxic activities respectively from extracts generated from selected fungal strains subjected to varied fermentation regimes.

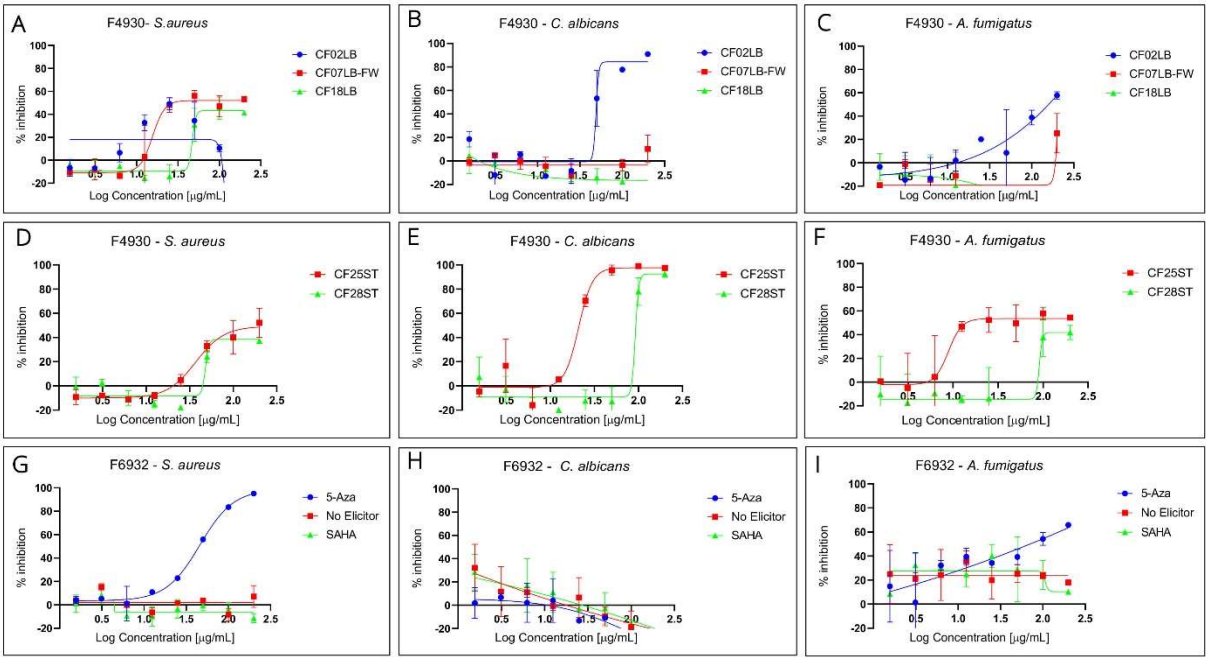


Figure 3:8 Differential expression of antimicrobial activity of extracts derived from two fungal strains against three test pathogens. [A – C] show the activity of extracts from F4930 grown in three different liquid media CF02LB, CF07LB-FW and CF18LB, [D – F] show activity of extracts derived from the same strain grown in two solid media, CF25ST and CF28ST, while [G – I] show the activity of extracts from strain F6932 grown in CF02LB media in the presence and absence of the chemical elicitors.

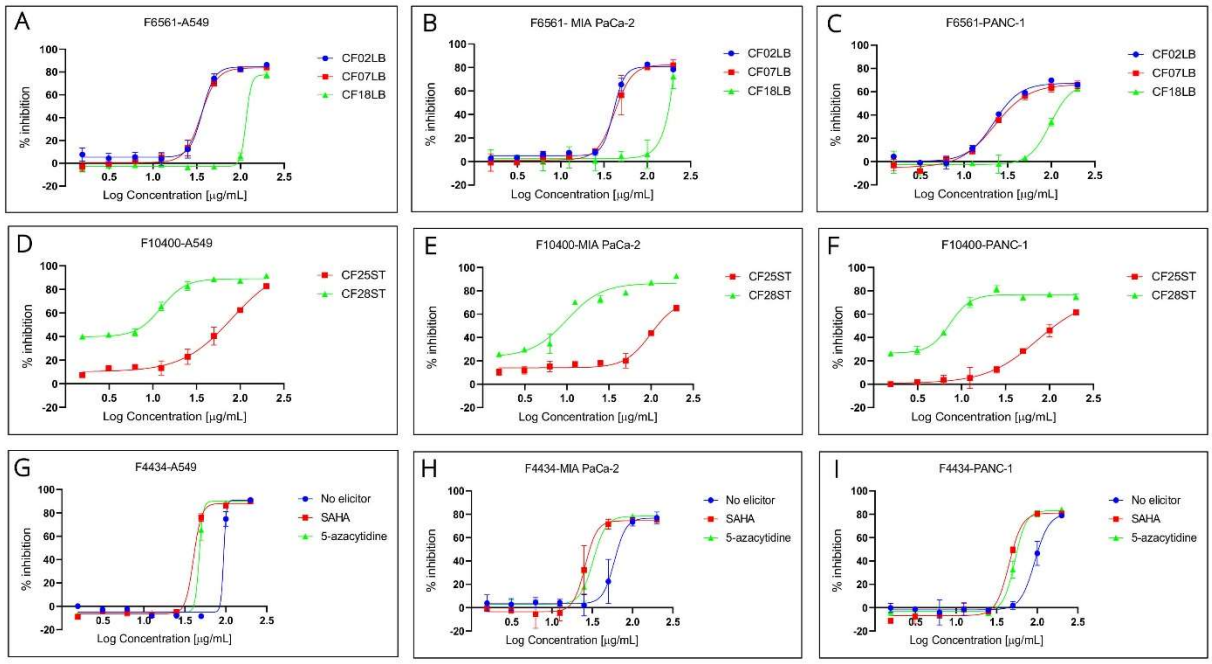


Figure 3:9 Differential expression of cytotoxic activity of extracts derived from three fungal strains against three cancer cell lines A549, MIA PaCa-2 and PANC-1. (A - C) show the activity of extracts from F6561 grown in three different liquid media CF02LB, CF07LB and CF18LB, (D - F) show activity of extracts derived from F10400 grown in two solid media CF25ST and CF28ST while (G - I) show the activity of extracts from strain F4434 grown in CF02LB in the presence and absence of the chemical elicitors (Gakuubi et al., 2022a).

In summary, antimicrobial and cytotoxic screening results revealed that 63 endophytic fungal strains representing 43% of all the fungal isolates screened had inhibitory activity. Out of these, 34 strains revealed exclusive cytotoxic activity against one or more of the studies cancer cell lines, 10 fungal strains exhibited exclusive antimicrobial activity against at least one tested microbial pathogens, while 19 strains exhibited both antimicrobial and cytotoxic activity against at least one of the tested panel of microbial pathogens and cells lines, respectively. The active fungal endophytes were distributed into 11 orders with *Diaporthales* accounting for the highest number of active strains (33%; 21 strains) followed by *Hypocreales* and *Glomerellales* which accounted

for 17% and 14% of all the active fungal strains respectively. The Genera *Diaporthe* and *Colletotrichum* accounted for the highest number of bioactive fungal strains with 30% and 14% of all the active fungal strains belonging to the two genera respectively. Figure 3.10 shows the phylogenetic relationship between different bioactive fungal endophytes.

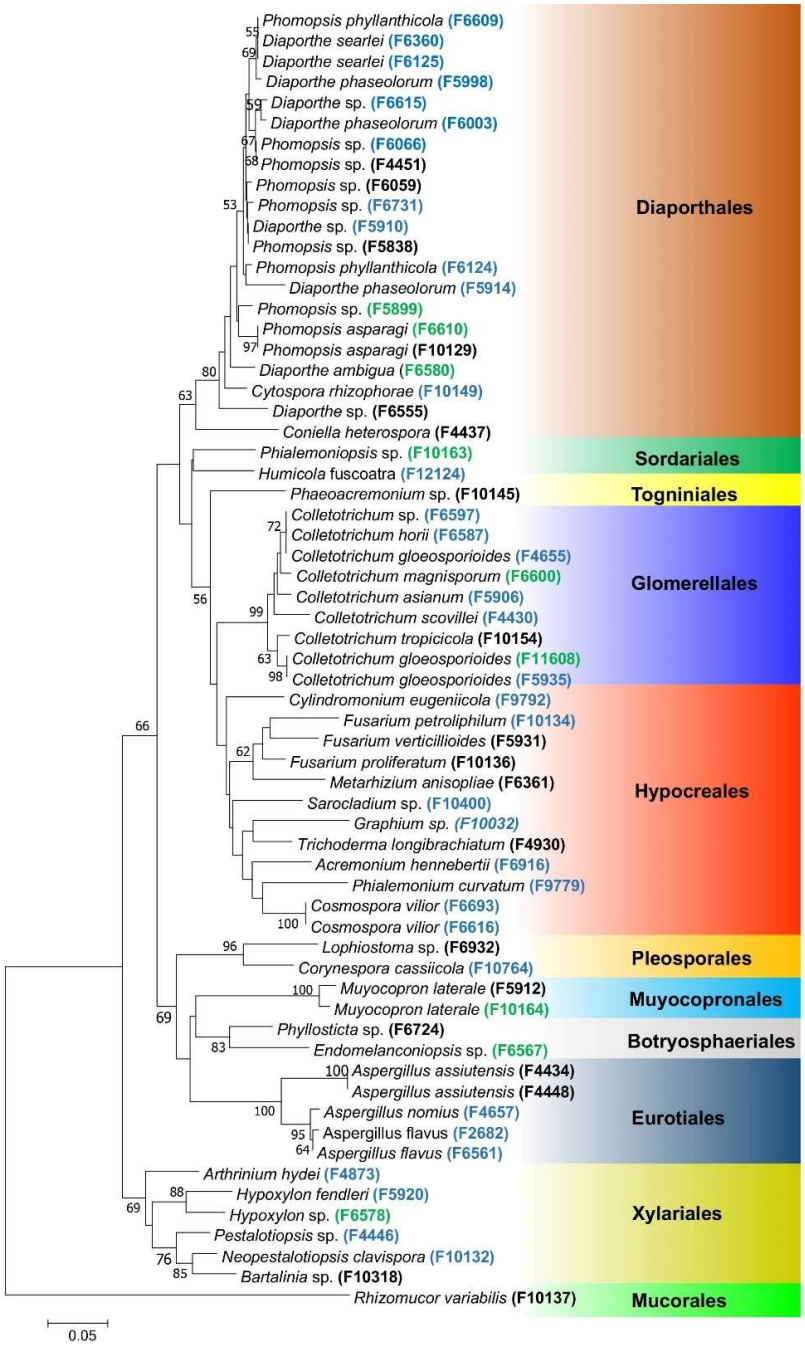


Figure 3:10 Phylogenetic tree of 63 active fungal strains based on internal transcribed spacer (ITS2) rDNA gene sequences. The tree was constructed using the neighbor-joining method with MEGA 7 software (Bootstrap value = 500; values below 50% are not shown). Strains whose ID codes are shown in green color exhibited antimicrobial activity, those shown in blue revealed cytotoxic activity while those shown in black revealed both inhibitory activities.

3.4: Discussion

Fungal endophytes are prolific producers of bioactive secondary metabolites including antimicrobial and cytotoxic compounds (Gupta & Shukla, 2020; Nisa et al., 2015). The absence of host plant defense reaction against fungal endophytes in addition to ability of some fungal endophytes to produce secondary metabolites associated with their hosts plants points to an interesting co-evolutionary history established over many years (Kumara et al., 2014). Because of this close fungus-host relationship, fungal endophytes occupy a distinctive ecological niche that allows them to produce highly specialized secondary metabolites (Caruso et al., 2020). In recent years, these microorganisms are increasingly receiving a lot of interest for their role in natural product discovery (Khiralla et al., 2016; Manganyi & Ateba, 2020; Patil et al., 2016). However, a number of limiting factors such as low yields of targeted molecules coupled with the fact that most of their biosynthetic gene clusters (BGCs) are never expressed under standard laboratory growth conditions limits the realization of full biosynthetic potential of these microbial factories (González-Menéndez et al., 2018). To overcome such challenges, numerous strategies including co-culture, the use of chemical elicitors, variation of various culture growth conditions and genetic engineering of the strains of interest among other strategies have been employed (Baral et al., 2018; Gakuubi et al., 2021; Kjærbølling et al., 2019).

In the current study, 146 fungal endophytes selected from among 222 strains following phylogenetic analysis were first grown in two liquid media for generation of crude extracts. Subsequently 45 of these strains were grown in the same two liquid in the presence of 50 µM of 5-azacytidine and 100 µM of suberoylanilide hydroxamic acid (SAHA). The second group of 101 fungal strains were grown in three addition media; the aim being to assess the media that would

promote the biosynthesis of active secondary metabolites. Deletion of histone deacetylase-encoding gene *HdaA* in *Aspergillus nidulans* strain A89 resulting in induced biosynthesis of sterigmatocystin and penicillin provided the first evidence that remodeling of chromatin landscape has an influence on the regulation of fungal secondary metabolism (Shwab et al., 2007). Since then, epigenetics has emerged as an important tool for unlocking fungi silent SMs biosynthetic gene clusters. In the current study, the crude extractable yields were significantly higher for fungal strains that were grown in the presence of the two chemical elicitors in comparison with those obtained from strains grown without the elicitors (Figure 3:11). These findings are in agreement with results obtained from a previous study where addition of SAHA to the cultures of an endophytic fungi, *Botryosphaeria mamane* at the seed and production stages and at the production stage only resulted in an increase in the yield of crude extracts by 50% and 32% respectively (Triastuti et al., 2019). Furthermore, growth of *B. mamane* in the presence of SAHA resulted in an induced biosynthesis of eight new metabolites that were not detected in the control cultures (Triastuti et al., 2019). Growth of numerous fungal strains in the presence of epigenetic modifiers, mainly histone deacetylase and DNA methyltransferases inhibitors, has been shown to have a profound effect on fungal growth and development and consequently their secondary metabolism (Ma et al., 2018). HDCA and DNMT have been employed to perturb fungal biosynthetic machinery leading to enhanced quantity of the resultant extracts in addition to induced biosynthesis of new chemical entities that are not produced in cultures grown in the absence of the elicitors (Dwibedi et al., 2019; González-Menéndez et al., 2019; Magotra et al., 2017). Chemical epigenetic manipulation of marine-derived fungus *Aspergillus versicolor* using histone deacetylase inhibitors namely SAHA, SBHA, nicotinamide, and sodium butyrate or/and DNA methyltransferase

inhibitors 5-Aza and 2′-deoxy-5-Aza significantly increased the chemical diversity of the SMs from this fungus when grown in Czapek-Dox medium (Wu et al., 2020). In another study, epigenetic treatment of endophytic fungus *Nigrospora sphaerica* with hydralazine hydrochloride and quercetin resulted in enhanced antimicrobial activity (Ramesha et al., 2021). The enhance antibacterial activity was attributed to induced production of otherwise cryptic compounds by the fungus when subjected to chemical epigenetic manipulation. In the current study, enhanced antimicrobial and cytotoxic activities were observed in numerous elicitor-derived extracts compared with the control. For example, crude extracts from strain F6932 when grown in CF02LB media in the presence of 5-azacytidine revealed strong antibacterial activity against *S. aureus.* Extract from the same fungal strain grown in the same media in the absence of chemical elicitors and in the presence of SAHA did not shown any antibacterial activity. Furthermore*,* extracts of the same strain grown in the second liquid media; CF18LB in the presence and absence of the two chemical elicitors did not show any antimicrobial activity. In the current study, preliminary studies had been carried out on the potential inhibitory activities of the two chemical elicitors on the the panel of microbial pathogens and cancer cell lines. Dose-response testing of antimicrobial and cytotoxic activities of the two chemical elicitors with a starting concentration of 50µM for 5-azacytidine and 100 µM for SAHA was carried out (Appendix IV). On the basis of the results obtained, it can be concluded that based on the estimated maximum amount of the chemical elicitor that would be present in any extract, any inhibitory activities in elicitor-derived crude extracts cannot be attributed to the presence of the elicitors in the extracts. Moreover, if the inhibitory activities of elicitor-derived extracts were as a result of the presence of the chemical elicitors in

the crude extracts, then it would be expected that all the extracts derived from 45 fungal strains grown in the presence the elicitors would exhibit inhibitory activities which is not the case.

While manipulation of fermentation media composition has traditionally been used as a tool to optimize the production of already known metabolites, this technique has recently found widespread application as a mean of awakening cryptic or poorly expressed BGCs in fungal endophytes (Gakuubi et al., 2021). Variation of media type and composition falls within the framework of the one strain many compounds (OSMAC) approach (Bode et al., 2002). OSMAC entails a systematic manipulation of various culture growth conditions with an aim of identifying the growth conditions that enhances the diversity and quantity of bioactive secondary metabolites (Bills et al., 2008; VanderMolen et al., 2013). Thus, variation of media type and composition can be employed to enhance the yield of a target bioactive compounds (García-Kirchner et al., 2005) or as an approach to maximize the chemical diversity from an individual strain or selected strains (Elias et al., 2006; VanderMolen et al., 2013). In the current study where 101 fungal strains were grown in five different media, the weight of crude extractables derived from the two solid media were significantly higher in comparison with those from the three liquid media cultures (Figure 3:12). Overall, fungal strains grown in CF28ST produced significantly more extracts compared to those derived from strains grown in the other four media types (Figure 3:13). Fungal strains grown in solid media have been found to produce crude extracts with masses that are one to two orders of magnitude higher than those derived from the same strains grown in liquid media (VanderMolen et al., 2013). A study focusing on isolation of aphid antifeedant from an endophytic fungus *Trichoderma* sp., found out that solid media (Pearl millet and corn-based media) produced higher

extract yields in comparison with liquid media such as potato dextrose broth (PDB) and Sabouraud dextrose broth (SDB) (Kaushik et al., 2020).

There was an increase in the number of hit strains as a results of growth of fungal strains in two chemical elicitors. An additional 4 and 5 hit strains showing antimicrobial and cytotoxic activities respectively. With regard to the use of three additional media, 9 and 16 additional hit strains with antimicrobial and cytotoxic activities respectively were discovered (Figures 3.4 and 3.6). These findings thus showed that chemical elicitation and variation of media composition are potential avenues for enhancing SMs biosynthesis in fungal endophytes. Among the strains whose inhibitory activities were uncovered following chemical elicitation is strain F6923 grown in the presence of 5-azacytidine (Figure 3.4). For the media diversification experiment, strain F5912 revealed inhibitory activity only when grown in two solid media CF25ST and CF28ST (Figure 3.6). These two strains are among the fungal strains that were progressed to large scale fermentation for compound isolation and structure elucidation following the identification of active constituents of interest during the chemical dereplication. In the last two decades, an array of interesting fungal secondary metabolites have been discovered from a wide range of fungal strains through the use of chemical elicitation using mainly HDAC and DNMT inhibitors (Pillay et al., 2022; Toghueo et al., 2020). This approach has gain popularity largely because of its simplicity compared to the more complex genome editing techniques. However, the use of chemical inhibitors of epigenetic modification has been shown to so often result in pleiotropic activation making it difficult to link a newly identified secondary metabolites or phenotype with any particular gene cluster (Gakuubi et al., 2021; Pillay et al., 2022). Further studies including but not limited to transcriptomic analysis may be required in order to assess the genes that are upregulated in the presence of specific

epigenetic modifier compounds. In the current study, further studies were required in order to ascertain whether the observed enhancement of inhibitory activities through epigenetic modification and variation of fermentation media were as a result of quantifiable changes in the biosynthesis of metabolites also produced in control cultures or if it was as a result of induced production of new chemical entities not produced in control cultures. Furthermore, other than its effects on secondary metabolism, chemical epigenetic manipulation of fungi has been reported to affect the fungal growth and virulence (González-Menéndez et al., 2020; Liu et al., 2015).

Among the liquid media, CF02LB performed better than the other two liquid media in enhancing the production of active crude fungal extracts while for the solid media, CF25ST and CF28ST performed more or less the same. From both the chemical elicitation and multiple fermentation media experiments, the highest antimicrobial and cytotoxic hits were observed in *A. fumigatus* and MIA PaCa-2 screens, respectively. While a good number of extracts revealed strong growth inhibitory activity against *S. aureus*, only a single extract derived from F10129 grown in CF18LB media revealed growth inhibitory activity (average % growth inhibition $\geq$ 50) against *K. aerogenes,* a Gram negative bacteria. However, this sample was not progressed for further studies because the dose-response testing did not produce promising results. The observed disparity in the activity of fungal extracts between two groups of bacteria can be attributed to the distinctive structure of Gram negative bacteria's cell wall principally the presence of the outer membrane which makes Gram negative bacteria more resistance to antimicrobial agents in comparison with the Gram positive bacteria (Yogabaanu et al., 2017). Low activity of extracts derived from fungal endophytes against Gram negative bacteria have been reported previously (An et al., 2020; Marcellano et al., 2017).

In total 63 endophytic fungal strains representing 43% of all the fungal isolates screened reavealed inhibitory activities. Out of these, 34 strains revealed exclusive cytotoxic activity against one or more of the studies cancer cell lines, 10 fungal strains exhibited exclusive antimicrobial activity against at least one tested microbial pathogens, while 19 strains exhibited both antimicrobial and cytotoxic activity against at least one of the tested panel of microbial pathogens and cells lines, respectively. One possible explanation for observed inhibitory activity of some fungal metabolites to bacteria, fungi and cancer cell line is that such metabolites share common targets and mechanisms of action i.e., they target metabolic pathways or cellular process that are common to all these cell types. These may include mode of actions such receptor-mediated or non-receptor mediated disruption of cell membrane, induction of DNA/RNA damage, efflux pump inhibition and inhibition of cellular growth by preventing the activation some proteins among others mechanisms (Chifiriuc et al., 2022). There is also the prospect of a natural product having the same targets and mechanisms displaying both antimicrobial and anticancer activities (Gao et al., 2020). A good example is the antineoplastic antibiotics such as anthracyclines, peptides, and quinolones many of which interact with topoisomerases both in prokaryotic and eukaryotic cells (Pommier et al., 2010).

While the largest fraction of the characterized fungal strains in the current study belong to the order *Glomerellales*, most of the active fungal strains were from the order *Diaporthales* consisting of mainly *Diaporthe* and its anamorph *Phomopsis*. *Diaporthe* is the teleomorph state while *Phomopsis* is the anamorph state. However, the divergent views on which of the two generic name

should be used continues to date (Udayanga et al., 2011; Xu et al., 2021). Due to the fact that *Diaporthe* was cited earlier than and also represents majority of the species in nature in comparison with *Phomopsis*, many mycologist suggest the use of the *Diaporthe* as the generic (Rossman et al., 2015; Udayanga et al., 2011). However, other authors especially those focusing on the role of the members of this the genus as phytopathogens use the anamorph name on the basis that this state is the most widely applied to many plant diseases (Udayanga et al., 2011). Still many authors continue to report the two as if they were distinct and separate species adding much to the confusion (Duan et al., 2019). In this study, we have adopted to use *Diaporthe* as the generic name in presenting the diversity results of this study among other discussion that relate to this Genera. However, for the construction of phylogenetic trees, we have retrained the two morphs based on the identity of the individual fungal isolates on the basis of BLAST searches on NCBI. *Diaporthe* have been reported to be not only among the most abundant members of endophytic fungal community but also producers a wider range of bioactive secondary metabolites (Chepkirui & Stadler, 2017; Xu et al., 2021). In the current study, 30% of the all the active fungal strains belong to this Genus with majority of these exhibiting exclusive cytotoxic activity and a few isolates showing exclusive antimicrobial or both of the inhibitory activities. These results are consistence with a review study whose analysis of the bioactive secondary metabolites from endophytic fungi from *Diaporthe* between 2010-2019 revealed that most of the identified bioactive compounds had cytotoxic activities followed with those exhibiting antibacterial and antifungal activities respectively (Xu et al., 2021). Moreover, out of the identified bioactive compounds, majority (64%) were from the polyketides group of chemical family (Xu et al., 2021). Even for fungal endophytes isolated from individual plant species, *Diaporthe* has been cited as a dominant

bioactive fungal genus. For example, a study focusing on the diversity and bioactivity of endophytic fungi isolated from *Distylium chinense* revealed that *Phomopsis* sp., accounted for the highest number of bioactive fungal isolates with majority of them exhibiting antibacterial, followed by antioxidant and anticancer activities respectively (Duan et al., 2019).

3.5: Conclusion

The classical bioassay-guided fractionation method is one of the most widely used approach in the discovery of bioactive natural products. This approach typically involves step-by-step separation of components from active crude extracts on the basis of their physiochemical characteristics followed by an assessment of their inhibitory activities and more rounds of separation of the active constituents and bioassays. Primary screening of the inhibitory activity of crude extracts is thus an important step that allows for the identification of 'hits' that warrants further investigations. From this step, only samples exhibiting a set threshold of inhibitory activity (average % inhibition became ≥ 50 in our case) are progressed to the next step. In this study, a total of 146 fungal endophytes were subjected to small scale fermentation for generation of extracts. All the fungal strains were initially grown in two liquid media CF02LB and CF18LB. Subsequently, the fungal strains were divided into two groups with the first group of strains grown in the presence of two chemical elicitors; 5-azacytidine and SAHA. The second group of strains were grown in three additional media. All the extracts generated were screening for their inhibitory activity. Antimicrobial activity assays were conducted against *Staphylococcus aureus* (ATCC 25923), *Klebsiella aerogenes* (ATCC 13048), *Candida albicans* (ATCC 10231) and *Aspergillus fumigatus* (ATCC 46645) while cytotoxic assays were conducted against three cell lines PANC-1, MIA PaCa-2 and A549. The yield of crude extracts was generally higher for fungal strains grown in the

presence of the chemical elicitors while generally solid media resulted in higher yields extractable yields when compared with liquid media. Out of the studied fungal endophytes, 63 strains representing 43% of all the tested strains exhibited inhibitory activity. Out of these, 34 and 10 fungal strains revealed exclusive cytotoxic and antimicrobial activities respectively while 19 strains exhibited both inhibitory activities. The genera *Diaporthe* and *Colletotrichum* accounted for the highest number of bioactive fungal strains with 30% and 14% of all the active fungal strains coming from the two genera respectively. The highest number of antimicrobial and cytotoxic hits were observed in *A. fumigatus* and MIA PaCa-2 screens respectively while only a single hit was observed against *K. aerogenes.* Significantly, the use of chemical elicitors 5-azacytidine and suberoylanilide hydroxamic acid (SAHA) and variation of fermentation media resulted in the discovery of more bioactive strains. This is an indication therefore that these two approaches can be used as an efficient tool for initial screening for bioactive secondary metabolite especially where a large number of fungal strains are involved before selection of strains of interest for further detailed studies.

Chapter 4: Chemical analysis of fungal secondary metabolites

4.1: Introduction

Microbial natural products or secondary metabolites (SMs) have played and continue to play an important role in drug discovery and development. One of the well-known natural product discoveries which ultimately revolutionized drug discovery was penicillin discovered from *Penicillium notatum* by Alexander Fleming in 1929. This discovery heralded a new era of natural product-based drug discovery resulting in intensive interest in microorganisms as a source of novel bioactive agents (Dias et al., 2012). This was followed by a period in which thousands of mostly soil microorganisms were screened resulting in discovery and commercialization of many classes of therapeutic agents with the vast majority of the microbial secondary metabolites known today discovered over a period of a few decades (Monciardini et al., 2014). The peak of this era was a period from 1950 to 1960 that has been christened 'the golden age of antibiotic discovery' during which one-half of the drugs in used today were discovered (Davies, 2006). This period of great success in natural product research was thereafter followed by a period of decline with the waning interest from the pharmaceutical industries from the 1990s (Atanasov et al., 2021; Pollastro & Minassi, 2021). The decrease in interest in natural product-based drug discovery can be attributed to diminishing hit rates, increased in rediscovery of known compounds among other technical barriers resulting in exhaustion of the 'low-hanging fruits' (Figure 4.1). In recent years however, there has been a renaissance of interest in microbes as a source of bioactives and in drug discovery. The renewed interest in microbial-based drug discovery can be attributed to a number of factors; chief among them the technological and scientific advances that have been witnessed in chemical analytical techniques, development of high-throughput sequencing and genome mining, new strain

engineering and genome editing technologies coupled with the increasing incorporation of synthetic biology in natural product research (Bachmann et al., 2015; Keller, 2019). All these have added to the optimism of the dawning of a new era of natural products discovery that will ultimately open-up new opportunities for exploring the 'high-hanging' underexplored natural products fruits.

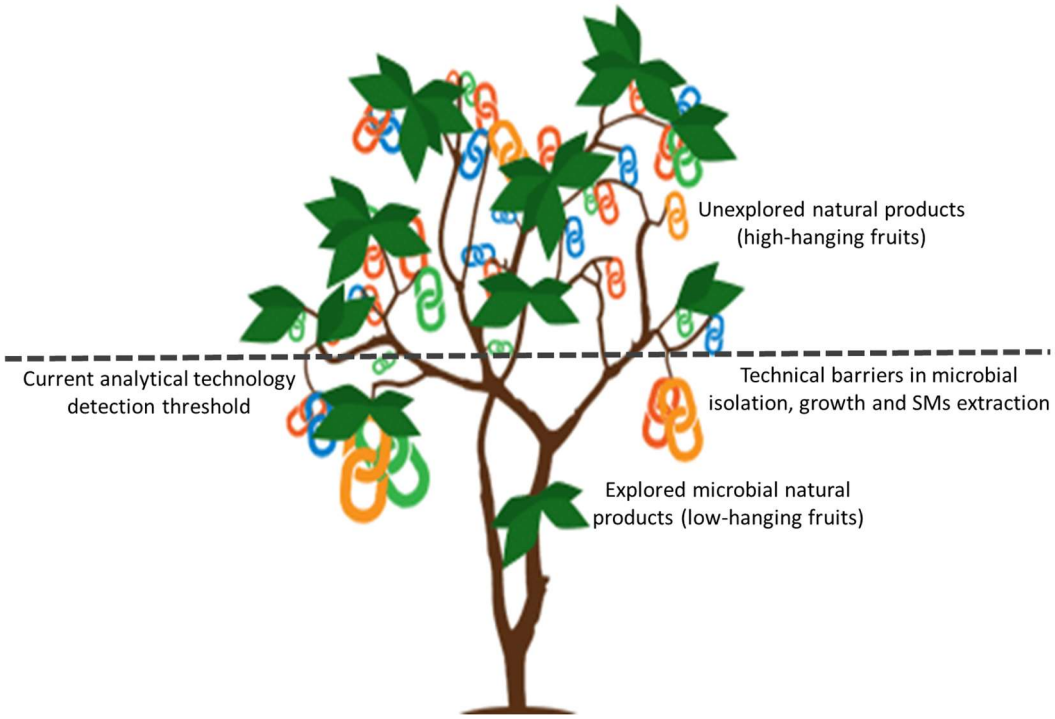


Figure 4:1 The concept of 'low-hanging' and 'high-hanging' fruits in reference to natural products discovery.

Fungal extracts are extremely complex given the enormous chemical diversity of the secondary metabolites that can be found within a single strain's extracts. For example, it has been estimated that fungi grown on rich media can produce in excess of 100 secondary metabolites (Nielsen & Larsen, 2015; Wolfender et al., 2015). Moreover, within a single extract can be found a wide range

of classes of secondary metabolites including but not limited to polyketides (PKs), non-ribosomal peptides (NRPs) and terpenoids (Nielsen & Larsen, 2015). Given this complex nature of fungal extracts, it is evident that no single analytical procedure or platform can achieve effective qualitative and quantitative analysis of fungal SMs. Consequently, numerous analytical separation techniques are employed followed by subsequent use of a wide range of spectroscopic analysis.

In this study, all the chemical analysis work including chemical analysis of bioactive extracts, compound isolation and structure elucidation were performed by Dr. Kuan Chieh Ching and Dr. Mario Wibowo both members of Dr. Yoganathan Kanagasundaram's Shared Analytics Group, Singapore Institute of Food and Biotechnology Innovation (SIFBI). In total, 28 fungal strains showing promising activity profiles were subjected to chemical dereplication following their screening for antimicrobial and cytotoxic activities. Based on the dereplication studies 8 promising fungal strains, 4 strains falling under the chemical elicitation experiment and 4 from the media variation experiment were subjected to large-scale fermentation for compound isolation and structure isolation (Table 4.1).

Table 4:1 Nine large-scale fermentation samples for compound isolation and structure elucidation.

S. No	Strain ID	Strain Identity	Media	Elicitor	Experiment
1	F4434	*Aspergillus assiutensis*	CF02LB	5-Azacytidine	Chemical elicitation
2	F4434	*Aspergillus assiutensis*	CF02LB	No elicitor	
3	F6916	*Acremonium hennebertii*	CF02LB	No elicitor	
4	F4448	*Aspergillus assiutensis*	CF02LB	No elicitor	
5	F6932	*Lophiotrema* sp	CF02LB	5-Azacytidine	
6	F5912	*Muyocopron laterale*	CF28ST		Media variation
7	F10154	*Colletotrichum tropicicola*	CF28ST		
8	F6610	*Phomopsis asparagi*	CF18LB		
9	F4930	*Trichoderma longibrachiatum*	CF05ST		

4.2: Materials and Methods

4.2.1: Chemical dereplication

One of the major hurdles encountered during the search for novel bioactive microbial natural products is re-isolation of already known bioactive compounds leading to wastage of both effort and resources (Carrano & Marinelli, 2015; Girão et al., 2019). Therefore, identification of known bioactive molecules from microbial extracts at an early stage through the process of chemical dereplication can help to speed up the process of discovery of new bioactive molecules saving both time and resources (Carrano & Marinelli, 2015). As such, to assess the extent to which the

observed inhibitory activities in crude extracts resulted from already known compounds, chemical dereplication need to be performed on the bioactive extracts. The purpose of chemical dereplication therefore is to facilitate rapid identification of known molecules which account for the activities observed in a particular extract.

In the current study, chemical dereplication of active extracts was performed according to the procedures described in literature (Butler et al., 2012). The dried extracts were soaked and resuspended with 20 mL MeOH, sonicated for 5 min and centrifuged to separate the insoluble from the soluble. The supernatants were transferred to 50 mL round bottom flasks and dried using Buchi rotary evaporator. The dried enriched samples were dissolved in 2.5 mL MeOH, centrifuged and the supernatants were then subjected to C_{18} reversed-phase preparative HPLC purification.

The separated 40 fractions (F39 and F40 contained 100 µg/mL and 200 µg/mL of the crude extracts respectively) arising from the chemical dereplication samples were subjected to antimicrobial and cytotoxic testing in order to identify the individual fraction(s) that were responsible for the observed activity in each of the active crude extracts. For each of the fraction, antimicrobial activity was evaluated at neat concentration and 10× dilution concentration with the fractions prepared as follows: A multidrop dispenser was used to add 10 µL of neat DMSO into the 96-deep well block containing the fractions (column 1-10). This was followed by 15 minutes of mixing at 40 rpm in a plate shaker. 90 µL of Milli-Q water was then added into each well followed by mixing in a plate shaker for a further 15 minutes. For antimicrobial screens, 5 µL aliquots of each of the prepared fractions was then transferred from the deep well block into 384-well clear flat bottom plates as the neat concentration of the fractions for the bioassay. To prepare for the 10× dilutions,

90 µL of 10% DMSO was added into 96-well round-bottom plates (column 1-10) using a Multidrop dispenser. This was followed by the addition of 10 µL of each of the prepared fractions from the deep well block and mixing using a semi-automatic 96-fold pipettor. 5 µL aliquots of the content from the 96-well round-bottom plates were then transferred into another set of 384-well clear flat bottom plates constituting 10× dilution of the fractions for the bioassay. For the cytotoxic assay, the reconstituted extracts were added to the cell-seeded plates after 24 hours of incubation. The rest of the antimicrobial and cytotoxic assays and associated controls were performed in a similar manner and conditions as described in Chapter 3 sections 3.2.3 and 3.2.4.

4.2.2: Compound isolation and structure elucidation

Following biological testing of the fractions arising from the chemical dereplication and analysis of the results, fungal strains whose extracts contained active constituents of interest were progressed to large-scale fermentation to obtain sufficient crude extracts for fractionation and structure elucidation of the purified active compounds using HR-MS/MS and 1D/2D-NMR techniques. Large-scale fermentation for compound isolation and structure elucidation was done by growing each of the fungal strain in 50 mL of the respective media in 250 mL flask with a minimum of 2 L of fermentation done for each fungal strain. Large-scale fermentation using liquid and solid media and isolation of crude fungal extracts were done according to the procedure described in section 3.2.1 and 3.2.2 respectively. Enriched sample obtained from *Lophiotrema* sp. F6932 was separated by the following condition (solvent A: H_2O + 0.1% HCOOH, solvent B: MeCN + 0.1% HCOOH; flow rate: 30-52 mL/min, gradient conditions: 85:15 isocratic for 5 min, 30 mL/min; followed by 85:15 isocratic for 5 min, 52 mL/min; 15% to 55% of solvent B over 30

min, 55% to 100% of solvent B over 22 min, and finally isocratic at 100% of solvent B for 10 min). to give 2.2 mg of palmarumycin CP_{30} (**1**) and 3.4 mg of palmarumycin C_8 (**2**). Enriched sample obtained from *Muyocopron laterale* F5912 was separated by the following condition (solvent A: H_2O + 0.1% HCOOH, solvent B: MeCN + 0.1% HCOOH; flow rate: 30-52 mL/min, gradient conditions: 75:25 isocratic for 5 min, 30 mL/min; followed by 75:25 isocratic for 5 min, 52 mL/min; 25% to 65% of solvent B over 50 min, 65% to 100% of solvent B over 10 min, and finally isocratic at 100% of solvent B for 17 min) to give 2.2 mg of muyocopronol A (**3**), 5.5 mg of muyocopronol B (**4**) and 1.6 mg of muyocopronol C (**5**). Enriched sample obtained from *Colletotrichum tropicicola* F10154 was separated by the following condition (solvent A: H_2O + 0.1% HCOOH, solvent B: MeCN + 0.1% HCOOH; flow rate: 30-52 mL/min, gradient conditions: 80:20 isocratic for 5 min, 30 mL/min; followed by 80:20 isocratic for 5 min, 52 mL/min; 20% to 50% of solvent B over 5 min, 50% to 100% of solvent B over 47 min, and finally isocratic at 100% of solvent B for 10 min) to give 8.3 mg tropicicolide (6). For chemical characterization of isolated compounds, specific rotations were measured using JASCO P-2000 polarimeter. NMR spectra were obtained from Bruker DRX-400 NMR spectrometer equipped with Cryoprobe. The NMR spectrometer used a 5-mm BBI (^{1}H, G-COSY, multiplicity-edited G-HSQC, and G-HMBC spectra) or BBO (^{13}C spectra) probe heads equipped with z-gradients. Agilent 1260 Infinity Preparative-Scale LC/MS Purification System equipped with Agilent 6130B single quadrupole mass spectrometer detector was used to conduct preparative HPLC analyses. Agilent 5 Prep C_{18} column (10 x 30 mm) was used for HPLC runs. HPLC-MS experiment was performed on an Agilent UHPLC 1290 Infinity coupled with a diode array detector (DAD) and an Agilent 6540 accurate-mass quadrupole time-of-flight (QTOF) mass spectrometer equipped with a splitter and

an ESI source. The analyses were conducted with Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) under standard gradient condition of 98% (0.1% formic acid) to 100% MeCN (0.1% formic acid) over 8.6 min, at a flow rate of 0.5 mL/min. The operating parameters for QTOF were the same as in (Sirota et al., 2018).

4.3: Results

4.3.1: Chemical dereplication of active extracts from selected fungal endophytes.

Among the active samples, extracts from 28 fungal strains grown under different conditions and exhibiting promising inhibitory activities were subjected to dereplication. The extracts were analyzed by high resolution mass spectroscopy for identification of molecules by matching their accurate molecular formula with the updated Dictionary of Natural Products (DNP) database for fungal natural products (http://dnp.chemnetbase.com). This resulted in the putative identification of numerous known compounds based on their accurate mass. Among the known compounds identified from the three *Aspergillus* strains are aspergiterpenoid A, asnovolin G, secalonic acid A and G and neoxaline. In the case of *Phomopsis*/*Diaporthe* sp., epicoccamide A, epicoccamide D, antibiotic M 6124, and 6-epicerevisterol were detected. Mycotoxins such as fumonisin B1 and fumonisin A1 in addition to one other known metabolite: glisoprenin E were dereplicated from two *Fusarium* strains. (Table 4.2**)**. The exact identification of several of these compounds was performed by HR-LCMS/MS comparison with our in-house authentic standard compounds library or with MS/MS spectra reported in the literature (Grigoletto et al., 2020; Tamura et al., 2014).

Table 4:2 Categorization of active fungal isolates subjected to chemical dereplication according to target pathogens and cancer cell lines. SA (*Staphylococcus aureus*), KA (*Klebsiella aerogenes*), CA (*Candida albicans*), AF (*Aspergillus fumigatus*), A5 (A549), MP (MIA PaCa-2), PC (PANC-1). Compounds tentatively identified by Dictionary of Natural Products database, authentic standard, and/or MS/MS literature data comparison.

Strain ID	Taxonomy	Primary Screening							Antimicrobial activity	Cytotoxic activity
		Antimicrobial activity				Cytotoxic activity			LC-MS Dereplication (Putative compounds)	LC-MS Dereplication (Putative compounds)
		SA	KA	CA	AF	A5	MP	PC		
F2682	*Aspergillus flavus*									No activity was observed in any fraction
F4434	*Aspergillus assiutensis*								Asnovolin G, Secalonic acids A* and G	Alterporriols F and G
F4437	*Coniella heterospora*									Chaetomugilin T, , Cylindrocladin B, Macrophorin A, Laccaridione B
F4448	*Aspergillus assiutensis*								Aspergiterpenoid A, neoxaline and secalonic acid A* and G	Alterporriols F and G
F4930	*Trichoderma longibrachiatum*								Paracelsin H, I and D, Trichocellin AII and AIV and Trilongin BIII*	Paracelsin H, I and D, Trichocellin AII and AIV and Trilongin BIII*
F5906	*Colletotrichum asianum*									Cytochalasin C and D*
F5910	*Diaporthe* sp.									PM 181110
F5912	*Muyocopron laterale*								No known molecule found	
F5914	*Diaporthe phaseolorum*									Phomopsichalasin D and Cytochalasin W
F5931	*Fusarium proliferatum*								Glisoprenin E	

Strain ID	Taxonomy	Primary Screening							Antimicrobial activity	Cytotoxic activity
		Antimicrobial activity				Cytotoxic activity				
		SA	KA	CA	AF	A5	MP	PC	LC-MS Dereplication (Putative compounds)	LC-MS Dereplication (Putative compounds)
F6361	*Metarhizium anisopliae*								Amauromine, conocandin, and coriolide	
F6578	*Hypoxylon* sp.								No activity was observed in any fraction	
F6580	*Diaporthe ambigua*								No activity was observed in any fraction	
F6609	*Phomopsis phyllanthicola*									Phomopsichalasin G and Cytochalasin O and H*
F6610	*Phomopsis asparagi*								Epicoccamide A*, Epicoccamide D*	
F6615	*Diaporthe phaseolorum*									Phomopsichalasin G and Cytochalasin O and H*
F6616	*Cosmospora vilior*									No activity was observed in any fraction
F6916	*Acremonium hennebertii*								Ergosta-6,22-dien-3-ol, 5,8-epidioxy-, 3-formate, (3β,5α,8α,22E)	
F6932	*Lophiotrema* sp.								No known molecule found	Palmarumycin C15, Palmarumycin C2* and Guignardin E
F10129	*Phomopsis asparagi*								6-epicerevisterol and antibiotic M 6124	
F10134	*Fusarium petroliphilum*								No activity was observed in any fraction	
F10136	*Fusarium proliferatum*								Fumonisin B1*, Fumonisin A1*	
F10145	*Phaeoacremonium rubrigenum*								No activity was observed in any fraction	

Strain ID	Taxonomy	Primary Screening						
		Antimicrobial activity				Cytotoxic activity		
		SA	KA	CA	AF	A5	MP	PC
F10154	*Colletotrichum tropicicola*							
F10163	*Phialemoniopsis curvata*							
F10164	*Muyocopron laterale*							
F10318	*Bartalinia* sp.							
F10400	*Sarocladium* sp.							
Total		**14**	**1**	**7**	**8**	**15**	**22**	**14**

Key

Active

Inactive

Antimicrobial activity	Cytotoxic activity
LC-MS Dereplication (Putative compounds)	**LC-MS Dereplication (Putative compounds)**
No known molecule found	
Gabusectin	
Lactariolide	
Secalonic acid A* and G	Secalonic acid A*
	Vaccinol G, H and O, apiosporamide

*Validated by LCMS authentic standard and MS/MS literature data

Active = Average % growth inhibition ≥ 50

Inactive = Average % growth inhibition <50

4.3.2: Bioactive compounds from *Lophiotrema* sp. F6932

Characterisation of bioactive compounds from selected fungal strains was carried out with high resolution electrospray ionization mass spectrometry (HR-ESI-MS) and Nuclear Magnetic Resonance (NMR). Two bioactive compounds were isolated from *Lophiotrema* sp. F6932 grown in CF02LB media supplemented with 50 μM of 5-azacytidine. Compound (**1**) was isolated as a brownish amorphous powder with $[\alpha]_D$ + 130 (c 0.6, MeOH). HR-ESI-MS measurement established the molecular formula of (**1**) as $C_{20}H_{14}O_5$. The ^{1}H NMR data revealed the presence of six naphthalene proton multiplets at δ_H 6.87–7.60. One multiplet at δ_H 4.20 was assigned to a methine proton of secondary hydroxy group. The assignment of the olefin group at C-3 and C-4 was supported by the observation of vinyl carbons at δ_C 124.9 and 124.6, as well as olefinic protons at δ_H 5.89 and 7.02 in the ^{1}H and ^{13}C NMR spectra respectively. The 1D and 2D-NMR data also revealed the presence of two adjacent aromatic protons, i.e. δ_H 6.84 and 6.71, found in a 1,4-methoxybenzene ring. Based on these data and the observed COSY and HMBC correlations, the structure of **1** was established as a new member of the spirobisnaphthalene palmarumycin family and named palmarumycin CP_{30} (Figure 4.2). The stereochemical determination at C-2 was unfeasible using spectroscopic method and Mosher's ester analysis. Hence, the stereochemistry in **1** remained undetermined.

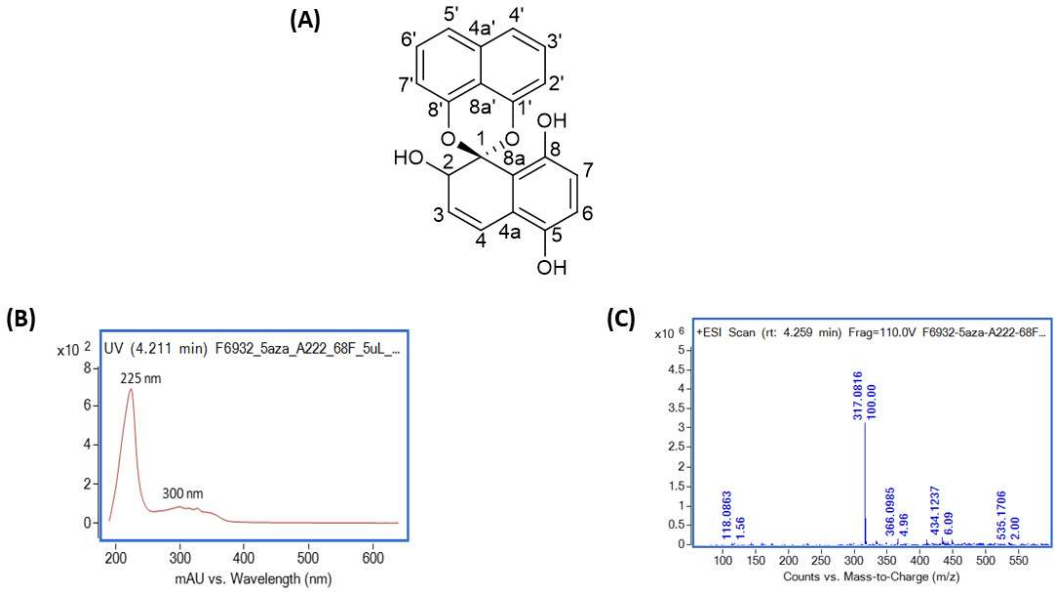


Figure 4:2 Structure of palmarumycin CP_{30} (A), UV spectrum of palmarumycin CP_{30} showing absorption at 225 nm and 300 nm (B), and (+)-HRESIMS spectrum of palmarumycin CP_{30} (C).

The newly discovered palmarumycin CP_{30} was subjected to antimicrobial and cytotoxic testing against a panel of three microbial pathogens; *S. aureus*, *C. albicans* and *A. fumigatus* and three cancer cell lines; A549, MIA PaCa-2 and PANC-1. Table 4.3 shows the IC_{90} values for compound **(1)** against the bacterial pathogen and IC_{50} values for the compound against the two fungal pathogens and three cell lines.

Table 4:3 Antimicrobial and cytotoxic activities of palmarumycin CP_{30}

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
S. aureus	>100
K. aerogenes	>100
C. albicans	>100

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
A. fumigatus	35
A549	>100
MIA PaCa-2	20.1
PANC-1	31.7

The second compound that was isolated from *Lophiotrema* sp. F6932 was compound (**2**) which was isolated as a brownish amorphous solid with $[\alpha]_D$ + 54 (c 1.2, MeOH) and its molecular formula established as $C_{20}H_{13}ClO_6$ on the basis of HR-ESI-MS measurement. The ^{13}C NMR data showed two oxy-quaternary carbons, positions C-4a and C-8a at δ_C 63.0 and 61.3, respectively. The resonance at δ_C 185.4 and 194.3 indicated two carbonyl carbons at C-4 and C-8 respectively. The presence of a 4-hydroxycyclohexanone moiety is supported by the COSY correlations from H-5 to H_2-7 and HMBC correlation from H_2-7 to carbonyl C-8. The carbonyl carbon atom at δ_C 185.4 was established at C-4 based on the HMBC correlation from H-5 to C-4 (Figure 4.3). Singlet for vinyl proton at δ_H 6.77 was established at C-2 while the signal of a chloro-substituted carbon atom at δ_C 131.4 in the ^{13}C NMR spectrum was established at C-3 based on the HMBC cross-peaks from H-2 to oxy-quaternary carbon C-1, vinyl carbon C-3 and carbonyl C-4 (Figure 4.3**)**. In addition, the ^{1}H and ^{13}C NMR data of **2** were consistent with those reported for palmarumycin C_8. The *cis* configuration of the epoxide oxygens and the stereochemistry of the proton at C-5 was assigned in agreement with (Krohn et al., 1994). Hence, compound **2** was identified as the known spirobisnaphthalene, palmarumycin C_8 (Figure 4.4). The NMR spectral data for palmarumycin CP_{30} (**1**) and palmarumycin C_8 (**2**) are shown on Appendix V.

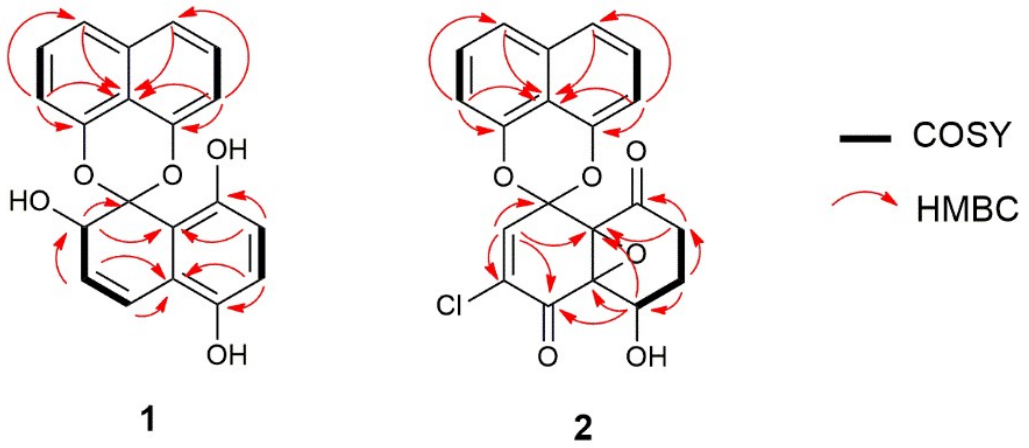


Figure 4:3 Selected COSY and HMBC correlations of palmarumycin CP_{30} (**1**) and palmarumycin C_8 (**2**).

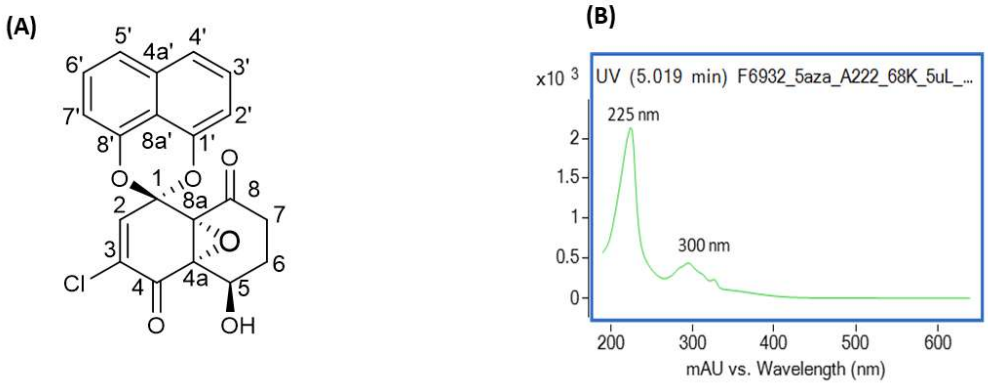


Figure 4:4 Structure of palmarumycin C_8 (A), UV spectra of palmarumycin C_8 showing absorption at 225 nm and 300 nm (B).

Palmarumycin C_8 revealed the most potent cytotoxic activity among all the tested compounds with IC_{50} values of 1.1 µg/mL, 2.1 µg/mL and 10.7 µg/mL against MIA PaCa-2, PANC-1 and A549 cells, respectively. Table 4.4 gives a summary of antimicrobial and cytotoxic activity of palmarumycin C_8 against the test microbial pathogens and cell lines respectively.

Table 4:4 Antimicrobial and cytotoxic activity of palmarumycin C_8

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
S. aureus	18
K. aerogenes	>100
C. albicans	58
A. fumigatus	7
A549	10.7
MIA PaCa-2	1.1
PANC-1	2.1

4.3.3: Bioactive compounds from *Muyocopron laterale* F5912

Three new compounds of polyketide origin were isolated from *Muyocopron laterale* F5912 grown in CF28ST media. Compound (**3**) was obtained as brownish amorphous powders with $[\alpha]_D$ + 68 (c 0.2, MeOH) and a molecular formula of $C_{32}H_{58}O_{11}$ as suggested by (–)-HRESIMS data. The ^{1}H, ^{13}C, and HSQC NMR data revealed the presence of eight methyl, five methylene, fifteen methine, and four non-protonated carbons. Following literature search, the NMR features of **3** were found to be similar to those of bionectriol C (Wang et al., 2014), except for the presence of an additional oxygenated methylened group in **3**, and that the hexopyranose, one methylene, and two methyl moieties in bionectriol C were missing in **3**. The ^{13}C NMR chemical shifts at δ_C 65.2, 68.0, 70.0, 70.1,70.4, and 73.0 indicated the presence of a mannitol unit in **3**, which was also supported by COSY correlations from H_2-1' along the chain to H_2-5' (Figure 4.5).

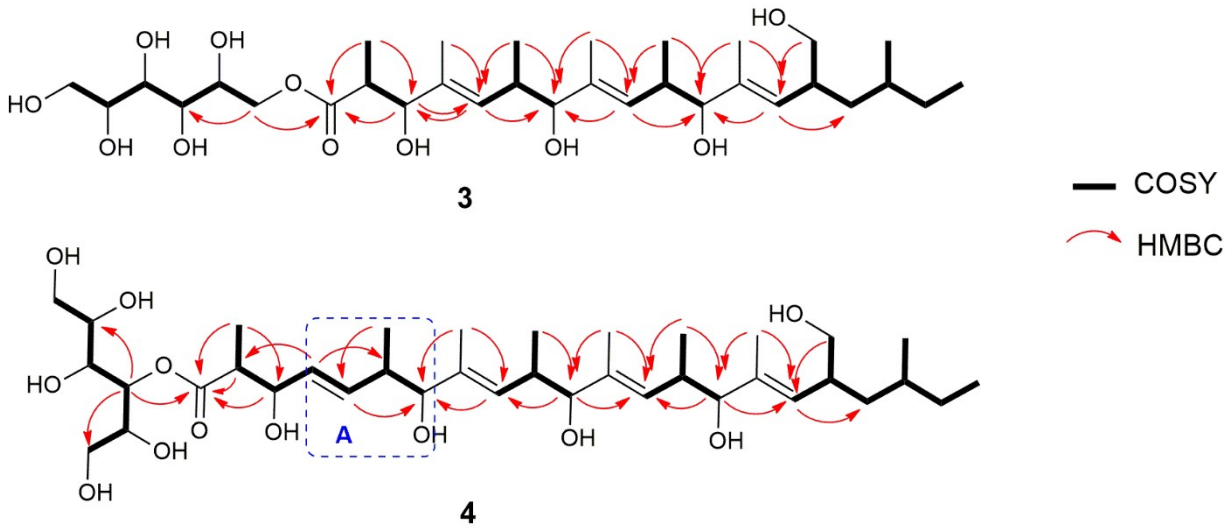


Figure 4:5 Selected COSY and HMBC correlations of muyocopronol A (**3**) and muyocopronol B (**4**).

The mannitol unit was connected to the ester carbonyl C-1 based on HMBC correlation from H_2-1' to C-1. Further COSY and HMBC correlations established the planar structure of **3**. The *E*-configurations of the three olefins in **3** were determined by NOESY correlations between H_3-20 and H-6, H_3-22 and H-10, as well as H_3-24 and H-14. The relative configuration of the mannitol in **3** were deemed to be the same to those of the corresponding part of bionectriol C, since the ^{1}H and ^{13}C NMR data and ^{1}H-^{1}H couplings for the mannitol unit in **3** and bionectriol C were identical (Wang et al., 2014). The large ^{1}H-^{1}H coupling constant (10.1 Hz) between H-2 and H-3 suggested an *erythro* configuration, which was also supported by NOESY correlation between H-3 and H_3-19 (Kasai et al., 2005). Based on these data, the structure of **3** was established as a new polyketide and named muyocopronol A (Figure 4.6).

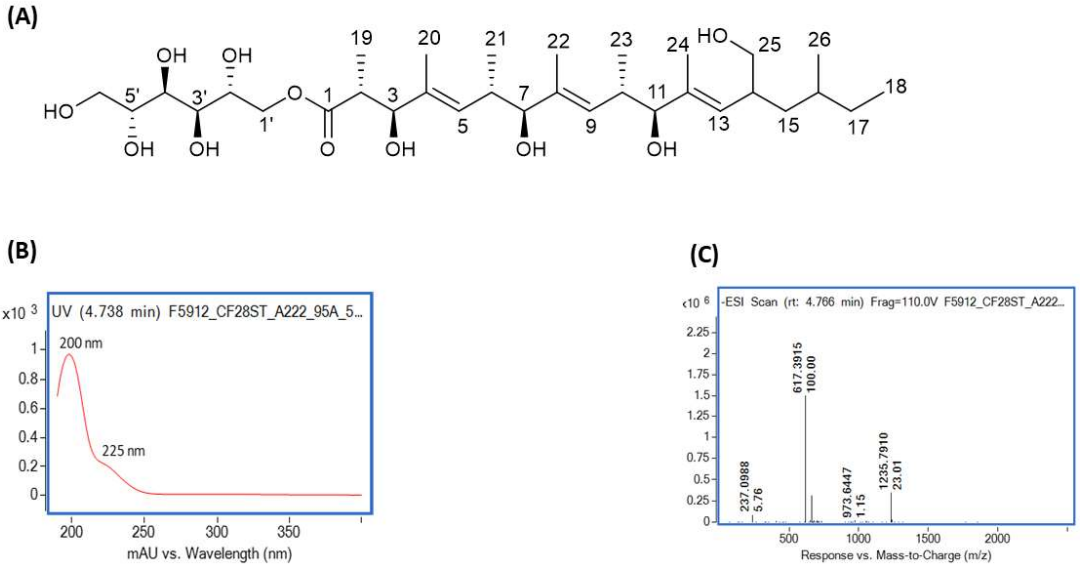


Figure 4:6 Structure of muyocopronol A (A), UV of spectrum muyocopronol A showing absorption at 200 nm and 225 nm (B), and (–)-HRESIMS spectrum of muyocopronol A (C).

Muyocopronol A (**3**) revealed varied levels of antimicrobial and cytotoxic activities against the test microorganisms and cell lines respectively. Muyocopronol A (**3**) did not exhibit antibacterial activity against either *S. aureus* or *K. aerogenes* but revealed weak antifungal activity against the two fungal pathogens with IC_{50} values of 26.3 and 99 µg/mL in *A. fumigatus* and *C. albicans*, respectively. Table 4.5 gives a summary of antimicrobial and cytotoxic activity of muyocopronol A (**3**).

Table 4:5 Antimicrobial and cytotoxic activity of muyocopronol A.

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
S. aureus	>100
K. aerogenes	>100
C. albicans	99
A. fumigatus	26.3

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
A549	97.4
MIA PaCa-2	96.4
PANC-1	97.6

Compound **4** was isolated as yellowish white powders with $[\alpha]_D$ + 33 (c 0.8, MeOH) and assigned a molecular formula $C_{37}H_{66}O_{12}$ based on (–)-HRESIMS analysis. Comparison of 1D and 2D NMR data of **4** with those of compound (**3**) revealed the presence of one additional methyl, two methine (one of which was oxygenated), and two olefinic methines. COSY correlations between H-4 and H-5, H-5 and H-6, H-6 and H_3-24, and between H-6 and H-7 constructed an additional C_5-substructure in **4**, as shown in (fragment A). The position of the extra C_5-fragment was suggested by HMBC correlations from H-4 to C-2 and from H_3-25 to C-7. The ^1H-^1H coupling constant of H-4/H-5 indicated a *trans*-configured double bond. The remaining part of the polyketide structural unit was assigned by detailed analysis of COSY and HMBC data (Figure 4.5). The ester moiety in **4** connected C-3' of the mannitol unit and the polyketide portion based on HMBC cross-peaks from H-3' to the ester carbonyl C-1(Ju et al., 2007). NOESY correlations between H_3-25 and H-10, H_3-27 and H-14, as well as between H_3-29 and H-18 indicated *E*-configured olefins in **4**. The *erythro* configuration between H-2 and H-3 was deduced from their ^1H-^1H coupling constant (8.2 Hz). Compound **4** was assigned the name muyocopronol B (Figure 4.7).

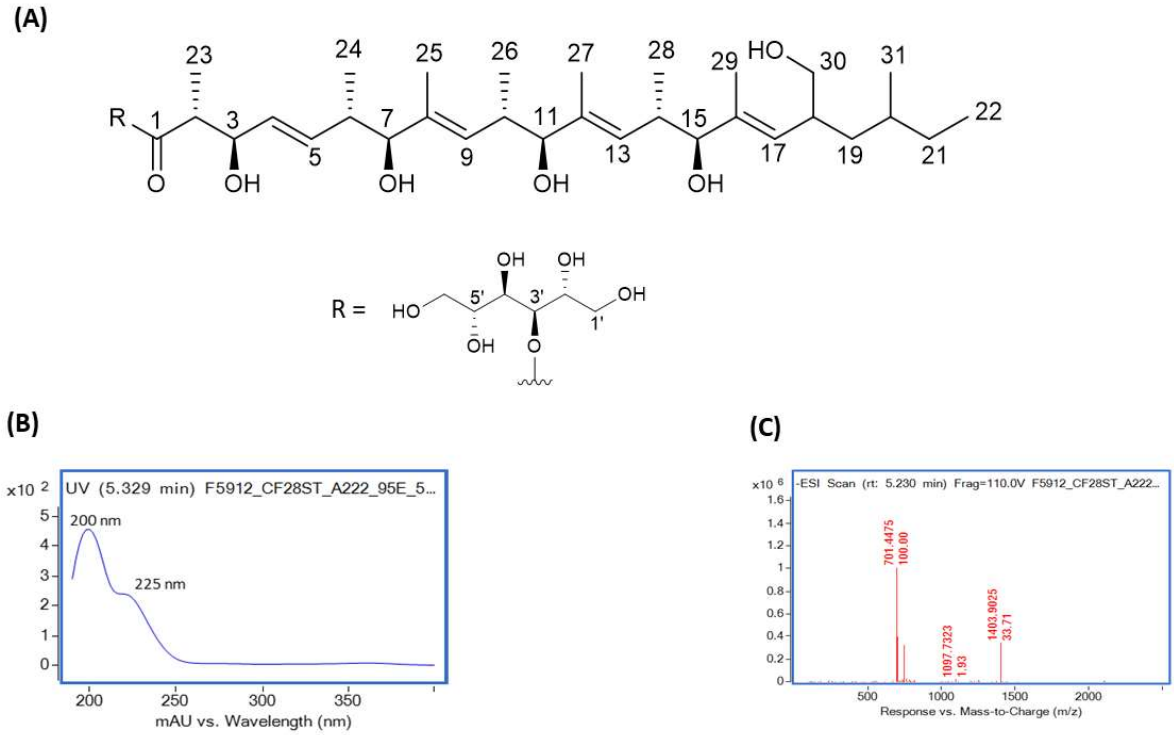


Figure 4:7 Structure of muyocopronol B (A), UV spectrum of muyocopronol B showing adsorption at 200 nm and 225 nm (B) and (–)-HRESIMS spectrum of muyocopronol B (C).

Muyocopronol B (**4**), similar to muyocopronol A (**3**) did not exhibit any significant antibacterial activity against any of the two tested bacterial pathogens but revealed promising antifungal activities with IC_{50} values of 3.4 and 4.5 µg/mL against *A. fumigatus* and *C. albicans*, respectively. Table 4.6 gives a summary of antimicrobial and cytotoxic activity of muyocopronol B against the test pathogens and cell lines respectively.

Table 4:6 Antimicrobial and cytotoxic activity of muyocopronol B.

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
S. aureus	>100
K. aerogenes	>100
C. albicans	4.5
A. fumigatus	3.4
A549	44.5
MIA PaCa-2	40
PANC-1	77.1

Compound (**5**) was isolated as yellowish white powders with $[\alpha]_D$ + 37 (c 0.2, MeOH) and assigned the molecular formula $C_{31}H_{54}O_7$ following (–)-HRESIMS data analysis. Comparison of NMR and MS data between **4** and **5** suggested that the latter natural product was missing a mannitol unit. HMBC correlation from H-2 to a carbonyl carbon at δ_C 182.7, that had not been detected in the ^{13}C NMR spectrum, confirmed the presence of a carboxylic acid moiety in 5. Analysis of 2D NMR data further supported the polyketide structural part in **5**. The same relative configuration previously determined for **4** was also assigned for **5** following analysis of $^1H–^1H$ coupling constants and the NOESY spectrum (Figure 4.8).

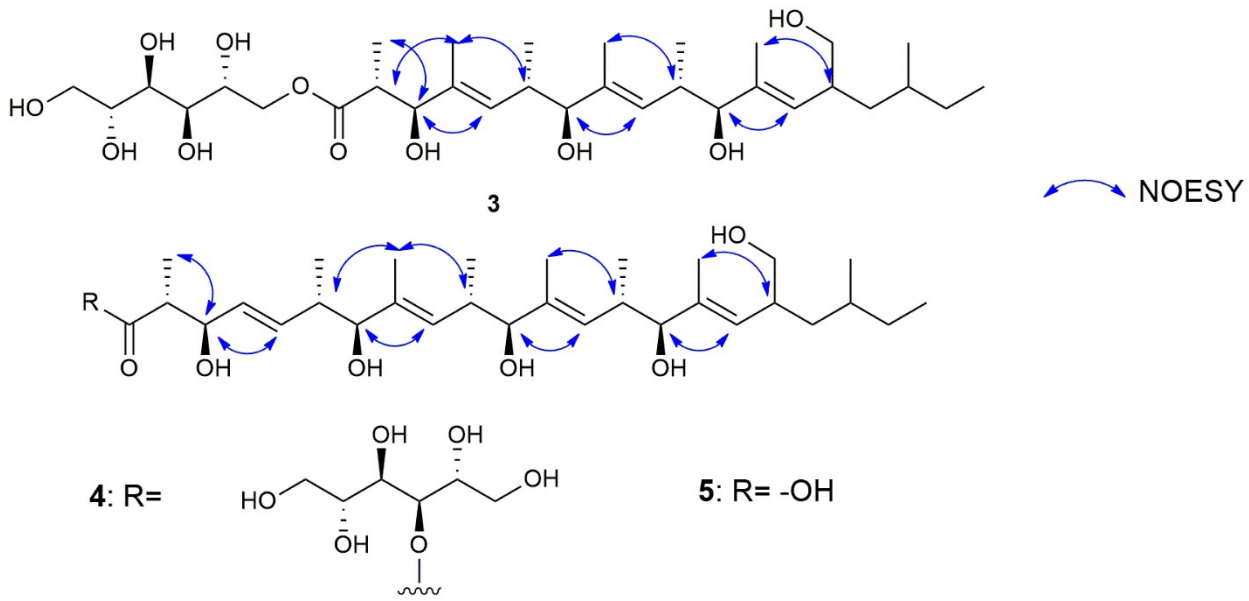


Figure 4:8 Selected NOESY correlations of muyocopronol A (**3**), B (**4**) and C (**5**) to illustrate the stereochemistry of the olefin groups in the muyocopronol analogues.

Compound 5 was designated muyocopronol C, and its structure, UV spectrum and HRESIMS spectrum are shown on Figure 4.9.

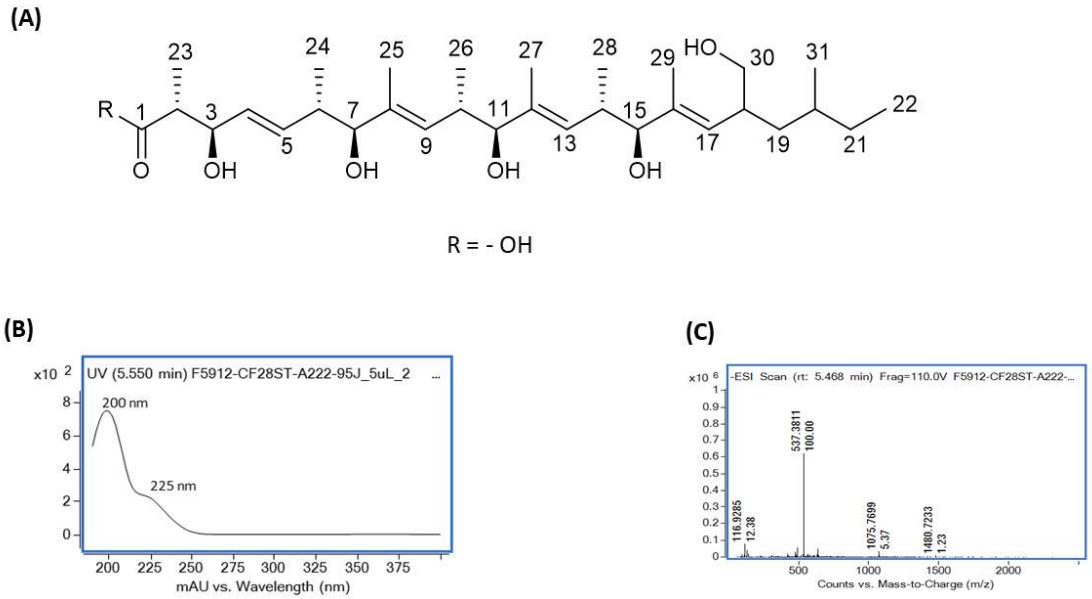


Figure 4:9 Structure of muyocopronol C (A), UV spectrum of muyocopronol C showing adsorption at 200 nm and 225 nm (B), and (–)-HRESIMS spectrum of muyocopronol C (C).

Muyocopronol C exhibited activity only against *C. albicans* with IC_{50} values of 9.2 µg/mL but did not have antimicrobial or cytotoxic activity against the rest of the tested microbial pathogens and cell lines respectively (Table 4.7). The NMR spectral data of muyocopronol A (**3**), B (**4**) and C (**5**) are shown on Appendix VI.

Table 4:7 Antimicrobial and cytotoxic activity of muyocopronol C.

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
S. aureus	>100
K. aerogenes	>100
C. albicans	9.2
A. fumigatus	>100
A549	>100
MIA PaCa-2	>100
PANC-1	>100

Other than muyocopronol A, B and C, a known compound eugenitin and a putatively identified depsipeptide compound were isolated from *Muyocopron laterale* F5912. Eugenitin is a member of a class of compounds known as chromones and has been previously isolated from endophytic fungi such as BCC 54265 (Botryosphaeriaceae), *Mycoleptodiscus indicus* and an endophytic mitosporic *Dothideomycete* sp. LRUB20 (Andrioli et al., 2014; Chomcheon et al., 2009; Isaka et al., 2018). Eugenitin revealed weak antifungal activity against *C. albicans* and *A. fumigatus* and did not exhibit any cytotoxic activity. The putatively identified depsipeptide compound did not reveal any antimicrobial or cytotoxic activity.

4.3.4: Bioactive compounds from *Colletotrichum tropicicola* F10154

From the extracts of *Colletotrichum tropicicola* F10154 grown in in CF28ST media, compound **6** was isolated as a pale-yellow sticky oil with $[\alpha]_D$ + 1.8 (c 1.2, MeOH) and assigned the molecular formula $C_{31}H_{54}NO_6P$, following analysis of HRESIMS and NMR data. The ^1H NMR spectrum of **6** was similar to that reported for eushearilide, suggesting their structural resemblance (Hosoe et al., 2006; Tonoi et al., 2019). Comparison of ^{13}C NMR and MS data of **6** and to those of eusharilide suggested that **6** was a 26-membered macrolide containing four non-conjugated diene and a choline phosphate ester group. The presence of the choline phosphate group was readily indicated by the characteristic $^{13}C/^1H$ resonances at δ_C /δ_H 54.7/3.22, 67.6/3.63, and 60.3/4.27. The positions of non-conjugated four double bonds in **6** were assigned based on ^{13}C NMR chemical shift and HMBC data. Detailed analysis of 2D NMR data further supported the remaining structural assignment in **6** (Figure 4.10). However, attempts to address the configuration of the double bonds of **6** proved unsuccessful due to the overlapping ^1H-NMR resonances of the olefins. Moreover, the stereochemical determination at C-3 and C-25 was unfeasible using spectroscopic method due to the rotatable nature of the methyl and the choline phosphate moieties. Thus, the planar structure of **6** was assigned and named tropicicolide (Figure 4.10). The structure of tropicicolide, its UV and HRESIMS spectra are shown in figure 4.11.

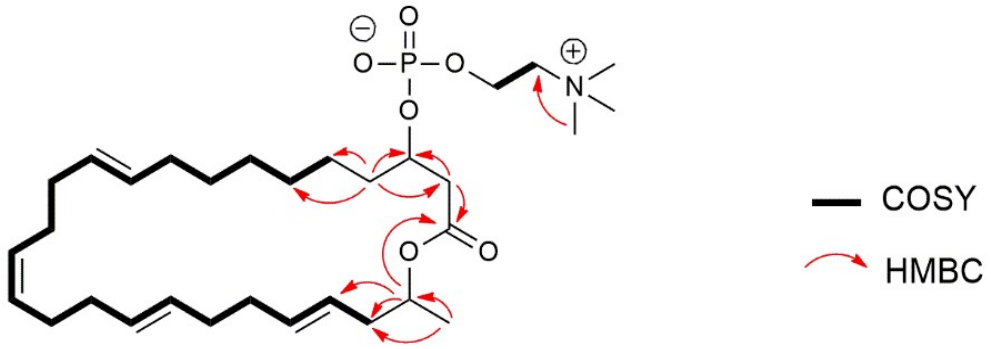


Figure 4:10 Selected COSY and HMBC correlations of tropicicolide.

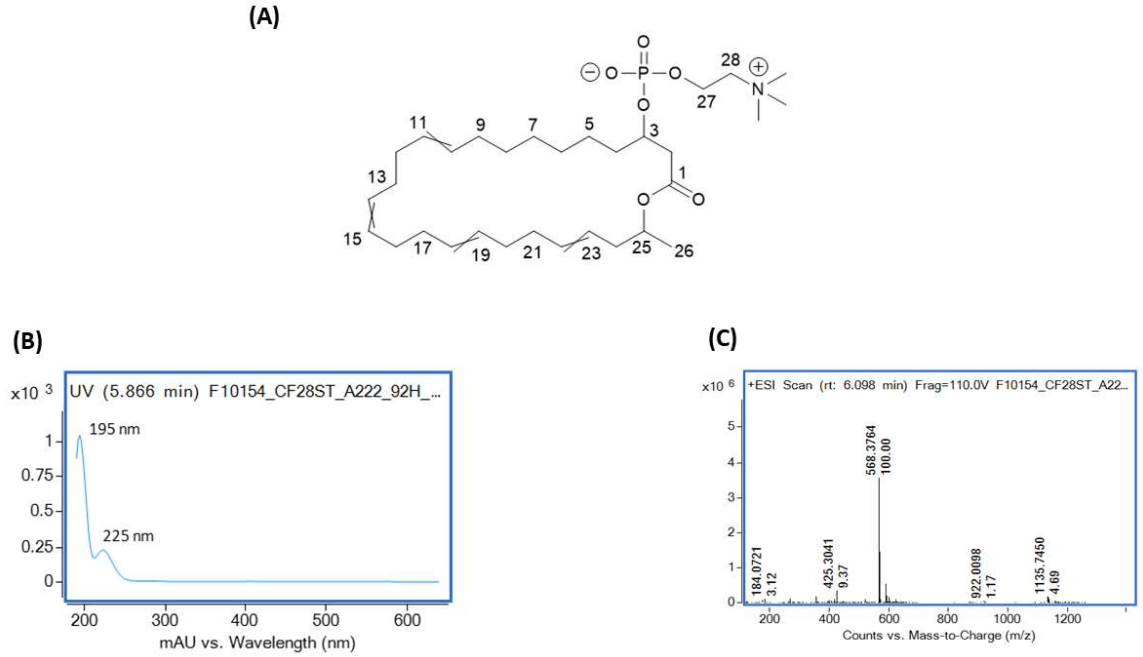


Figure 4:11 Structure of tropicidolide (A), UV spectrum of tropicidolide showing adsorption at 195 nm and 225nm (B) and (+)-HRESIMS spectrum of tropicidolide (C).

Tropicicolide presented the strongest antifungal activity against *A. fumigatus* of all the tested compounds with an IC_{50} of 1.8 µg/mL but with a lesser activity of an IC_{50} value of 7.1 µg/mL

against the yeast pathogen *C. albicans*. The compound however revealed no antibacterial or antiproliferative activity against *S. aureus* and the three cancer cell lines respectively (Table 4.8). The NMR spectral data for tropicicolide are shown on Appendix VII while the ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC spectra for the 6 compounds are shown on Appendix VIII**.**

Table 4:8 Antimicrobial and cytotoxic activity of tropicicolide.

Test microorganism / Cancer cell line	IC_{90} (*S. aureus* and *K. aerogenes*) and IC_{50} (fungal pathogens and cancer cell lines) in µg/mL
S. aureus	>100
K. aerogenes	>100
C. albicans	7.1
A. fumigatus	1.8
A549	>100
MIA PaCa-2	>100
PANC-1	>100

In addition to tropicidolide, three other known compounds namely; zearalenol (putative), monocillin IV and monorden E were isolated from *C. tropicicola* F10154. Monocillins and monordens are well known fungal secondary metabolites that have been isolated from a wide range of fungi such as *Colletotrichum graminicola* (Wicklow et al., 2009), nematophagous fungus *Pochonia chlamydosporia* strain 170 (Yip et al., 2019) and saprobic filamentous fungus *Paecilomyces* sp.SC0924 (L. Xu et al., 2017). Monocillin IV exhibited weak antifungal activity against *C. albicans* and *A. fumigatus* and no antibacterial or cytotoxic activities. Zearalenol (putative) is a derivative of zearalenone, a mycotoxin with estrogenic effects on mammals and has been isolated mostly from *Fusarium* sp. Zearalenol exhibited weak antifungal activity against *A. fumigatus* and weak antiproliferative activity against the three tested cell lines (Table 4.9).

4.3.5: Bioactive compounds from *Acremonium hennebertii* F6916

From large-scale fermentation of *Acremonium hennebertii* F6916 grown in CF02LB media, only one known compound fusidic acid was isolated. Fusidic acid is a member of fungal fusidane-type antibiotics also represented by helvolic acid and cephalosporin and is the only fusidane-type antibiotic that has been clinically used. It is primarily used for the treatment of topical and systemic infections caused by Gram-positive bacteria including the methicillin-resistant *Staphylococcus aureus* strains (Cao et al., 2019). Its mode of action is through the inhibition of protein synthesis by binding to EF-G-GDP, resulting in the disruption of peptide translocation and ribosome disassembly (Fernandes, 2016). Fusidic acid was first isolated from *Fusidium coccineum* and has recently been isolated from an endophytic fungi *Acremonium pilosum* F47 (same genus as *A. hennebertii* F6916). As expected, fusidic acid exhibited very strong antibacterial activity against *S. aureus* presenting an IC_{90} value of 0.24 µg/mL. This IC_{90} value is in agreement with the value 0.25 µg/mL that has been obtained for fusidic acid against a wide range of *S. aureus* strains such *S. aureus* (7339), methicillin-resistant *S. aureus* (MRSA) (3876), methicillin-susceptible *S. aureus* (MSSA) (3463) and Coagulase-negative *S. aureus* (1352) (Jones et al., 2011). Predictably, fusidic acid did not shown any cytotoxic activity against any of the three tested cell lines (Table 4.9).

4.3.6: Bioactive compounds from *Phomopsis asparagi* F6610

From large-scale fermentation samples of *Phomopsis asparagi* F6610 grown in CF18LB media, two known compounds epicoccamides A and D were isolated. Epicoccamide was first isolated from the fungus *Epicoccum purpurascens* isolated from inner tissue of the jellyfish *Aurelia aurita.* Epicoccamides are a unique class of compounds in that they contain three biosynthetically distinct subunits namely; glycosidic, fatty acid and tetramic acid (amino acid) (Wright et al., 2003).

Epicoccamide A exhibited moderate antifungal activity with IC_{50} values of 9.0 and 13.3 µg/mL against *C. albicans* and *A. fumigatus* respectively. Epicoccamide D on the other hand revealed strong antifungal activity against *C. albicans* with an IC_{50} of 1.7 µg/mL. In a previous study, epicoccamide A was reported to have moderate antifungal activity against *Ustilago maydis* AB33 presenting MIC value of 1.8 mM (Harwoko et al., 2020). Epicoccamide D has been previously reported to have antiproliferative activity towards mouse fibroplast (L-929) and human leukemia cell lines (K-562) with growth inhibition (GI_{50}) of 50.5 and 33.3 µM, respectively (Kemami Wangun et al., 2007). In the current study however, none of the two epicoccamides exhibited cytotoxic effects against any of the three tested cell lines (Table 4.9).

Two other fungal strains; *Aspergillus assiutensis* F4434 grown in CF02LB in the presence and absence of 5-azacytidine and *Aspergillus assiutensis* F4448 grown in CF02LB media were subjected to large-scale fermentation. However, these three samples were not progressed further to compound isolation and structure elucidation since their activities were attributed to the presence of already known molecules. As for *Trichoderma longibrachiatum* F4930 grown on CF05ST media, the work on characterization of the bioactive compounds were inconclusive due to the very low amount of the active fractions obtained. Figure 4.12 shows the structures of known compounds that were isolated from different fungal strains.

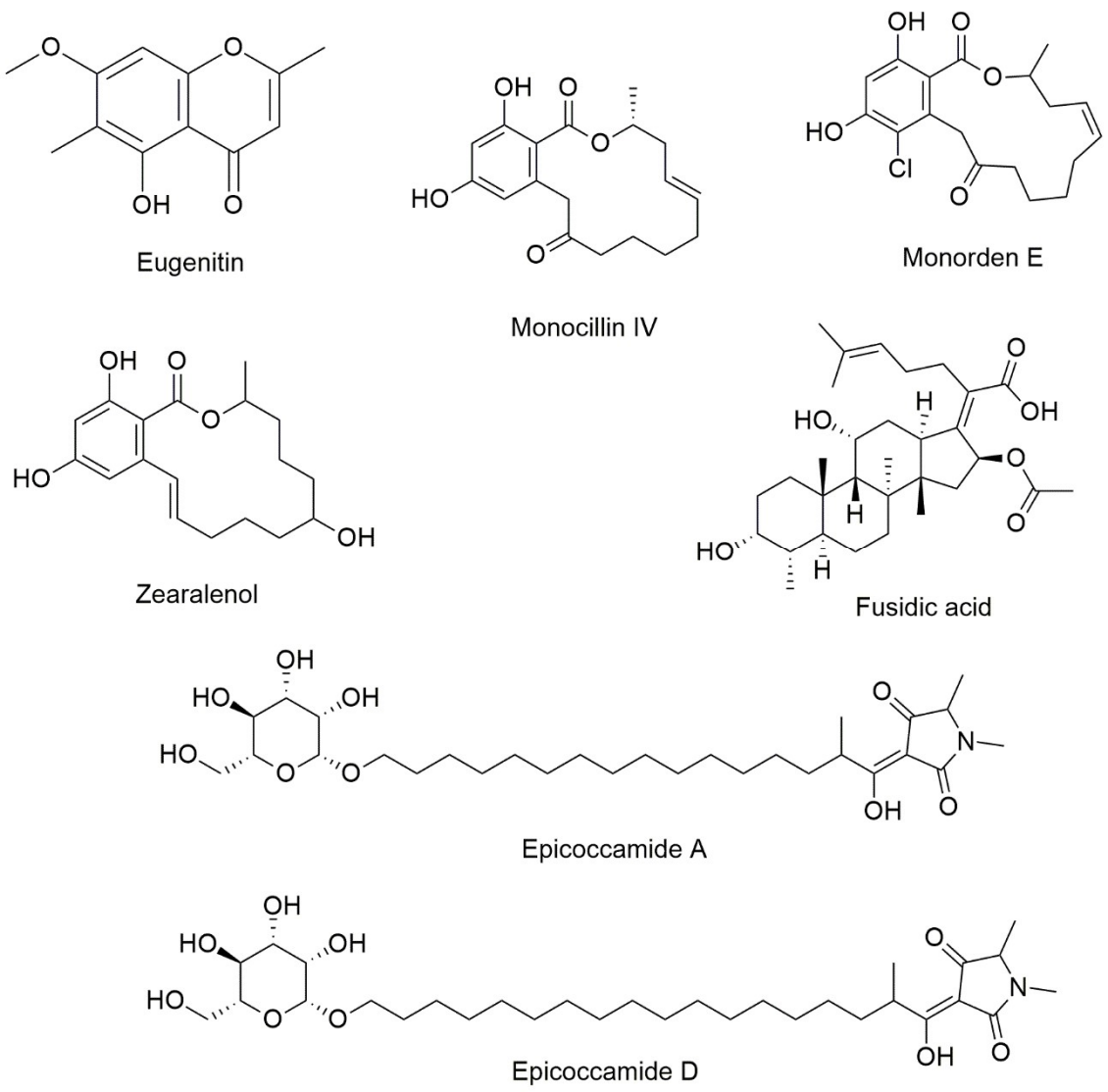


Figure 4:12 Some known compounds that were isolated from different endophytic fungal strains.

All the isolated new and known compounds were subjected to biological testing for antimicrobial and cytotoxic activities against the panel of microbial pathogen and cancer cell lines. Table 4.9 gives a summary of the bioactivity all the compounds isolated in this study and a comparison of their inhibitory activities with those of the reference standards compounds used in the study.

Table 4:9 A comparison of antimicrobial and cytotoxic activities of all the isolated compounds with reference standards used in the study.

Compound	*S. aureus*	*K. aerogenes*	*C. albicans*	*A. fumigatus*	A549	MIA PaCa-2	PANC-1
	IC_{90} (µg/mL)		IC_{50} (µg/mL)				
Palmarumycin CP_{30}	>100	>100	>100	35	>100	20.1	31.7
Palmarumycin C_8	18.0	>100	58	7.0	10.7	1.1	2.1
Muyocopronol A	>100	>100	99	26.3	97.4	96.4	97.6
Muyocopronol B	>100	>100	4.5	3.4	44.5	40	77.1
Muyocopronol C	>100	>100	9.2	>100	>100	>100	>100
Eugenitin	>100	>100	64.2	17.8	>100	>100	>100
Depsipeptide (Putative)	>100	>100	>100	>100	>100	>100	>100
Tropicicolide	>100	>100	7.1	1.8	>100	>100	>100
Monocillin IV	>100	>100	41.5	22.7	71.8	>100	>100
Monorden E	>100	>100	>100	11.6	>100	9.1	>100
Zearalenol (Putative)	>100	>100	>100	18.7	57.6	48.8	72.1
Fusidic acid	0.24	>100	>100	>100	>100	>100	>100
Epicoccamide A	>100	>100	9.0	13.3	>100	>100	>100
Epicoccamide D	7.2	>100	1.7	8.6	>100	>100	>100
Gentamicin	0.15	0.21	-	-	-	-	-
Amphotericin B	-	-	0.29	0.26	-	-	
Puromycin	-	-	-	-	0.31	0.18	0.18

4.4: Discussions

Fungal endophytes are prolific producers of bioactive molecules including structurally uncommon molecules and those with remarkable bioactivities (Gupta & Shukla, 2020; Nisa et al., 2015; Ortega et al., 2021). However, several limiting factors such as low yields of targeted compounds and weak expression of many of their biosynthetic gene clusters (BGCs) under standard laboratory growth conditions limits the realization of full biosynthetic potential of these microbial factories (González-Menéndez et al., 2018; Motoyama et al., 2021). To overcome such challenges, numerous strategies such as co-culture, chemical elicitation, variation of culture growth conditions and genetic engineering of strains of interest among other strategies have been employed (Baral et al., 2018; Gakuubi et al., 2021; Kjærbølling et al., 2019). In the current study, chemical elicitation using 5-azacytidine and SAHA and variation of fermentation media were assessed for their potential in enhancing secondary metabolite biosynthesis and bioactivity of crude fungal extracts.

From the dereplication studies, extracts obtained from *Lophiotrema* sp. F6932 grown in CF02LB media supplemented with 50 µM of 5-azacytidine and those of *Muyocopron laterale* F5912 and *Colletotrichum tropicicola* F10154 both grown in CF28ST media were selected for further investigation because no known molecules were found that could explain the activity observed in these extracts. Chemical investigation of the extracts led to the identification a novel compound palmarumycin CP_{30} (**1**) and its known analogue palmarumycin C_8 (**2**) from *Lophiotrema* sp. F6932. Three new polyketides, muyocopronols A (**3**), B (**4**), and C (**5**) were isolated from *Muyocopron laterale* F5912 while a novel 26-membered macrolide designated tropicicolide (**6**) was isolated from *Colletotrichum tropicicola* F10154. Palmarumycins belong to the spirobisnaphthalenes

family of secondary metabolites characterized by the presence of a 1,8-dihydroxynaphthalene-derived spiroketal unit connected to a second oxidized naphthalene unit (Mou et al., 2015). Palmarumycins which have been shown to possess a wide range of inhibitory activities including antibacterial, antifungal and antitumor activities are produced mainly by filamentous fungi (Kanehara et al., 2021). These compounds have been isolated from fungi from a wide range of Genera. For example, nine palmarumycins, B_1−B_9 were isolated from endophytic fungi *Berkleasmium* sp with two more new palmarumycin C_{12} and C_{13} isolated from the same fungi (Mou et al., 2015; Shan et al., 2014). Palmarumycins CP_4 and CP_5 were isolated from *Phaeoseptum* sp. (Kanehara et al., 2021) while palmarumycin CE_4, palmarumycin CP_4, palmarumycin CP_1 were discovered from and endophytic fungi *Anteaglonium* sp. (Mafezoli et al., 2018). Muyocopronols A - C are closely related to bionectriols, which are fungal secondary metabolite of polyketide origin and assign to a class of glycosylated, polyunsatured polyols (Freinkman et al., 2009). The first of this type of molecules designated bionectriol A was isolated from *Bionectria* sp., associated with the fungus-growing ant *Apterostigma dentigerum* (Freinkman et al., 2009). Three related compounds (bionectriols B–D) were later discovered from *Bionectria ochroleuca* and all exhibited strong anti-biofilm activity against *C. albicans* in addition to synergistic antifungal activities when used in combination with amphotericin B (Wang et al., 2014). Significantly, only these two references exist regarding the isolation of bionectriols from fungi and in both cases, these compounds were isolated from members of the *Bionectria* sp. which are within the class *Sordariomycetes*. This makes it an interesting finding the discovery of these three new compounds from *Muyocopron laterale* which belong to a different fungal class i.e. Dothideomycetes. From *C. tropicicola*, tropicicolide (**6**) was discovered. Structure of tropicicolide (**6**) shared similarity with

the structure of eushearilide. Eushearilide is a 20-membered macrolide with a non-conjugated diene and a choline phosphate ester moiety was for the first time isolated from cultures of *Eupenicillium shearii* and revealed antifungal activity against a wide range of human pathogens including *Aspergillus fumigatus, Aspergillus niger* and *Candida albicans* (Hosoe et al., 2006). In the current study, the newly discovered tropicicolide (**6**) had the most potent activity against *A. fumigatus* among all compounds with an IC_{50} of 1.8 µg/mL. A closely related compound designated as eushearilide was discovered some years back from the fungus *Eupenicillium shearii*, and since then, only total synthesis of the compound and its various stereoisomers and/or related compounds have been reported (Tonoi et al., 2016, 2019). This, therefore is the second time that this class of compounds is being reported from Nature.

4.5: Conclusion

Bioassay-guided isolation and structure elucidation of bioactive compounds from fungal endophytes isolated from diverse habitats of Singapore using chemical elicitation and media diversification resulted in the discovery of five new compounds from three fungal strains. Significantly, antimicrobial hits from crude extracts derived from two of the three prioritized fungal strains were uncovered following their growth in the presence of 5-azacytidine (*Lophiotrema* sp. F6932) use of additional growth media (*Muyocopron laterale* F5912). Consequently, had our investigation on the discovery of bioactive secondary metabolites from fungal endophytes been limited to the use of two initial liquid media (CF02LB and CF18LB), four of the five newly discovered compounds associated with the two aforementioned strains would not have been discovered. This study therefore demonstrates how diversification of growth media and the use of chemical epigenetic manipulation using molecules such as 5-azacytidine and

suberoylanilide hydroxamic acid (SAHA) can enhance the discovery of new bioactive microbial natural products. Three fungal strains that were subjected to large-scale fermentation could not be progressed further to compound isolation and structure elucidation because their activity was attributed to presence of already known molecules. This is one of the unavoidable drawbacks of bioassay-guided approach to discovery of bioactive compounds.

Chapter 5: A CRISPR/Cas9 RNP-mediated system for genome editing of *Lophiotrema* sp. F6932

5.1: Introduction

Lack of a versatile genetic engineering system that can be employed across the vast majority of non-model filamentous fungi has been a long-standing challenges toward a broader exploitation of fungal secondary metabolite biosynthetic capabilities (Nødvig et al., 2015). Many of the early genetic engineering protocols in filamentous fungi were developed for the common model species such as *Aspergillus oryzae, Aspergillus nidulan,* and *Trichoderma reesei* among others (Jiang et al., 2021; Jin et al., 2021; Wang et al., 2020). The emergence of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system, a programmable gene editing technology that is convertible with all cell types from prokaryotes to eukaryotes has greatly expanded the avenue for genetic manipulation of living organisms including fungi. Derived from bacterial adaptive immune system, CRISPR-Cas9 technology is a powerful molecular tool that allows for precise DNA editing and has led to accelerated pace of research on genetic manipulation of filamentous fungi (Deng et al., 2017; Krappmann, 2017). Type II CRISPR-Cas9, the most common of the CRISPR systems consist of two main components (a) a CRISPR-associated Cas9 endonuclease derived from *Streptococcus pyogenes* and (b) a single-guide RNA (sgRNA) created by fusion of CRISPR RNA(crRNA) and trans-activating CRISPR RNA (tracrRNA) (Deng et al., 2017; Ferrara et al., 2019). CRISPR-Cas9 system relies on bacteria-derived Cas9 protein and for this reason, codon optimization of the *cas9* gene is in most cases required for optimized expression of the system in eukaryotes (Nødvig et al., 2015; Zou et al., 2020). Moreover, it has shown that constitutively expressed Cas9 can have detrimental effect on the some host genome structure in

addition to causing unexpected phenotypic changes such as delayed growth and loss of fitness (Enkler et al., 2016). Furthermore, rearrangements or off-target mutations and toxic effects on the cells associated with *Cas9* overexpression have been reported (Foster et al., 2018; Jacobs et al., 2014).

Recently, an alternative approach; CRISPR/Cas9 RNP-mediated genome editing has been adopted in numerous fungal studies. This approach entails *in vitro* assembly followed by delivery of ribonucleoprotein complex consisting of the Cas9 protein and the gRNA to the cell. This approach has a number of advantages among them the elimination of the need to find appropriate promoters for expression of Cas9 protein and gRNAs (Abdallah et al., 2017; Ouedraogo & Tsang, 2020). Besides, CRISPR/Cas9 RNP-mediated genome editing has been shown to reduce the rate of off-target effects (Foster et al., 2018). This approach furthermore allows for *in vitro* assessment of the efficiency of gRNAs to cleave the target region before the actual transformation (Abdallah et al., 2017; Ouedraogo & Tsang, 2020). CRISPR/Cas9 RNP-mediated genome editing has already been successfully employed in numerous filamentous fungi such as *Aspergillus fumigatus* (Abdallah et al., 2017), *Magnaporthe oryzae* (Foster et al., 2018), *Aspergillus niger* (Kuivanen et al., 2019), *Fusarium proliferatum* (Ferrara et al., 2019), *Trichoderma reesei* and *Cordyceps militaris* (Zou et al., 2020) and *Penicillium polonicum* (Valente et al., 2021). There is however no report in literature on the application of CRISPR/Cas9 gene editing in L*ophiotrema* genus.

L*ophiotrema* is a genus of ascomycetous fungi within *Lophiotremataceae* family. Members of this genus have been isolated from plant debris where they are thought to function as saprobes and also as endophytes in a wide range of marine and terrestrial host plants. *Lophiotrema* sp. F6932 was

isolated from white mangrove (*Avicennia officinalis*) and its crude extracts exhibited strong antibacterial activity against *S. aureus*. Bioassay-guided isolation and structure elucidation of active constituents led to the isolation of a novel palmarumycin CP_{30} and it known analogue palmarumycin C_8 (see Chapter 4). In this chapter, we describe the development of a CRISPR/Cas9 RNP-mediated genome editing system that enabled us to characterize a type 1 iterative polyketide synthases (iPKS), that is involved in the biosynthesis of the palmarumycins and melanin in *Lophiotrema* sp. F6932. To verify the involvement of palmarumycin (*PAL*) gene cluster in the biosynthesis of these compounds, we employed ribonucleoprotein (RNP)-mediated CRISPR-Cas9 to induce targeted deletion of the ketosynthase (KS) domain in *PAL*.

5.2: Materials and methods

5.2.1: Fungal strain

Lophiotrema sp. F6932 was isolated from white mangrove also known as the Indian mangrove (*Avicennia officinalis*) in Pulau Ubin Island, Singapore and stored at A*STAR's Natural Product Library (NPL), Singapore Institute of Food and Biotechnology Innovation (SIFBI). Molecular identification of the fungal isolate was previously performed through amplification of the internal transcribed spacer 2 (ITS2) region of the rDNA gene using primer set ITS86F/ITS4 (Turenne et al., 1999; White et al., 1990). The ITS sequence generated was submitted to BLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the analysis of sequences similarity and the strain showed a match score of 99% with *Lophiotrema* sp. (MK587671.1). The ITS2 sequence for this strain is available in the GenBank database under the accession number OM791904. In the current work, attempt was made to further establish the identity of *Lophiotrema* sp. F6932 and its phylogenetic relationships with closely related species. Multilocus sequence analysis (MLSA) was

performed using five molecular markers, namely, three nuclear ribosomal genes, i.e. 18S nuclear small subunit ribosomal DNA (nrSSU), nuclear large subunit (nrLSU), and the entire internal transcribed spacer region (ITS), and two protein-coding loci, i.e. RNA polymerase II second largest subunit (RPB2) and translation elongation factor 1-alpha (*TEF1-α*). The sequences for the five molecular markers were obtained from *Lophiotrema* sp. F6932 genome data while those of closest relatives were retrieved from NCBI database and recent references (Hashimoto et al., 2017; Andreasen et al., 2021). DNA gene sequences were aligned using the ClustalW algorithm in MEGA 7 software (Kumar et al., 2016). For each of the five loci, sequences were aligned individually and then concatenated for phylogenetic analyses based on Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods.

5.2.2: Whole genome sequencing and bioinformatics analyses

Whole genome sequencing of *Lophiotrema* sp. F6932 was done by Macrogen (South Korea) using a combination of PacBio single molecule, real-time (SMRT) sequencing and illumina platforms followed by *de novo* assembly using bioinformatics software FALCON for PacBio long-reads and assembly polishing with Arrow. Illumina reads were applied for accurate genome sequence using Pilon for error correction. *Lophiotrema* sp. F6932 was found to have a 51.4% GC content with 37.1 MB spread over 17 contigs. The quality of the genome was assessed using two approaches namely; the N50 parameter which defines assembly quality in terms of contiguity and represents the the length of the shortest contig for which longer and equal length contigs cover at least 50% of the assembly (Alhakami, 2017). The N50 for F6932 genome was 1,955,251 bp. The second parameter used was the Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis. BUSCO is a tool that searches for the presence or absence of highly conserved genes in the

assembly and is used for measuring the completeness of genome assembly data and annotated gene sets (Simão et al., 2015). From a set of 303 universal single copy orthologs in 100 different species (bacteria or eukaryota), 99.01% were found to be complete, 0.33% were found fragmented and 0.66% missing while no orthologs were found duplicated. F6932 genome was therefore considered to be of high quality given that BUSCO score above 95% is considered good. The genome assembly data was submitted to antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) server for biosynthetic gene clusters (BGCs) prediction (Medema et al., 2011).

5.2.3: Antibiotics sensitivity test

In order to assess the suitability of using hygromycin B resistance gene (*hph*, hygromycin B phosphotranferase) as a selectable marker for knockout mutants, the sensitivity of *Lophiotrema* sp. F6932 wild-type strain to hygromycin B was evaluated by growing the fungus in potato dextrose agar (PDA) plates amended with different concentrations of hygromycin B (InvivoGen, US). Three agar plugs (3mm in diameter) were cut from the periphery of an actively growing 14-day old cultures of and grown in PDA plates amended with 5, 10, 15, 20 and 50 µg/mL of hygromycin B. After 14 days of incubation, the concentration of antibiotic that resulted in 100% growth inhibition was assessed visually.

5.2.4: Protoplast isolation

A two-week-old plate of *Lophiotrema* sp. F6932 was flooded with 5 mL of sterile water and scraped gently using a cell spreader. The dislodged conidia were filtered through a layer of miracloth and 500 µL of conidia inoculated into 50 mL of potato dextrose broth (PDB) in a 250mL flask and incubated at 24°C and 150 rpm for 72 h to allow the conidia to germinate. Mycelia were

collected by filtration using miracloth and washed with 4 volumes of TPC buffer (50 mM potassium phosphate, pH 7.0, 0.8 M NaCl and 20 mM $MgSO_4$). The harvested mycelia were aseptically transferred to a 250 mL Erlenmeyer flask containing 100 mL of TPC buffer supplemented with 10 mM Dithiothreitol (DTT) and incubate for 1h at 30°C and 200 rpm. The mycelium was collected by filtration using Miracloth and resuspended in 30 mL of protoplasting solution in a 250 mL flask and incubated at 30°C and 90 rpm. The protoplasting solution consisting of 1.2 M KCl and 20 mg/mL of cell wall-digesting enzyme was prepared an hour prior to protoplast isolation and had been stirred for 30 minutes followed by filtered through a 0.45µm filter. The release of protoplasts was checked from 10 µL aliquots taken hourly.

Three commercial cell wall-digesting enzymes: Lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich, USA), yatalase (Takara Bio Inc, Japan) and snailase (Sigma-Aldrich, USA) were evaluated for their efficacy in protoplast generation with two of the best performing enzymes additionally tested in a cocktail consisting of 10 mg/mL of each enzyme. Protoplast yield was determined as the total number of protoplasts generated in protoplasting solution at the end of the incubation period divided by the fresh weight of the mycelia used (i.e. protoplasts/g FW*).* After the incubation period and when most of the mycelia had been digested and maximum concentration of protoplast could be recovered, protoplasts were filtered from the protoplasting solution first using 100 µm cell strainer followed by 20 µm cell strainer to remove residual mycelia. The solution containing the protoplast was centrifuged at 1000 × g for 5 min at 4°C. The protoplasts were gently resuspended in 10 mL chilled STC buffer (10 mM Tris-HCl; pH 7.5, 1M sorbital, 20 mM $CaCl_2$) using a wide orifice pipet tip and centrifuged at 1000 × g for 5 min at 4 °C. This centrifugation and resuspension step was repeated twice with a known volume of STC

buffer used in the last resuspension step. Protoplast concentration was determined using a hemocytometer and the concentration adjusted to a range of 1 - 5 × 10^8 protoplasts/mL followed by addition of 7% DMSO. Aliquots of 200 µL in 1.5 mL Eppendorf tubes were stored at -80°C until when required for the transformation process.

5.2.5: Design of sgRNA and amplification of the HygR repair template

The ketosynthase (KS) domain of the putative palmarumycin gene cluster (*PAL*) is 1290 bp long (Figure 1). The sgRNAs for *PAL* KS deletion were designed using PhytoCRISP-Ex, a stand-alone program for the identification of target sequences for CRISPR-CAS9 editing which was also used to check for potential off-targets (Rastogi et al., 2016). The design of the sgRNAs was based on the following parameters: (i) Two sgRNA cleavage sites located approximately 100 bp upstream and downstream of the KS domain (ii) the protospacer adjacent motif (PAM) sequence for the sgRNAs was 5′-NGG-3′ ('N' being any nucleotide base). Hygromycin phosphotransferase (*hph*) gene under the control of *Aspergillus nidulans trpC* promoter and terminator and which had been previously cloned into vector pUC19 in *E. coli* was used as a selectable marker. Plasmid DNA was isolated from *E. coli* harboring the plasmid of interest using QIAprep Spin Miniprep kit (Qiagen, Germany) following the manufacturers' instructions. For generation of the repair template for CRISPR/Cas9 RNP-mediated gene editing, hygromycin B phosphotransferase expression cassette was PCR-amplified using primer set 50-bp-HomoF/50-bp-HomoR. The resultant PCR fragments were purified using MEGAquick-spin total fragment DNA purification kit (iNtRON Biotechnology, South Korea). The purified PCR products were sequenced to check for any error and then used as a repair template consisting of 1892 bp hygromycin B resistance cassette flanked on both side by 50 bp microhomology arm targeting the two RNP cleavage sites (Figure 5.1).

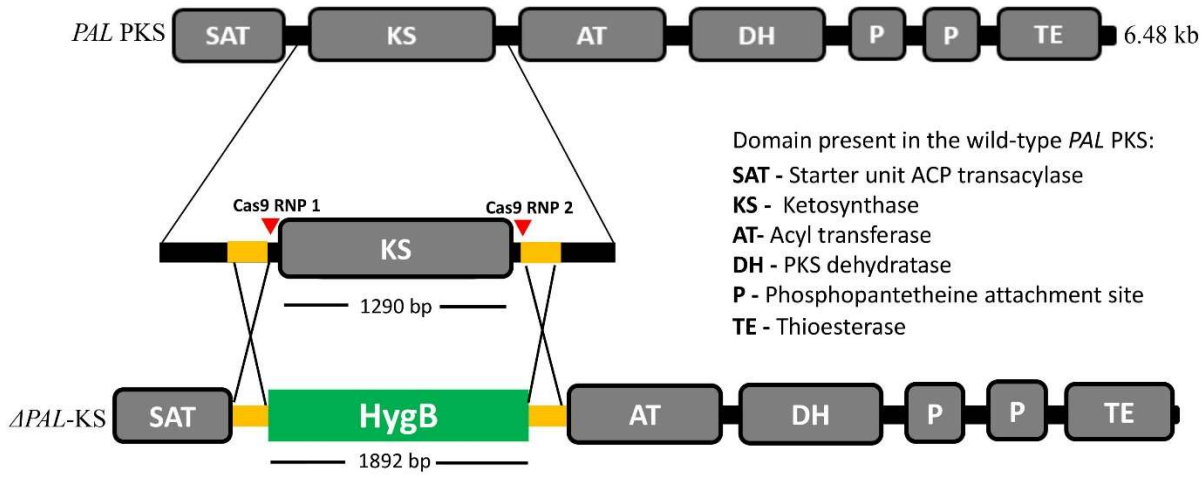


Figure 5:1 Schematic illustration of *PAL* ketosynthase (KS) domain deletion strategy by *in vitro*-assembled dual Cas-9 ribonucleoprotein coupled with homology-directed repair. The red triangles indicate the cleavage sites of the two Cas9-RNPs, while the orange and green segments indicate the 50-bp microhomology arms and the hygromycin B resistance cassette, respectively (Gakuubi et al., 2022b).

5.2.6: Assessment of sgRNA efficiency by in vitro DNA cleavage

Prior to use of the designed sgRNA for CRISPR-Cas9 mediated gene editing, it is important to test their efficiency in cleaving the target DNA. To do this, an *in vitro* Cas9 cleavage assay is performed. The assay entails three steps, namely, (1) PCR-amplification of a target DNA template containing one or several sgRNA target cleavage sites; (2) *in vitro* cleavage of the target sequence by recombinant Cas9 and candidate sgRNA; (3) separation of cleavage products by agarose gel electrophoresis. The procedure described by (Bente et al., 2020; Mehravar et al., 2019) with some modifications were followed for the *in vitro* pre-validation of the designed sgRNAs. *Lophiotrema* sp. F6932 genomic DNA was isolated from mycelia harvested from fleshy subcultured MEA plates. Approximately 100 mg of mycelia were scraped using sterile toothpick and grounded to

powder using a sterile mortar and pestle. DNA was extracted using DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturers' instructions. The cleavage activity was assessed on a 1664-bp PCR product encompassing the *PAL* KS domain amplified from the genomic DNA using primer set ChkF and ChkR (Table 5.1). Duplex RNA (crRNA and tracrRNA) were resuspended in nuclease-free duplex buffer (IDT, USA) to a stock solution of 100 µM. The two were then mixed in equimolar concentration to a final concentration of 10 µM using nuclease-free duplex buffer, annealed by heating at 95°C for 5 min in a thermomixer followed by cooling to room temperature for 15 mins. To assemble the CRISPR RNP complex, crRNA:tracrRNA duplex was combined with Alt-R *S. pyogenes* Cas9 nuclease (IDT, Singapore) in equimolar amounts and incubated at room temperature for 10 min. The cleavage reaction was performed by mixing the following: 1 µL of 10X Cas9 nuclease reaction buffer (200 mM HEPES, 1M NaCl, 50 mM $MgCl_2$, 10 mM EDTA, pH 6.5), 1 µL of 1 µM Cas9 RNP, 3 µL of 10 nM of target DNA template and 5 µL of nuclease-free water. The mixtures were incubated at 37°C for 1 hr. After incubation, 1 µL of proteinase K (20 mg/mL) was added to the reaction followed by incubation of the mixture at 56°C for 10 min to release the DNA from the Cas9 endonuclease. The products of each reaction were then visualized by electrophoresis on 1% agarose gel stained with SYBR safe DNA gel stain. All the oligonucleotides used in the current study were purchased from Integrated DNA Technologies (IDT, Singapore) and are shown on Table 5.1.

Table 5:1 Primers and oligonucleotides used in the study. Microhomology arms used for homology directed repair are underlined while sequence specific for the amplification of HygR cassette are in lower case.

Name	Sequence (5'-3')	Type or purpose
gRNA1	GTAACGGTTTGCATCGACCT-GGG	5' crRNA (Sequence – PAM)
gRNA2	GAGTCCACACGTTGTGATCG-CGG	3' crRNA (Sequence – PAM)
50-bp-HomoF	AACAGCCTGGCATCGGCCCTAAA GGCAGGTGGCCAGACCTCGATCTC TGTgctagtggaggtcaacac	Amplification of HDR-HygR repair template with 50-bp microhomology arm
50-bp-HomoR	CTTCAACGAAGCAATAGACCTGGC AGTAACCGTAACGGTTTGCATCGA CCaacccaggggctggtgac	Amplification of HDR-HygR repair template with 50-bp microhomology arm
ChkF	TTCAGCAACAGCGAGTGC	Verification of knockout mutants, and amplification of template for *in vitro* Cas9 cleavage assay to check for gRNA efficiency
ChkR	GAGACACAGACAGCGACG	Verification of knockout mutants, and amplification of template for *in vitro* Cas9 cleavage assay to check for gRNA efficiency
KSF1	AGGAGAGCTGGTCCACCATC	Verification of knockout mutants
KSR1	GTGCTAACGTCAGTGCCATC	Verification of knockout mutants
HyF1	TTTCGGCTCCAACAATGTCC	Verification of knockout mutants (Hygromycin resistance cassette)
HyR1	GCTGCTCCATACAAGCCAAC	Verification of knockout mutants (Hygromycin resistance cassette)

Name	Sequence (5'-3')	Type or purpose
ITS86F	GTGAATCATCGAATCTTTGAA	Amplification of the internal transcribed spacer 2 (ITS2) region in rDNA gene
ITS4	TCCTCCGCTTATTGATATGC	Amplification of the internal transcribed spacer 2 (ITS2) region in rDNA gene

5.2.7: PEG-mediated protoplast fungal transformation

PEG-mediated protoplast transformation was performed following the procedure describe by (Abdallah et al., 2017) with some modifications. The two gRNAs were generated by separately hybridizing each crRNA to the tracrRNA using nuclease-free duplex buffer to a final concentration of 33 µM. The mixtures were heated at 95°C for 5 min in a thermomixer followed by cooling to room temperature for 15 min. To generate the Cas9-NRP complexes, 1.5 µL of each gRNA was separately mixed with 0.75 µL of 1 µg/µL Cas9 enzyme and 11 µL of 1× PBS buffer at room temperature and the mixtures incubated at room temperature for 10 min. For the control, Cas9 enzyme was replaced with PBS. The two reaction mixtures were then combined to a final volume of 26.5 µL at room temperature and used for protoplast transformation. Protoplasts stored at -80°C were thawed on ice and centrifuged at 3000 × *g* for 5 min at 4 °C to remove DMSO. The protoplasts were resuspended in 400 µL chilled STC buffer, centrifuged and finally resuspended in 200 µL STC buffer before transferring into a sterile 15 mL conical tube containing 26.5 µL Cas9-NRP complex. This was followed by the addition of 5 µg of purified repair template and 25 µL of PEG-$CaCl_2$ buffer (30% [wt/vol] PEG 3350, 1M $CaCl_2$, 50 mM Tris-HCl, pH 7.5), then incubation on ice for 90 min. Subsequently, 1.25 mL of PEG-$CaCl_2$ buffer was added followed by incubation at

room temperature for 20 min. The mixture was diluted to a total volume of 3 mL with STC buffer and transferred into 7 mL of warm molten malt extract agar (1.5% wt/vol agar) supplemented with 1.2 M sorbitol in a 50 mL conical tube. The mixture was swirled gently and poured into petri dish, allowed to cool and incubated at 24°C for 72 hours. Subsequently, the plates were overlaid with 10 mL molten MEA (1.5% wt/vol agar) supplemented with 1.2 M sorbitol and 50 µg/mL of hygromycin B. The negative control plate (lacking Cas9 RNP) was overlaid with MEA without the antibiotic while the positive control plate (with the Cas9 RNP) had MEA containing the antibiotic. The plates were incubated at 24° C for 4 more days with the growth of colonies on the plates monitored regularly. Single colonies growing in the experimental plates were subculture on fresh MEA plates containing 50 µg/mL of hygromycin B.

5.2.8: Screening for stable transformants

Putative CRISPR/Cas9-induced deletion mutants that grew through the overlay MEA supplemented with hygromycin B were transferred to new MEA plates containing the antibiotic. After 10 days of growth the mutants were subcultured into new MEA plates containing the selection antibiotics. Mycelia were cut from the periphery of an actively growing 10-day old cultures of these plates and subcultured in non-selective media (MEA without hygromycin B) and incubated for 10 days. This step was repeated for three successive times. Finally, putative mutants growing on non-selective medium were subcultured back to selective media to confirm their resistance towards hygromycin B. Growth of the putative mutants in MEA plates supplemented with 50 µg/mL of hygromycin B at this stage indicated that they were mitotically stable (Ibragimova et al. 2020). Successful deletion of the KS domain and integration of hygromycin B resistance gene *hph* into the transformants genome was then confirmed by PCR and sequencing.

5.2.9: Fungal cultures extraction and liquid chromatography-mass spectrometry (LC-MS) analysis

The wild-type strain and the two arbitrarily selected *Lophiotrema* sp. F6932 iPKS$^{\Delta KS}$ mutants were fermented in CF02LB media for generation of crude extracts. The fungal strains were grown in 50 mL media in 250 mL conical flasks by inoculating three mycelial discs (5 mm in diameter) into each of the media flask. The cultures were incubated for 14 days in a shaking incubator at 24°C and 200 rpm for 21 days. The cultures were then frozen at −80°C and freeze-dried in a vacuum freeze-dryer to expel all moisture before extracted using MeOH. The crude extracts were dissolved in 1:1 ratio of DMSO and 50% MeCN/H_2O (0.1% formic acid) and prepared in 20 mg/mL concentration. After centrifugation at 14,600 rpm for 5 min, the supernatants were transferred to LC vials and a volume of 2 µL of the extract was subjected to LC-HRESIMS. LC-HRESIMS was performed on an Agilent UHPLC 1290 Infinity coupled to Agilent 6540 accurate-mass quadrupole time-of-flight (QTOF) mass spectrometer equipped with a splitter and ESI source. Liquid chromatography was carried out using an Agilent Poroshell 120 SB-C18 (4.6 × 75 mm, 2.7 µm) using MeCN and H_2O, both containing 0.1% formic acid, as mobile phase. Initially, an isocratic system of 5% of MeCN/H_2O was employed for 2 min, followed by a linear gradient to 100% MeCN over 16 min and an isocratic wash of 100% MeCN for 5 min; all at a flowrate of 2 mL/min. Mass spectrometer parameters were set as previously described (Sirota et al., 2018). MS data were analyzed and processed using Agilent MassHunter Qualitative Analysis Version 10.0. The presence of palmarumycins was confirmed by comparison with our in-house standard compounds library (Gakuubi et al., 2022a). All the solvents for extraction and LCMS were Fisher Chemical HPLC or LCMS grade.

5.2.10: Effects of culture conditions on production of bioactive secondary metabolites in *Lophiotrema* sp. F6932.

Following the identification of the antibacterial secondary metabolites from *Lophiotrema* sp. F6932, we aimed to assess how variation of culture conditions would affect the production of the antibacterial secondary metabolites form this fungal strains. To achieve this, the fungal strain was grown in two different media CF02LB and PDB in the presence of two chemical elicitors; 50 µM 5-azacytidine and 100 µM SAHA. Furthermore, a previous study had revealed that addition of Cu^{+2} in cultures of endophytic fungus *Berkleasmium* sp. Dzf12 grown in PDB resulted in enhanced production of palmarumycins C_{12} and C_{13} (Mou et al., 2015). Thus, *Lophiotrema* sp. F6932 was grown in PDB media in the presence of 250 µM Cu^{2+}. Additionally, it was observed that *Lophiotrema* sp. F6932 sporulation and formation of the dark colour associated with melanin biosynthesis occurred around the 5^{th} day of growth. Thus we aimed at assessing whether the antibacterial secondary metabolites are produced before or after this stage of fungal growth. Thus, the antibacterial activities of *Lophiotrema* sp. F6932 extracts isolated on the fourth day of fermentation was compared with those of the fungus subjected to the normal 14 days of fermentation. Lastly, we aimed at assessing whether *Lophiotrema* sp. F6932 antibacterial secondary metabolites are produced intracellularly or extracellularly. Therefore, both biomass and supernatant-derived extracts were prepared from *Lophiotrema* sp. F6932 and tested for their antibacterial activities.

5.3: Results

5.3.1: Genomic DNA isolation from *Lophiotrema* sp. F6932

Genomic DNA samples from *Lophiotrema* sp. F6932 were first isolated using DNeasy PowerSoil Kit (Qiagen, Germany) and sent for whole genome sequencing at Macrogen (South Korea). Following quality control (QC) assessment by Macrogen, this first batch of sampled did not meet the required QC standards because of having low DNA Integrity Number (DIN) values. DIN is a numerical measure of the gDNA integrity and ranges from 1 to 10, where 1 indicates highly degraded gDNA and 10 represents highly intact gDNA (Macrogen, 2015). A DIN of ≥9 is considered the threshold QC pass before gDNA samples can be processed for the next steps of sequencing. After optimization of the various step of gDNA extraction all aimed at minimizing DNA shearing and degradation, a second batch of gDNA samples were extracted using the same DNA extraction kit and protocol and resubmitted to Macrogen. However, once again, all the submitted samples failed to pass the QC test with regard to the DIN values.

Thereafter, different methods were employed for gDNA extraction from the fungus in order to establish the method that would result in the extraction of better quality gDNA. These methods included; the conventional phenol-chloroform method for extraction of gDNA from filamentous fungi and the use of additional commercial DNA extraction kits including; Dneasy Powersoil Pro kit, (Qiagen, Germany), Dneasy Powersoil kit (older version) (Qiagen, Germany) and MasterPure Yeast DNA Purification Kit (Lucigen, Singapore). Figure 5.2 shows gDNA electrophoresis profile of samples isolated using different methods. After submission of the gDNA sample to Macrogen, the samples extracted using the phenol-chloroform method had the best DIN value and passed the QC and thus were selected for whole genome sequencing. It is worth noting however that gDNA

extracted via phenol-chloroform method had RNA contamination and required further treatment for removal of RNA contamination while samples extracted using the three commercial kits were free of RNA and did not require RNase treatment (Figure 5.2).

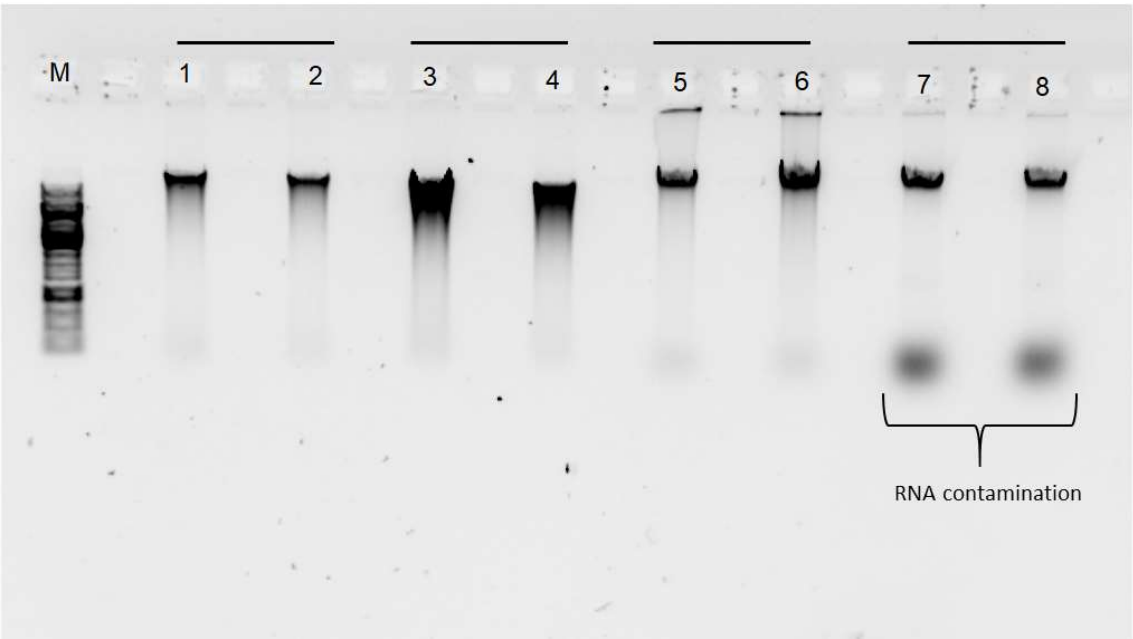


Figure 5:2 Gel electrophoresis profile of genomic DNA samples from *Lophiotrema* sp. F6932 obtained using different extraction protocols. 1 and 2 (Dneasy Powersoil kit), 3 and 4 (Dneasy powersoil pro kit), 4 and 5 (MasterPure yeast DNA purification kit) and 7 and 8 (phenol-chloroform method), M (1kb DNA ladder). 1µL of aliquots of each sample were run on a 1% agarose gel.

5.3.2: Characterization of *Lophiotrema* sp. F693 by phylogenetic analysis

Lophiotrema sp. F6932 was previously identified on the basis of sequencing analysis of ITS2 region of the rDNA gene. To further establish the identity of this isolate and its phylogeny, multilocus sequence analysis was conducted using an aligned sequence dataset comprising of 1014 nucleotide positions from nrSSU, 256 from ITS, 1116 from nrLSU, 1004 from RPB2 and 899 from *TEF1-α*. Phylogenetic analysis was performed using a data set of 21 taxa representing 9 genera in

the family *Lophiotremataceae* with *Crassiparies quadrisporus* HHUF 30409 as the outgroup. The resultant ML and NJ phylogenetic tree analyses of the combined datasets yielded the best scoring trees for *Lophiotremataceae* (Figure 5.3 and Figure 5.4) and show that F6932 is close to *L. eburnoides* HHUF 30079, with 91.97% identity score in the ITS locus. Based on MLSA analysis of the five loci, *Lophiotrema* sp. F6932 differs from *L. eburnoides* HHUF 30079 by 20 nucleotides (nt) in the D1/D2 domains in the ITS region. Variations are also observed in other gene sequences such as LSU (11 nt), nrSSU (3 nt), *TEF1-α* (40 nt), and RPB2 (137 nt) when compared to *L. eburnoides* HHUF 30079. These results indicate that *Lophiotrema* sp. F6932 represents a potentially novel species within the Genus *Lophiotrema.* The sequences for the nrSSU, nrLSU, ITS, RPB2 and *TEF1-α* for this strain are available in the GenBank database and their accession numbers are given in Appendix IX.

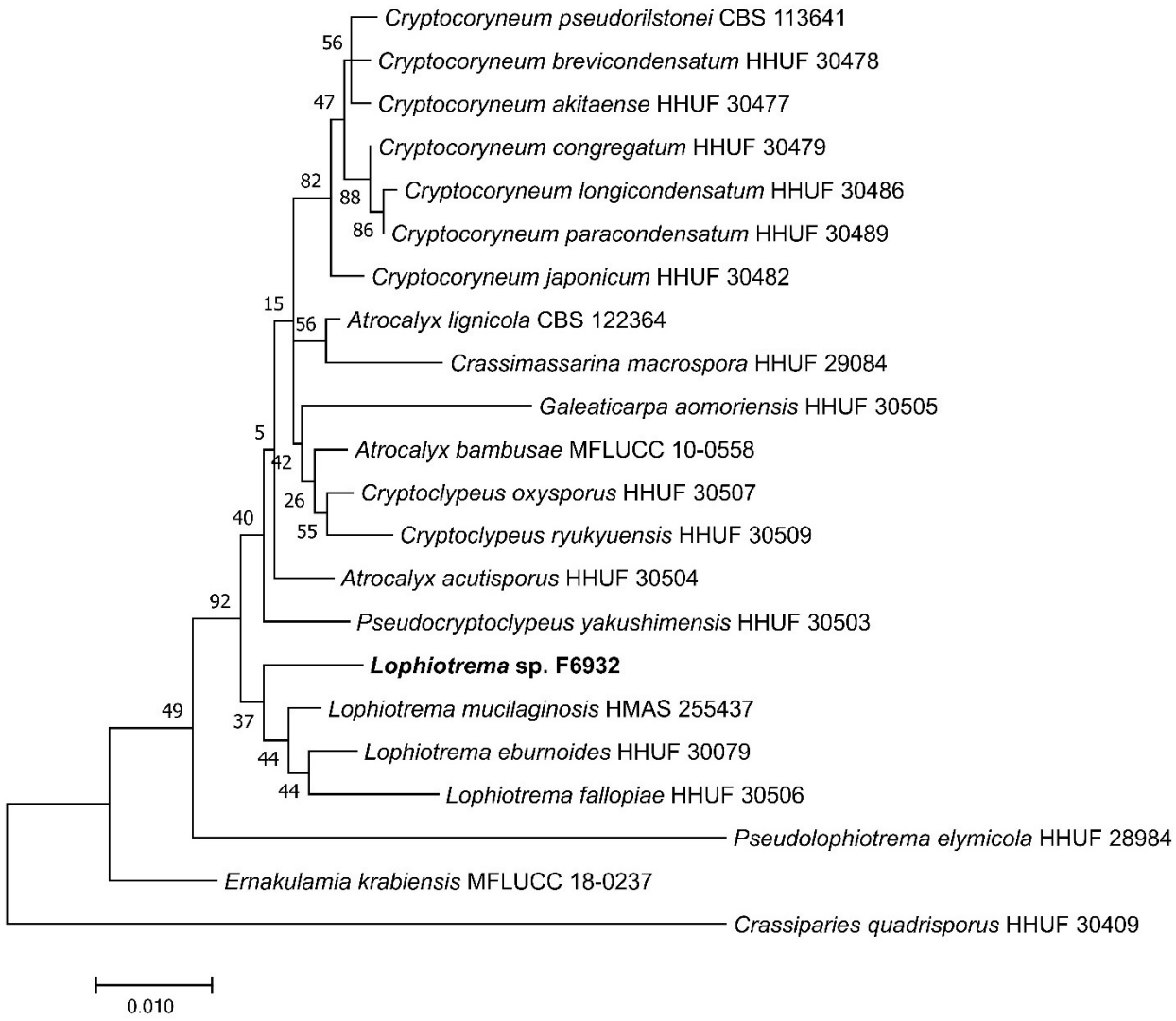


Figure 5:3 Molecular phylogenetic analysis by maximum likelihood method showing selected members of the family *Lophiotremataceae. Crassiparies quadrisporus* HHUF 30409 was used as the outgroup in the analysis (1000 bootstrap replications).

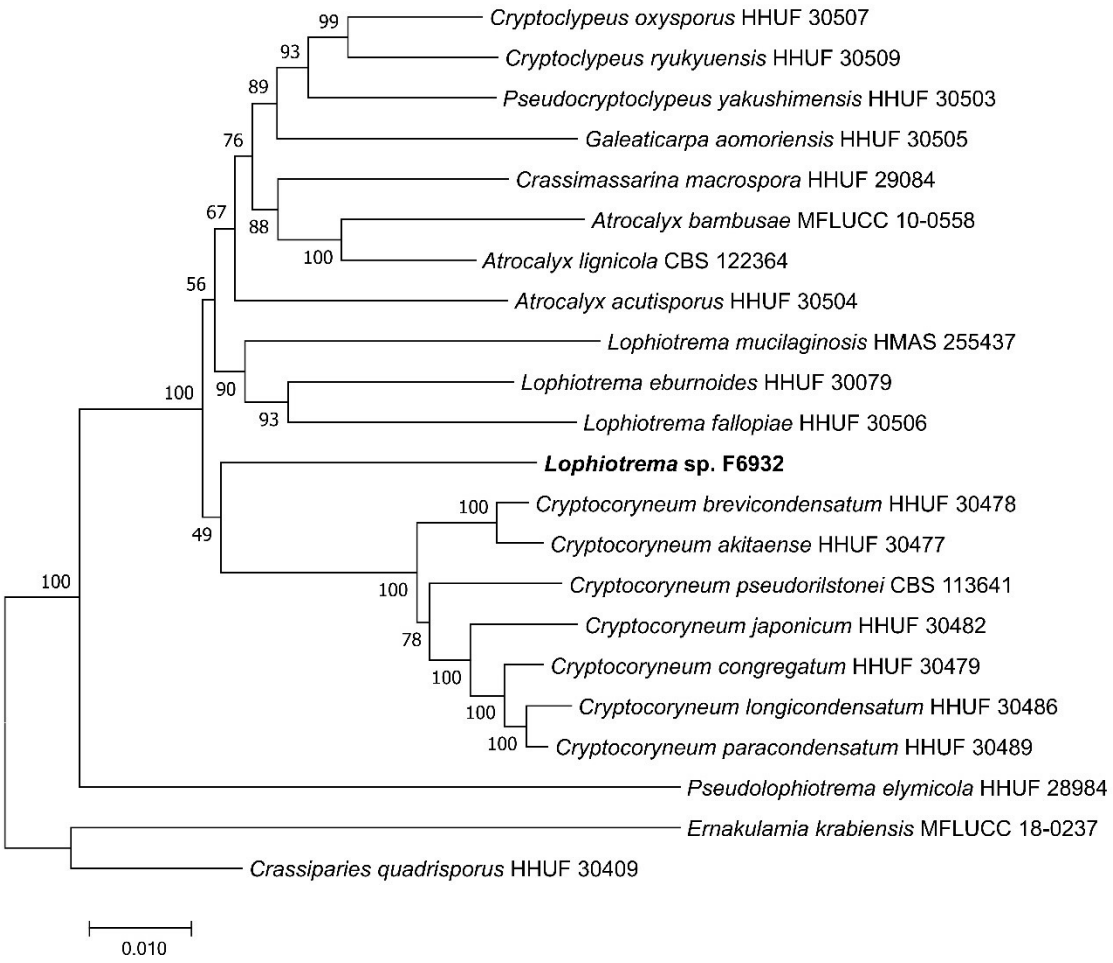


Figure 5:4 Molecular phylogenetic analysis by neighbor-joining method showing selected members of the family *Lophiotremataceae. Crassiparies quadrisporus* HHUF 30409 as the outgroup (1000 bootstrap replications).

5.3.3: *Lophiotrema* sp. F6932 secondary metabolites biosynthetic potential

Genome sequencing data revealed that *Lophiotrema* sp. F6932 has a 51.4% GC content with 37,108,200 bp (37.1MB) spread over 17 contigs. Table 5.2 gives a summary of genome assembly and annotation results for the fungal strain. *Lophiotrema* sp. F6932 genome assembly data were submitted to antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) server for biosynthetic gene clusters (BGCs) prediction. The genome sequencing data revealed the presence of

a large number of secondary metabolite biosynthetic gene clusters (BGCs) with majority of the BGCs being of unknown type (Figure 5.5 and Appendix X). *Lophiotrema* sp. F6932 was predicted to contain 14 T1PKS, 8 NRPS-like, 6 NRPS and 6 terpene gene clusters in addition to a single T3PKS and NRPS-terpene hybrid cluster. Majority of the predicted biosynthetic gene clusters in *Lophiotrema* sp. F6932 were found to bear no similarity with any known gene clusters (Appendix X**).**

Table 5:2 Genome assembly and annotation results summary for *Lophiotrema* sp. F6932

Strain identity	Contigs	Total length (bp)	GC content (%)	N50	Genome annotation	
					Hit	No hit
Lophiotrema sp. F6932	17	37,108,200	51.37	1,955,251	12,937	1,140

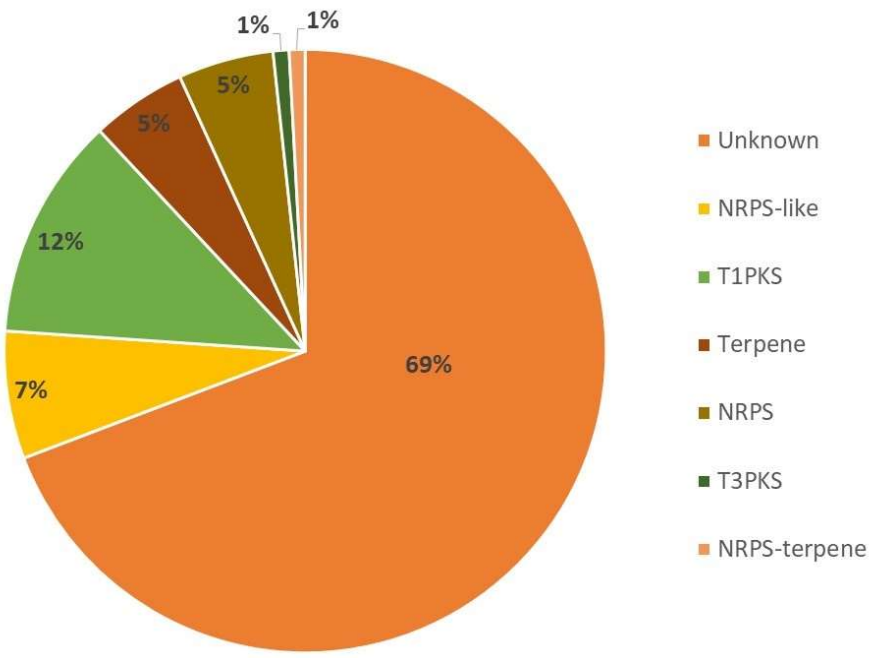


Figure 5:5 A summary of the classes of secondary metabolite gene clusters in *Lophiotrema* sp. F6932 genome as predicted by antiSMASH.

Analysis of antiSMASH data resulted in the identification of a putative 6.48 kb type 1 PKS (*PAL*) (Genbank accession number ON768617) that was predicted to be involved in the biosynthesis of palmarumycins in *Lophiotrema* sp. F6932. Moreover, *PAL* showed a 100% similarity to the melanin biosynthetic gene cluster from phytopathogenic fungus *Bipolaris oryzae* based on antiSMASH analysis results (Moriwaki et al., 2004). Therefore, *PAL* was likewise predicted to be involved in the biosynthesis of melanin in this fungus with the two compounds believed to share the same precursor DHN (Mafezoli et al., 2018).

5.3.4: Sensitivity of *Lophiotrema* sp. F6932 to hygromycin B antibiotic

In order to assess the suitability of using hygromycin B resistance gene (*hph*, hygromycin B phosphotranferase) as a selection marker, the sensitivity of *Lophiotrema* sp. F6932 wild-type to

hygromycin B was evaluated by growning the wild-type strain in plates amended with different concentrations of the antibiotic**.** Plates amended with 20 µg/mL hygromycin B resulted in complete growth inhibition of the fungus, an indication that hygromycin B resistance gene would be suited for used as a selectable marker for *Lophiotrema* sp. F6932 knockouts mutants (Figure 5.6).

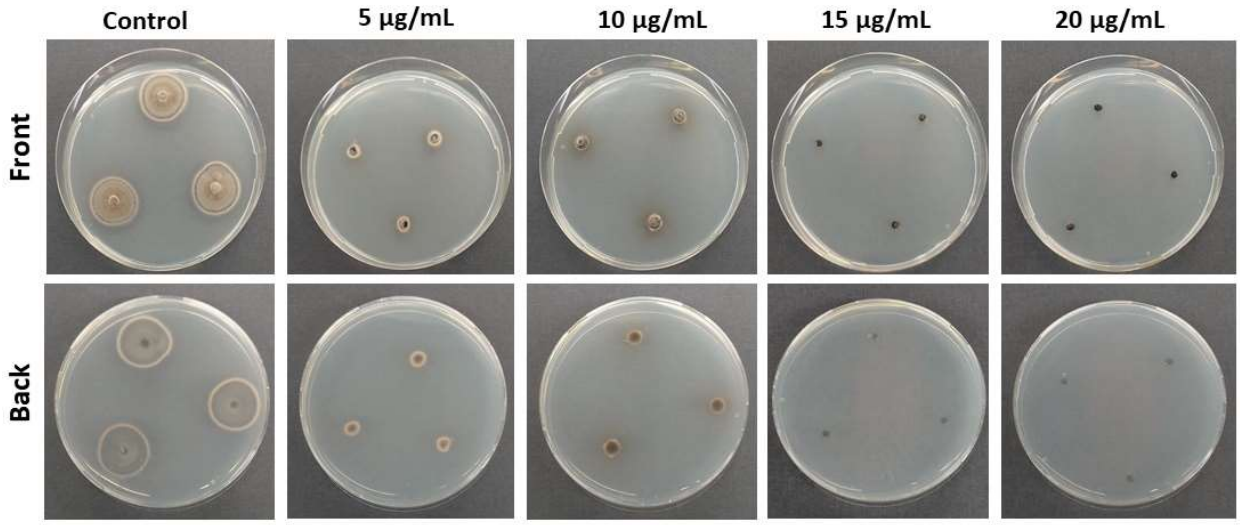


Figure 5:6 Sensitivity of wild-type *Lophiotrema* sp. F6932 grown in PDA with different concentrations of hygromycin B after 14 days.

5.3.5: Protoplast isolation from *Lophiotrema* sp. F6932

No protocol exists in literature for protoplast isolation from members of *Lophiotrema* genus or even the closely related genus *Lophiostoma*. Thus, we used several existing protoplasts isolation methods as the basis for developing a simple yet efficient method for protoplast generation from *Lophiotrema* sp. F6932 (Abdallah et al., 2017; Ning et al., 2022; Roth & Chilvers, 2019). Preliminary studies had shown that potato dextrose broth (PDB) was the best media for generating mycelia for protoplast isolation from this fungus compared with two other media; malt extract broth (MEB) and Sabouraud dextrose broth (SDB). Three enzymes; lysing enzymes from *T.*

harzianum, yatalase, and snailase at the rate of 20 mg/mL were tested for their efficacy in protoplast generation. After 5 h of incubation, most of the mycelia was digested and maximum concentration of protoplast was recovered. The best performing of the three enzymes was yatalase with a mean yield of 5.2×10^7 protoplasts/g FW followed by lysing enzymes from *T. harzianum* with a mean yield of 2.9×10^7 protoplasts/g FW. Snailase enzyme had the lowest protoplast generation capacity with a mean yield of 7.8×10^6 protoplasts/g FW (Figure 5.7). Protoplast yield by yetalase was significantly higher than that obtained with either lysing enzymes from *T. harzianum* or a cocktail of lysing enzymes from *T. harzianum* and yatalase. There was, however, no significant differences in the mean yield of protoplasts between lysing enzymes from *T. harzianum* and the cocktail of the two enzymes (Figure 5.7).

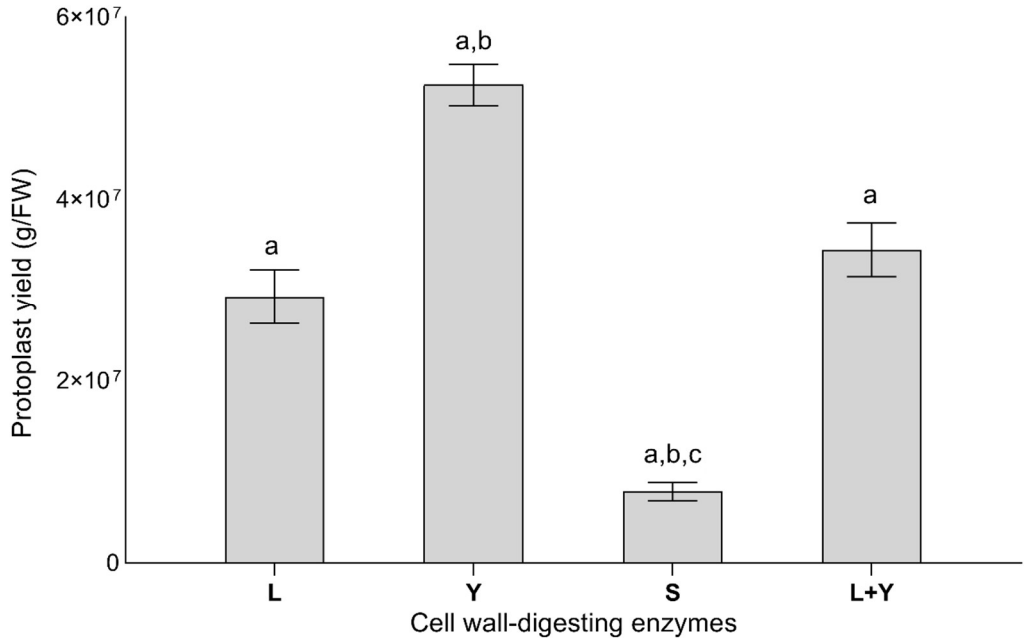


Figure 5:7 Protoplasts yield from *Lophiotrema* sp. F6932 using different cell wall-digesting enzymes. (L= lysing enzymes from *T. harzianum*, Y = yatalase, S = snailase and L+Y= cocktail of L and Y). Results are expressed as means ± SEM from three biological replicates. Different lowercase letters (a-c) among the treatments indicate statistically significant differences between means by one-way ANOVA with Tukey–Kramer multiple comparison test ($P < 0.05$).

5.3.6: *In vitro* cleavage efficiency of sgRNAs

The two sgRNAs (Table 5.1) that were designed for use in this study were subjected to *in vitro* cleavage assay to assess their ability to cleave the target 1664 bp PCR fragment encompassing the *PAL* KS domain. Analysis of the cleavage reaction using agarose gel electrophoresis resulted in the expected band sizes (Figure 5.8). Cleavage using gRNA1 produced expected fragments of approximately 1494 bp and 170 bp, gRNA2 produced fragments of approximately 1557 bp and 107 bp while the use of the two gRNAs together resulted in expected bands of 1387 bp, 107 bp and 170 bp (Figure 5.7). On the basis of these results, the two gRNA were deemed to be efficient

in the cleavage of the target sites during the polyethylene glycol (PEG)-mediated transformation experiment.

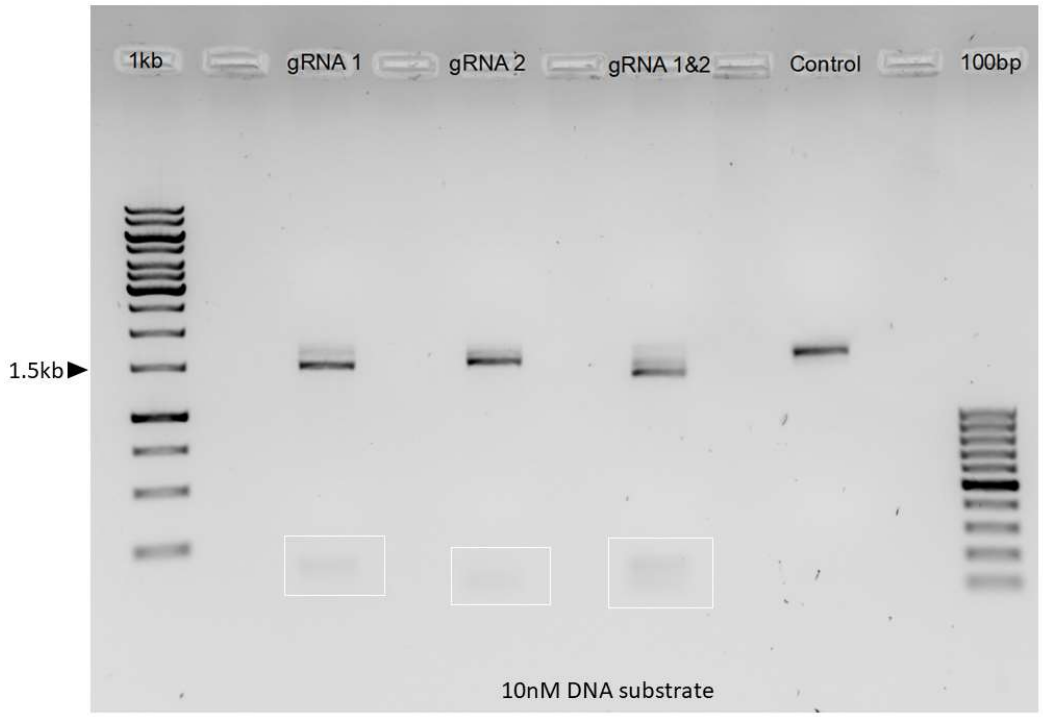


Figure 5:8 sgRNA-mediated *in vitro* cleavage of the target region encompassing the ketosynthase domain of *PAL*. 10 nM of 1664 bp PCR fragment was mixed with Cas9 enzyme and each of the two sgRNAs individually and then together. For the negative control, a reaction with all the reagents minus the Cas9 RNP was included for comparison. Bands of lower size (<200 bp) appears as smears at the bottom of the gel photographs and have been highlighted using white rectangles and their sizes can be estimated using the 100 bp ladder.

5.3.7: Morphological characteristics of RNP-mediated CRISPR-Cas9 deletion mutants

The morphology of *Lophiotrema* sp. F6932 iPKS$^{\Delta KS}$ mutants was evaluated after 7 and 14 days of growth on three common media; malt extract agar (MEA), potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA). All the deletion mutants displayed completely different morphological characteristics from the wild-type strain. In all the three tested media, the mutants

had a white to creamy appearance in contrast to characteristic dark coloration of the wild-type strain after 7 days (Figure 5.9A). However, by the 14th day of incubation, the mutants' pigmentation changed in all the three media. In SDA, the colonies showed three distinct regions; creamy white peripheries surrounding an orange-red middle section and a creamy white patch at the center. In PDA, the colonies had orange-red peripheries surrounding a creamy white/yellowish in the middle while in MEA exhibited mixed morphologies with some colonies retaining the creamy white appearance while others had in addition some orange-red patches. (Figure 5.9A). When the mutants were grown beyond three weeks, they retained the three distinct shades of colour in SDA, while in PDA the mutants retained the creamy white peripheries with concentric rings and orange-red the middle and yellowish and orange-red patches at the centre of the colony. In MEA, the orange-red colour spread to the entire colony (Figure 5.9B).

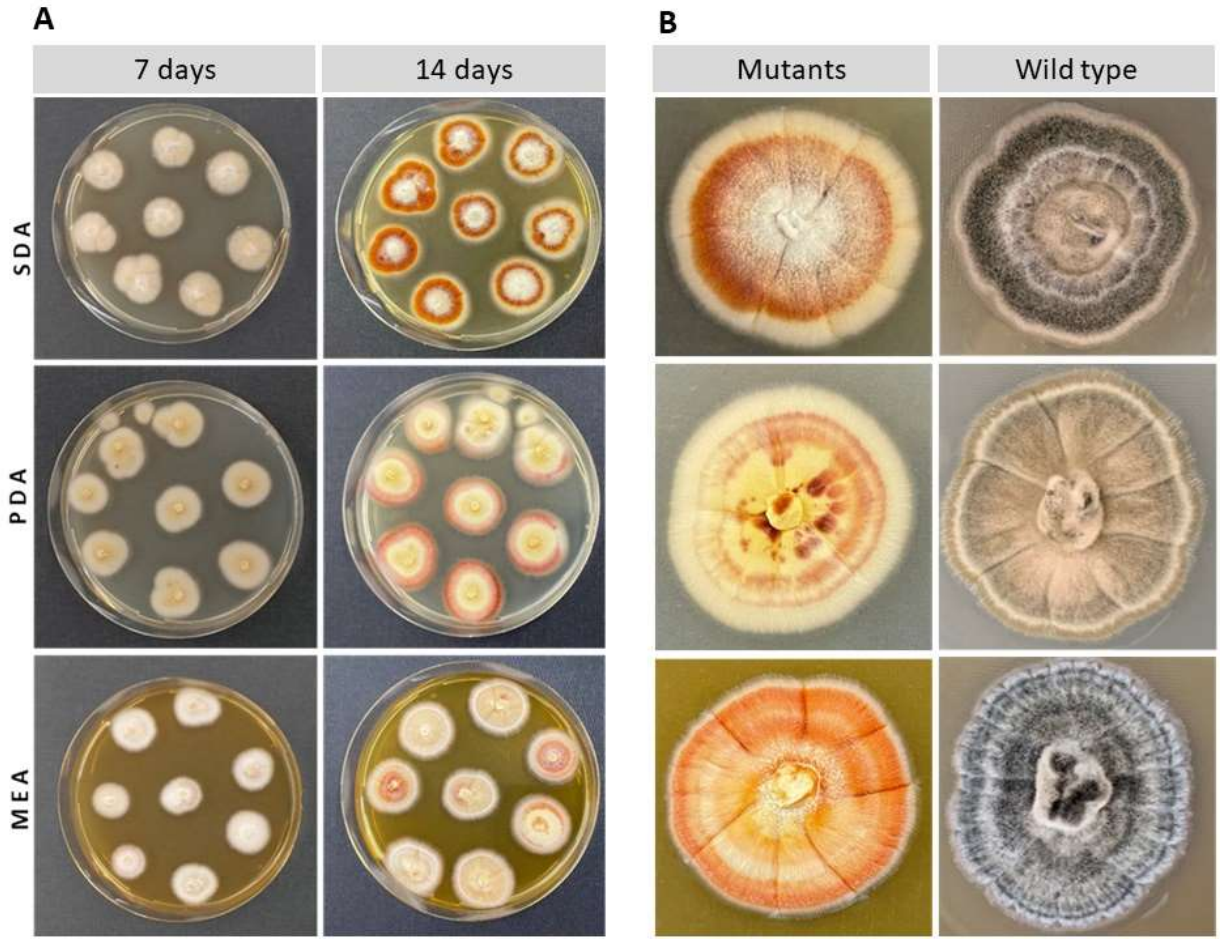


Figure 5:9 Morphological characteristics of *Lophiotrema* sp. F6932 CRISPR/Cas9-induced mutants. (A) Morphology of the deletion mutants after 7 and 14 days of growth in SDA, PDA and MEA media (B) close up view comparison of the colony morphology of the deletion mutants and the wild-type strain after 21 days of growth in SDB, PDA and MEA media (Gakuubi et al., 2022b).

In addition to the observed differences in colony morphology between the deletion mutants and the wild-type strain, the deletion mutants were moreover able to grow in the selective media even after the concentration of hygromycin B was increased four-fold of what was used in the transformation experiment (200 µg/mL). There were furthermore clear differences in the colour of mutant-derived crude extracts when compared with those derived from the wild-type strain (Figure

5.10). However, no growth differences were observed between the radial growth rate of the deletion mutants in comparison with the wild-type strains.

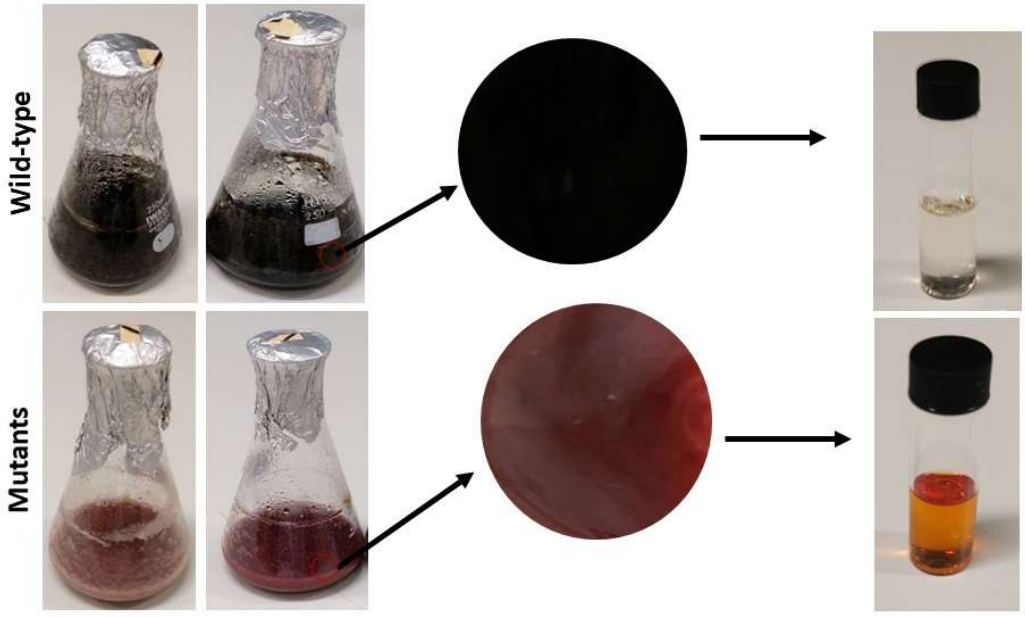


Figure 5:10 Differences in the coloration of crude extracts derived from wild-type and CRISPR/Cas9-induced *Lophiotrema* sp. F6932 mutants grown in CF02LB media.

To evaluate the pathway that *Lophiotrema* sp. F6932 uses in melanin biosynthesis, the wild-type strain was grown in the presence of two 1,8-dihydroxynaphthalene (DHN) melanin synthesis inhibitors phthalide and tricyclazole, and two 3,4-dihydroxyphenylalanine (DOPA) melanin synthesis inhibitors kojic acid and tropolone. The results showed inhibition of melanin biosynthesis when the fungus was grown in the presence of phthalide and tricyclazole, confirming that this fungus, similar to *B. oryzae,* produces melanin through the DHN pathway (Figure 5.11). However, cultures of the deletion mutants grown in PDB lacked the characteristic dark colour of the wild-type strain suggesting that they were unable to produce melanin. Thus, there were no

observable differences in the appearance of deletion mutants' cultures when grown in the presence all the four melanin synthesis inhibitors (Figure 5.11).

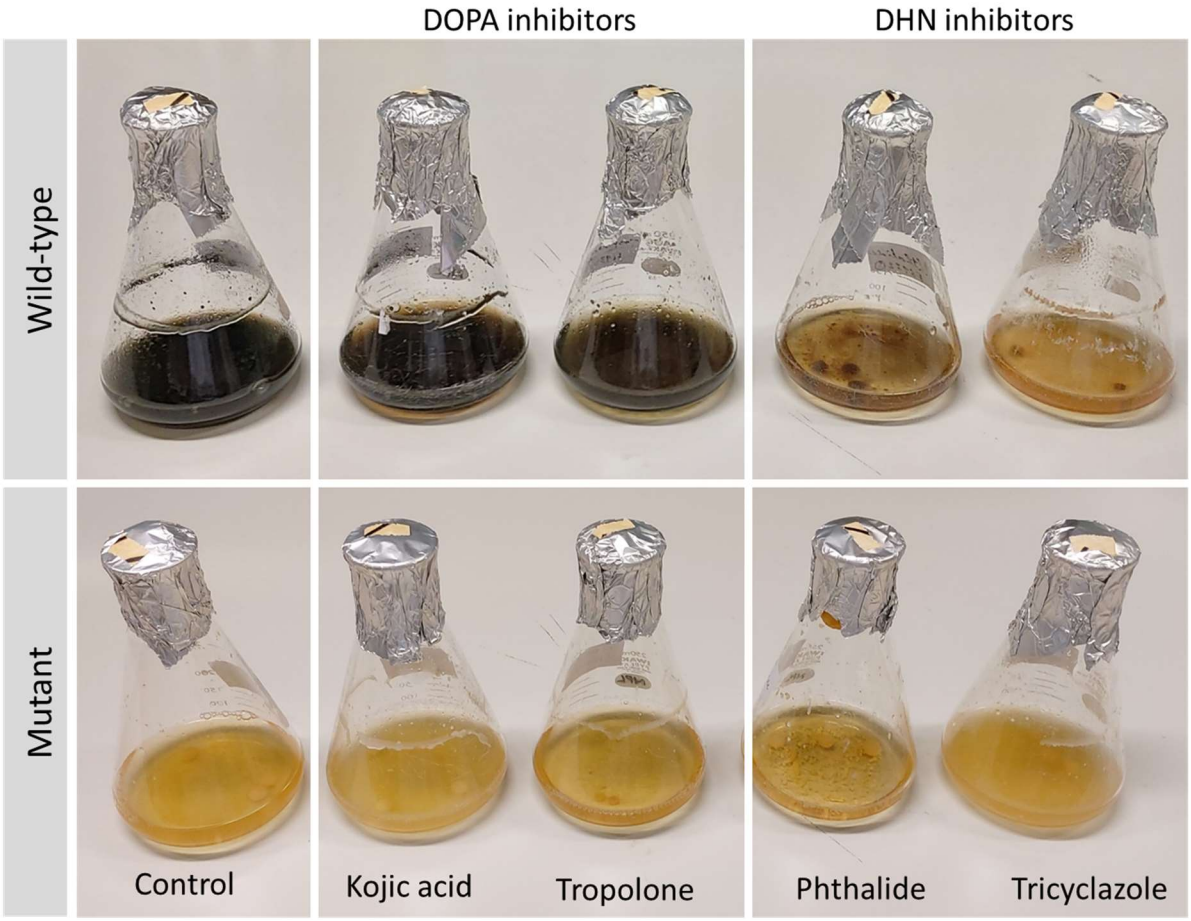


Figure 5:11 Growth of *Lophiotrema* sp. F6932 wild-type strain and CRISPR/Cas9-induced mutants in PDB in the presence of different melanin synthesis inhibitors after 6 days. The concentration of kojic acid, phthalide and tricyclazole used was 100 µg/mL while 12.5 µg/mL of tropolone was used. The results show that melanin biosynthesis in wild-type was inhibited only in the presence of two DHN melanin synthesis inhibitors (Gakuubi et al., 2022b).

5.3.8: Molecular analysis of hygromycin-resistant mutants

The identity of the deletion mutants was confirmed through sequencing of the ITS2 region using primer set ITS86F/ITS4. The putative iPKS$^{\Delta KS}$ deletion mutants were then screened by PCR amplification and sequencing of the PCR products. PCR amplification of the genomic sequence upstream and downstream of the two Cas9-RNPs cleavage sites was performed from genomic DNA isolated from ten randomly selected mutants using ChkF/ChkR primer set (Figure 5.12A). This resulted in the expected fragment of 2169 bp consistent with the correct integration of the HygR cassette within the cleavage sites in all the 10 mutants, while a 1664 bp band was obtained in the wild-type strain (Figure 5.12B). Analysis of the sequencing results of the resultant PCR products revealed the presence of the entire HygR cassette sequence in all the ten mutants. Furthermore, to confirm the orientation of the integrated HygR cassette, PCR amplification the 10 deletion mutants and wild-type strain was performed using primer sets KSF1/HyR1 and HyF1/KSR1 (Figure 5.12A). This resulted in the expected bands of approximately 1629 bp and 1388 bp in all the 10 deletion mutants respectively. As would be expected, no band was obtained in the wild-type strain since HyR1 and HyF1 primers are located with the HygR cassette (Figure 5.12 C and D).

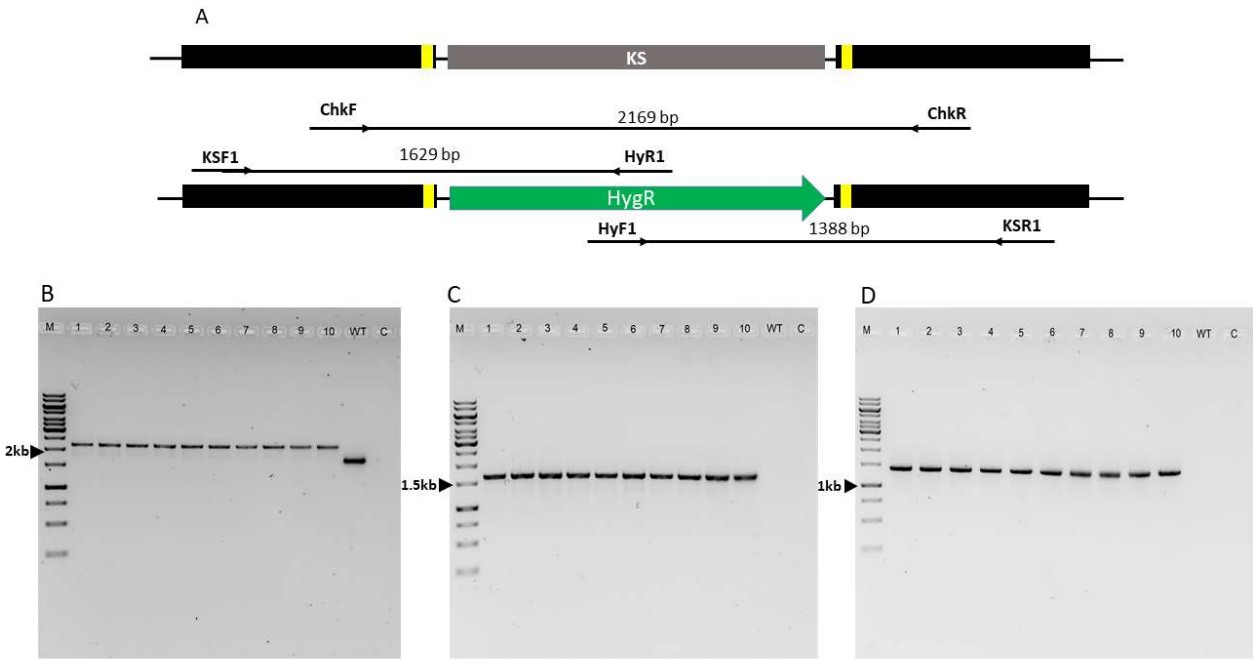


Figure 5:12 PCR analysis of *Lophiotrema* sp. F6932 iPKS$^{\Delta KS}$ deletion mutants. (A) An overview of the PAL KS domain locus in the wild-type strain and integration of HygR after CRISPR-Cas9 RNP-mediated deletion of the KS domain*;* (B) PCR amplification of HDR-HygR repair template integration locus spanning the cleavage sites of Cas9-RNPs complexes using ChkF/ChkR primer set showing the expected 2169 bp and 1664 bp bands in the mutants and wild-type strain, respectively; (C) PCR amplification of the deletion mutants and wild-type strain using primer sets KSF1/HyR1 and (D) HyF1/KSR1. M = 1kb DNA ladder, 1-10 = iPKS$^{\Delta KS}$ deletion mutants resistant to hygromycin B, WT = wild-type strain, and C = Control. The full-length gel pictures are shown in appendix XI (Gakuubi et al., 2022b).

5.3.9: Palmarumycin analysis results

The target gene for this study was the polyketide synthase, *PAL* that was predicted to be involved in the biosynthesis of palmarumycins. We hypothesized that a knockout of the KS domain in *PAL* would disrupt the functioning of the gene and consequently the production of this group of compounds in *Lophiotrema* sp. F6932. Therefore, LC-MS analysis was performed on MeOH

extracts obtained from the cultures of *Lophiotrema* sp. F6932 wild-type strain and two arbitrarily selected hygromycin B resistant mutants grown under the same conditions. The LC-MS analysis results confirmed the presence of palmarumycins in wild-type strain extracts but not in mutant-derived extracts (Figure 5.13). These results thus confirm the involvement of *PAL* in palmarumycins biosynthesis.

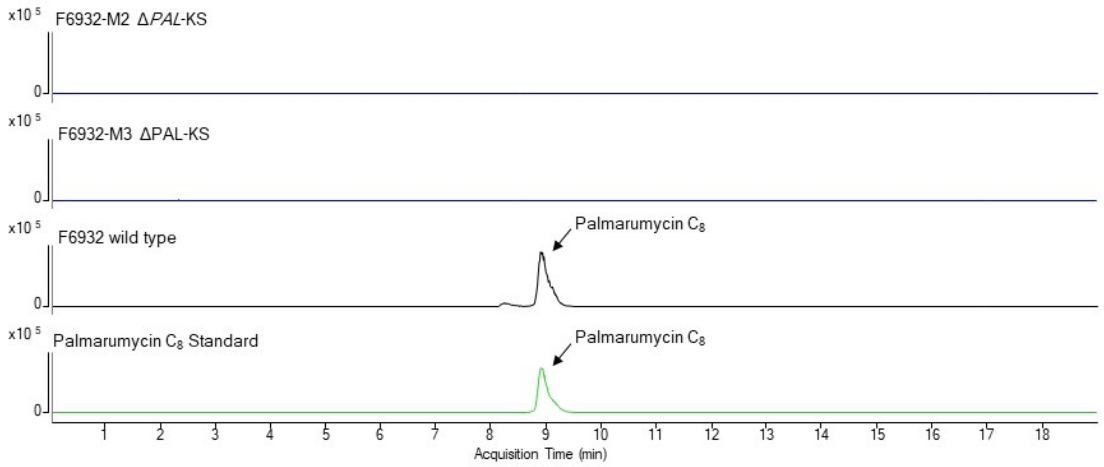


Figure 5:13 Extracted ion chromatograms (EIC) for palmarumycin C_8 of cultures extracts from wild-type *Lophiotrema* sp. F6932 (black line) and two arbitrarily selected iPKS$^{\Delta KS}$ mutants (blue lines). Palmarumycin C_8 (*m/z* =385.0473 ± 10 ppm, RT 8.8 min).

5.3.10: Biosynthesis of antibacterial SMS in *Lophiotrema* sp. F6932 under different culture conditions.

Lophiotrema sp. F6932 extracts generated from the fungus grown in CF02LB and PDB media in the presence of the two chemical elicitors; 5-azacytidine and SAHA and Cu^{2+} were tested for their antibacterial activities against *S. aureus*. The result revealed that both 5-azacytidine and SAHA resulted in a significant increase in the production of antibacterial metabolite(s) in CF02LB (Figure 5.14). There was however no significant change in the production of antibacterial metabolite(s) in

Lophiotrema sp. F6932 when grown in two different media (CF02LB and PDB) in the presence of the two chemical elicitors 5-azacytidine and SAHA (Figure 5.14). With regard to the stage at which the antibacterial secondary metabolites are produced in *Lophiotrema* sp. F6932, extract derived from four-days old culture exhibited significantly lower antibacterial activity compared with those derived from 14 days of incubation, an indication that the compounds of interest are produced at a later stage of the fungal growth. With regard to whether antimicrobial SMs from *Lophiotrema* sp. F6932 are produced intracellularly or extracellularly, extracts derived from both culture supernatant and biomass revealed strong antibacterial activities. However, there was no significant difference in the antibacterial activity of extracts derived from the culture supernatant and those derived from the biomass (Figure 5.14).

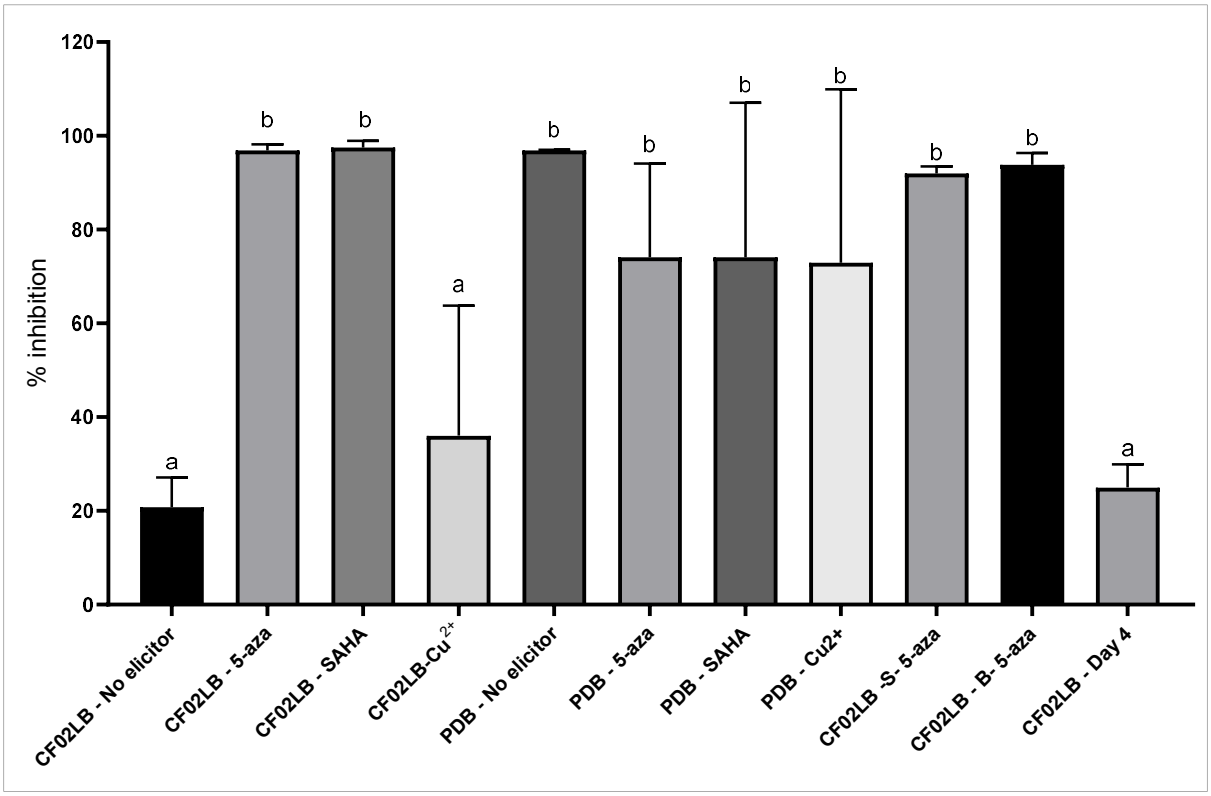


Figure 5:14 Effects of different culture conditions on the production of antibacterial secondary metabolites in *Lophiotrema* sp. F6932. Different lowercase letters (a–d) among the treatments indicate statistically significant differences between means by one-way ANOVA with Tukey–Kramer multiple comparison test ($p < 0.05$). (S= supernatant, B= biomass and 5-aza = 5-azacytidine).

5.4: Discussion

The emergence of CRISPR-Cas9 genetic editing technology has opened up new avenues for genetic manipulation of practically all type of fungal strains thus overcoming the many challenges that are associated with the classical methods of genome engineering of this group of microorganisms (Nødvig et al., 2015; Schuster & Kahmann, 2019). In this study, we report the successful CRISPR/Cas9-based genome editing in *Lophiotrema* sp. F6932 using *in vitro*

assembled Cas9-RNPs coupled with homology-directed repair. We used a similar approach employed in transformation of *Aspergillus fumigatus* using *in vitro*-assembled Cas9-gRNA RNP coupled with microhomology repair template (Abdallah et al., 2017). This method does not require generation of a plasmid harboring the Cas9 expression cassette but rather, entails the use of a complex of purified and commercially available Cas9 enzyme and sgRNAs to direct double strand breaks (DSBs) upstream and downstream of the targeted region (Abdallah et al., 2017; Ferrara et al., 2019; Valente et al., 2021). This is followed by HDR using repair templates harboring homology arms on both sides. We successfully employed this approach in the characterization of palmarumycin biosynthetic gene cluster (*PAL*) in *Lophiotrema* sp. F6932 through deletion of *PAL* ketosynthase domain followed by homology-directed repair using hygromycin resistance cassette flanked by 50 bp of homology on both side of the DSBs.

Among the many barriers towards genetic transformation of filamentous fungi is the difficulty encountered in generating high-quality protoplasts from some fungal strains (Roth & Chilvers, 2019). This is compounded by the fact that some fungal strains are not amenable to protoplasting (McCluskey & Baker, 2017). Different species of fungal strains possess cell walls with unique properties requiring the use of specific protoplast isolation conditions including the types and concentrations of cell wall-digesting enzymes (Li et al., 2017; Roth & Chilvers, 2019). Moreover, selection of an appropriate type and concentration of an osmotic stabilizer in the protoplasting medium is very critical in preventing lysis or shriveling of protoplast due to osmotic stress (Ren et al., 2018; Wu & Chou, 2019). Other than the enzymes and osmotic stabilizers, other factors such as enzymatic hydrolysis temperature, duration of incubation, fungal age and mycelium status need to be optimized for each strain (Jin et al., 2020; Ning et al., 2022). For example, depending on

whether the fungal strain is slow or fast-growing, very short culture time may yield less mycelium and thus low yield of protoplast. On the other hand, prolonged culture time may result in aged mycelia whose thick cell wall may be resistant to enzymatic hydrolysis (Ning et al., 2022; Wei et al., 2012).

Because of the many differences that exist among fungal strains regarding the cell wall composition, most often, the existing protoplast isolation protocols have to be optimized, and at times completely new protocols developed for efficient protoplast isolation and eventual transformation especially among the uncommon fungal strains (Li et al., 2017). *Lophiotrema* sp. F6932 is a slow-growing and heavly-melanized fungal strain; two characteristics that have been shown to contribute significantly to recalcitrant to protoplast generation and amenability to genetic manipulation in filamentous fungi (Ishikawa et al., 2010; Voigt et al., 2020). No protoplast isolation protocol exists in literature for fungi belonging to *Lophiotrema* genus or even the closely related phylogenetic groups such as *Lophiostoma.* Our attempt to use available protoplast isolation methods failed to generate the quantity of protoplasts needed for the transformation process for *Lophiotrema* sp. F6932. Therefore, through optimization of various growth parameters and used of different types and concentrations of cell wall-digesting enzymes and osmotic stabilizers, and borrowing from existing protoplast isolation protocols (Abdallah et al., 2017; Ning et al., 2022; Roth & Chilvers, 2019), we designed a simple yet efficient method for protoplast isolation from *Lophiotrema* sp. F6932. Of all the factors that we optimized during the design of protoplast isolation protocol from this fungus, protoplast yield from different enzymes was studied in detailed. To this end, three enzymes; lysing enzymes from *T. harzianum*, yatalase, and snailase at the rate of 20 mg/mL were tested for their efficacy in protoplast generation. Two of the best

performing enzymes were additionally tested in a cocktail consisting of 10 mg/mL of each enzyme. There were significant differences in protoplast yield among the three enzymes used. Yetalase resulted in significantly higher yield of protoplasts compared with lysing enzymes from *T. harzianum* and snailase enzyme. Furthermore, a cocktail of yetalase and lysing enzymes from *T. harzianum,* two of the best performing enzymes produced significantly lower yields of protoplasts than yetalase used alone. Generally, different fungal strains will vary in their sensitivity to different enzyme because of the differences in the cell wall composition and structure (Jin et al., 2020). Therefore, depending on the nature of the fungi cell wall, different cell-wall digesting enzymes will act on different sites of the cell wall resulting in variations in the level of cell wall hydrolysis and consequently the quantity of protoplasts released (Ning et al., 2022). Thus, the observed difference in the yield of protoplasts in the current study is indicative of the differences in the sensitivity of *Lophiotrema* sp. F6932 to different enzymes due to the fungus cell wall composition.

A number of factors have been found to have an influence on the overall success rate of homologous recombination in PEG-mediated fungal transformation. For example, a study by (Abdallah et al., 2017) revealed that the concentration of the repair template, length of microhomology arms and concentration of Cas9 nuclease have an influence on the efficiency of CRISPR-Cas9 mediated gene editing in wild-type and a *ΔakuB* strains of *A. fumigatus.* The study found out that the use of 2 µg of repair template franked by either 35 bp or 50 bp homologous sequence resulted in high efficiency gene deletion (up to 97%) in *ΔakuB* strain. However, using the same concentration of the repair template flanked by 35 bp and 50 bp homologous sequence resulted in 46% and 74% gene deletion efficiency in the wild-type strain, respectively (Abdallah et al., 2017). In another study, a modified CRISPR/Cas9 gene editing system was developed for

A. niger in which the efficiency of gene replacement was tested using repair templates with 100 bp and 500 bp arm lengths. Interestingly, the results revealed that using 100-bp homology arms was sufficient to obtain recombination at high frequency (Zhang et al., 2019). In another study focusing on the development of a CRISPR-Cas9 RNP-mediated co-editing and counter selection system for rice blast fungus *Magnaporthe oryzae,* two lengths of homology sequences namely; 30 bp and 40 bp were used. The results demonstrated that 30 bp homologous sequence was sufficient in promoting homologous recombination of the repair template resulting in successful generation of *ALB1* mutants that were unable to produce melanin (Foster et al., 2018). In the current study, homologous recombination frequency of 71.4 % was achieved using 50 bp homology arms and 5 µg of the repair template. These results are comparable to those obtained in a study on RNP-directed genome editing of *Trichoderma reesei,* where homologous recombination frequency of 73.9% was obtained with 50 bp homology sequences (Zou et al., 2020). It would be interesting to assess how variation of these among other factors would impact on the overall success rate of homologous recombination in the transformation of *Lophiotrema* sp. F6932 in the future.

The *PAL* gene cluster in *Lophiotrema* sp. F6932 shows high similarity (100%) to the melanin biosynthetic gene cluster from phytopathogenic fungus *Bipolaris oryzae* based on antiSMASH analysis results. *Bipolaris oryzae* produces melanin via the 1,8-dihydroxynaphthalene (DHN) pathway using acetate as a precursor (Moriwaki et al., 2004). *Lophiotrema* sp. F6932 is a heavily-melanized fungi and growth of the fungus in different melanin synthesis inhibitors revealed that similar to *B. oryzae,* it produces melanin via the DHN and not the DOPA (3,4-dihydroxyphenylalanine) pathway. *Lophiotrema* sp. F6932 produces spirobisnaphthalene palmarumycin C_8 among other analogues such as palmarumycin CP_{30} (Gakuubi et al., 2022a).

Significantly, the biosynthesis of palmarumycins has been proposed to involve phenolic oxidative dimerization of 1,8-dihydroxynaphthalene, the same precursor that is involved in melanin biosynthesis in many filamentous fungi (Bode & Zeeck, 2000; Mafezoli et al., 2018). It is on this basis therefore that we predicted the involvement of *PAL* in the biosynthesis palmarumycins in addition to melanin in *Lophiotrema* sp. F6932. To confirm this, we deleted the ketosynthase domain of the *PAL* followed by homology-directed repair using hygromycin *resistance* gene cassette. The ketosynthase domains catalyzes polyketide chain elongation via decarboxylative Claisen condensation of acyl and malonyl (Robbins et al., 2016; Yun et al., 2020). The resultant *Lophiotrema* sp. F6932 deletion mutants were no longer able to produce palmarumycins and other related compounds, confirming that *PAL* is responsible for biosynthesis of this group of compounds. Moreover, the mutants were similarly unable to produce melanin when grown in PDB confirming that indeed, 1,8-dihydroxynaphthalene (DHN) is the immediate precursor for biosynthesis of both palmarumycins and melanin (Mafezoli et al., 2018). Results from this study thus shows that deletion of the KS domain rather than the entire PAL PKS is sufficient to disrupt the genes involved in the biosynthesis of these compounds. Because the biosynthesis of dimeric pentaketide spirobisnaphthalenes such as palmarumycins that involve the phenolic oxidative coupling of DHN has been well-documented, we therefore, propose the biosynthetic routes for melanin, palmarumycin C_8, and palmarumycin CP_{30} from their shared precursor DHN in *Lophiotrema* sp. F6932 (Figure 5.15).

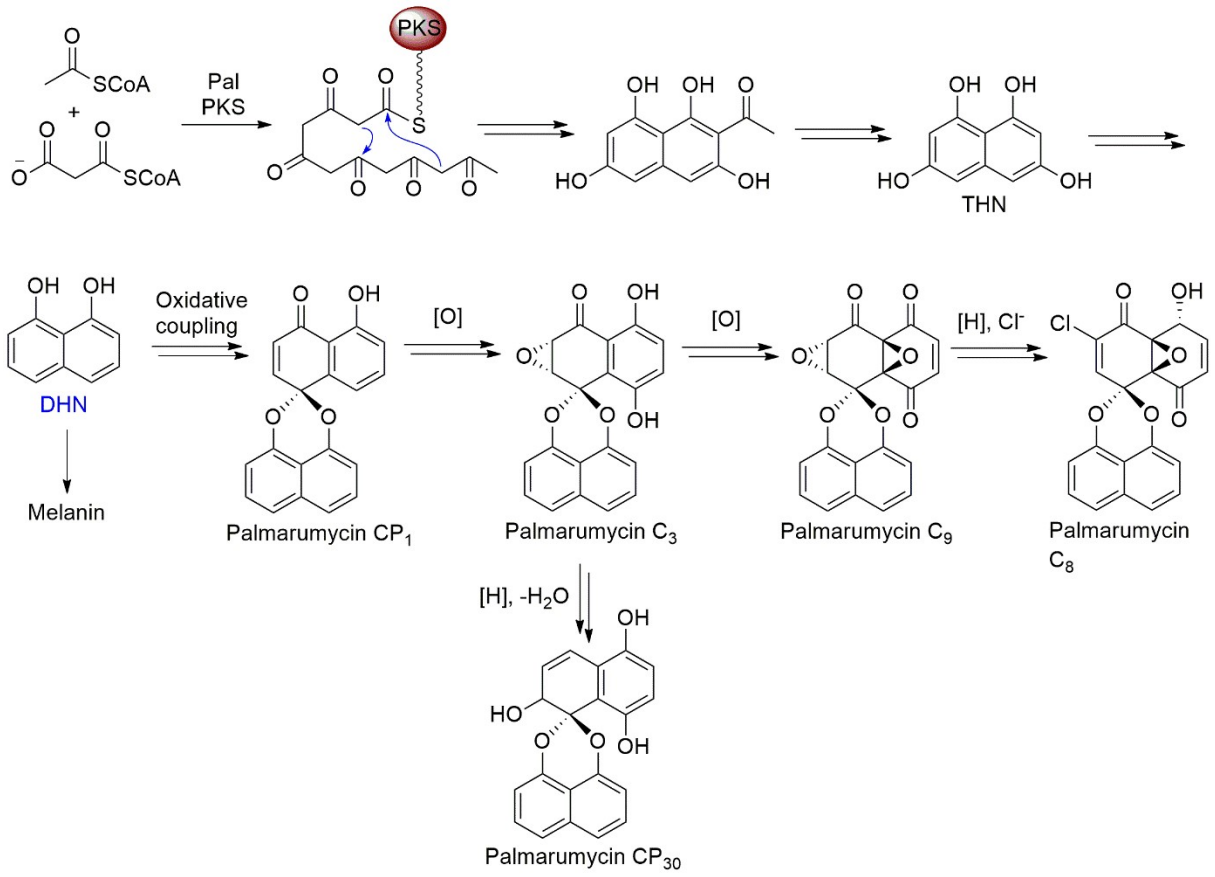


Figure 5:15 Proposed biosynthetic routes to melanin, palmarumycin C_8, and palmarumycin CP_{30} from the shared precursor DHN in *Lophiotrema* sp. F6932 (Gakuubi et al., 2022b).

Results of the testing for antibacterial activity of crude extracts from *Lophiotrema* sp. F6932 grown in CF02LB and PDB media in the presence of the two chemical elicitors and Cu^{2+} revealed that 5-azacytidine and SAHA greatly enhanced the production of antibacterial metabolite(s) in CF02LB but slightly reduced their production in PDB media. A previous study had reported that Cu^{2+} enhanced the production of palmarumycins C_{12} and C_{13} in the fungus *Berkleasmium* sp. Dzf12 (Mou et al., 2015). It has been suggested that Cu^{2+} enhances the activity of lacasses which are fungal multi-copper oxidases that are involved in melanin biosynthesis and thus may also play a key role in the biosynthesis of spirobisnaphthalenes such as palmarumycins (Mafezoli et al., 2018).

Indeed, Cu^{2+} had previously been shown to enhance the production of laccases by the white-rot fungus *Pleurotus pulmonarius* (Tychanowicz et al., 2006). In the current study however, Cu^{2+} at a concentration of 250 µM slightly increased the production of antibacterial secondary metabolites in CF02LB media but appear to have a suppressive effect on the biosynthesis of such metabolites in PDB media. Regarding the stage of growth at which the antimicrobial secondary metabolites are produced in *Lophiotrema* sp. F6932, it was observed that no such metabolites were produced at day 4 before the sporulation of the fungi. A study investigating the biosynthesis of palmarumycin C_{13} in the fungus *Berkleasmium* sp. Dzf12 found out that production of this compound remain very low within the first 5 days of incubation and then increased gradually with the highest amount of the compound produced around day 13 (Zhao et al., 2013). Similarly, a study investigating the enhancement of palmarumycin C_{12} and C_{13} biosynthesis through addition of oligosaccharides from the fungus host plant revealed that maximum yield of the two compounds was achieved on day 15 (Li et al., 2012). The same study also reported that the largest proportion of palmarumycin C_{13} was produced extracellularly with the rest produced intracellularly. Interestingly, palmarumycin C_{12} was not detected in the broth; an indication that it was produced intracellularly but not secreted into the media (Li et al., 2012). In the current study, antibacterial secondary metabolites were found in extracts derived from both the supernatant and the biomass. This may be an indication that while these metabolites may have been produced intracellularly, a large fraction of these metabolites are secreted in the media with an approximately an equal amount being retained in the fungal mycelia.

5.5: Conclusion

We have established an efficient CRISPR-Cas9 genome editing system for *Lophiotrema* sp. F6932 which we have employed to characterize the polyketide synthases (*PAL*) involved in palmarumycin and melanin biosynthesis in this fungal strain. To achieve this, we applied *in vitro*-assembled Cas9 RNP to induce targeted deletion of ketosynthase (KS) domain of the *PAL* followed by homology directed repair with hygromycin resistance cassette flanked by 50 bp of homology arms on both sides of the DSBs. The resultant deletion mutants displayed different phenotype to that of the wild-type strain; they had different colony morphology and where no longer able to produce palmarumycins or melanin, an indication that the knockout of KS domain had ablated the ability of the fungi to produce these molecules. This confirms that *PAL* is the gene cluster responsible for palmarumycin and melanin biosynthesis in this fungus. To the best of our knowledge, this is the first report of successful CRISPR-Cas9 mediated gene editing of a fungus belonging to *Lophiotrema* genus. This study therefore paves the way for implementing similar approach in characterization of other BGCs of interest in this largely understudied fungal strain.

Chapter 6: Conclusion and Future Study

The main objective of this study was to investigate the diversity and bioactive potential of fungal endophytes from A*STAR's Natural Product Library (NPL) previously isolated from eight sites corresponding to four different types of habitats in Singapore. On the basis of the study's findings, the following conclusion and recommendations are made:

6.1: Conclusion

6.1.1: Diversity and characterization of fungal endophytes

Endophytic fungal diversity is influenced by a myriad of biotic and abiotic factors, such as the dominant vegetation types and age, climatic and weather factors such as precipitation, variation and exposure to solar intensity, various edaphic factors among others factors. The fungal isolates used in the current study were originally isolated from eight different sites that corresponds to four different habitats in Singapore and preserved at the A*STAR's Natural Product Library. Since field isolation of fungal isolates was not part of this study, nor are the sample sizes from each of the study habitat equal, we have limited ourselves with regards to making generalized conclusion per se regarding the species richness and diversity in this study. Instead, we give a general picture of the diversity of the strains that were successfully revived and used in this study. On the basis of the study findings therefore, we can conclude that:

1) The largest fraction of the identified fungal endophytes (99%) belongs to the phylum *Ascomycota* with two strains belonging to the phylum *Basidiomycota*, and only one strain was from *Mucoromycota*.

2) *Colletotrichum* was the most dominant genera representing 27% of all the identified strains. However, members of this genus were found in habitats 2, 3 and 4 but not among the strains from habitat 1.
3) Majority of the identified genera (63%; 37 genera) were represented by a single fungal strain with the bulk of the identified fungal genera (73%; 43 genera) occurring in only one of any of the four studied habitats.
4) A total of 21 strains, an equivalent of 9% of all the studied fungal strains had < 97% sequence similarity in the ITS2 rDNA gene region when compared with reference strains from NCBI and thus some of these strains may potentially be new undescribed fungal endophytes strains.

6.1.2: Inhibitory activity of fungal endophytes

Extracts from fungal endophytes subjected to varied growth regimes were tested for their inhibitory activity namely; antibacterial, antifungal and cytotoxic activities against a panel of test microbial pathogen and cancer cell lines respectively. The following conclusions are made based on the results obtained:

1) Generally, the weight of crude extractable was higher for cultures grown in the presence of the chemical elicitors when compared with extracts grown in the absence of the chemical elicitors. Similarly, higher extract yields were obtained from strains grown on solid media compared to those derived from liquid cultures.
2) Chemical elicitation using 5-azacytidine and suberoylanilide hydroxamic acid (SAHA) resulted in increased in the number of bioactive strains. In total, 4 and 5 strains revealed antimicrobial and cytotoxic activity respectively when grown exclusively in the presence of one or both of the chemical elicitors.

3) Growth of fungal strains in three additional media likewise resulted in the discovery of more bioactive strains. In total 16 fungal strains representing 34% of all the active strains obtained from different fermentation media experiment exhibited inhibitory activity only when grown in the three additional media.
4) Among the two chemical elicitors, 5-azacytidine performed better in enhancing the production of bioactive secondary metabolites. For the media diversification study, CF02LB performed better than the other two liquid media while for the solid media, CF25ST produced the higher number hits compared to CF28ST.
5) A total 63 endophytic fungal strains representing 43% of all the fungal screened presented inhibitory activity. Out of these, 34 fungal strains revealed exclusive cytotoxic activity, 10 fungal isolates exhibited exclusively antimicrobial activity while 19 strains exhibited both antimicrobial and cytotoxic activity against at least one of the tested panel of microbial pathogens and cancer cells lines, respectively.

6.1.3: Chemical analysis of fungal secondary metabolites

Endophytic fungal extracts showing promising inhibitory activity during the primary screening were subjected to dose-response testing for confirmation of activity. Samples showing better inhibitory activity following dose-response testing were subjected to chemical dereplication and where active constituents of interest were detected, these samples were progressed to large-scale fermentation to obtain sufficient crude extracts for fractionation and structure elucidation of the purified active compounds. From this work, the following conclusions are made:

1) Based on the chemical dereplication studies, three fungal strains were progressed to large scale fermentation for compound isolation following the identification of active

constituents of interest. These three strains are *Lophiotrema* sp. F6932 fermented in CF02LB supplemented with 5-azacytidine, and *Muyocopron laterale* F5912, and *Colletotrichum tropicicola* F10154 both grown in CF28ST media.

2) A known compound palmarumycin C_8 and a new analogue palmarumycin CP_{30} were isolated from *Lophiotrema* sp. F6932. Palmarumycin C_8 exhibited strong cytotoxic activity and weak to moderate antimicrobial activities.
3) From *Muyocopron laterale* F5912, three new compounds of polyketide origin designated as muyocopronol A - C were isolated. Muyocopronol B exhibited moderate antifungal activity and weak cytotoxic activity.
4) A novel 26-membered macrolide containing four non-conjugated diene and a choline phosphate ester group and designated as tropicicolide was isolated from *Colletotrichum tropicicola* F10154. Tropicicolide exhibited the most potent antifungal activity against *A. fumigatus* with an IC_{50} of 1.8 µg/mL and moderate activity against *C. albicans* with an IC_{50} of 7.1 µg/mL.
5) Among the five novel compounds isolated from the aforementioned three fungal strains, four were uncovered through chemical elicitation and diversification of fermentation media. Therefore, had our investigation been limited to the use of two initial liquid media only, these four compounds would not have been discovered.
6) Re-isolation of already known molecules is one of the challenges that face bioassay-guided discovery of natural products. Numerous known molecules were isolated from a total of nine samples that were subjected to large-scale fermentation for compound isolation including some that were putatively identified.

6.1.4: CRISPR/Cas9 RNP-mediated genome editing of *Lophiotrema* sp. F6932

With the isolation of palmarumycin C_8 and a new analogue palmarumycin CP_{30} from *Lophiotrema* sp. F6932, we sought to identify and validate the biosynthetic gene cluster involved in the production of these compounds. With no known protocol available for genome editing in *Lophiotrema*, we developed a CRISPR/Cas9 RNP-mediated system for genome editing of this strain that we have used to validate a type 1 iterative polyketide synthases (iPKS) involved in the production of palmarumycins in this fungal strain. The following are the conclusion arising from this study:

1) Multilocus sequence analysis (MLSA) performed using five molecular markers namely; 18S nuclear small subunit ribosomal DNA (nrSSU), nuclear large subunit (nrLSU), the internal transcribed spacer region (ITS), RNA polymerase II second largest subunit (RPB2) and translation elongation factor 1-alpha (*TEF1-α*) shows that *Lophiotrema* sp. F6932 represents a potentially novel species within the Genus *Lophiotrema.*
2) Protoplast generation in *Lophiotrema* sp. F6932 is influence by numerous factors among them the type of cell wall-digesting enzymes. The observed difference in the yield of protoplasts from different enzyme may be indicative of differences in the sensitivity of *Lophiotrema* sp. F6932 to different enzymes due to the fungus cell wall composition.
3) A type 1 iterative PKS (*PAL*) BGC (6.48 kb) is responsible for biosynthesis of both palmarumycin and melanin in *Lophiotrema* sp. F6932 through phenolic oxidative dimerization of 8-dihydroxynaphthalene (DHN). Moreover, *Lophiotrema* sp. F6932 produces melanin via the DHN pathway.

4) CRISPR/Cas9 RNP-mediated genome editing of *Lophiotrema* sp. F6932 was successful with homologous recombination frequency of 71.4 % achieved using 50 bp homology arms and 5 μg of hygromycin repair template. The resultant deletion mutants were morphologically different from the wild-type strain and were no longer able to produce palmarumycin or melanin.
5) Deletion of ketosynthase (KS) domain in *PAL* rather than the entire polyketide synthases is sufficient to disrupt this gene cluster and consequently the biosynthesis of both palmarumycin and melanin in *Lophiotrema* sp. F6932.

6.2: Future study

Due to the limitation of time, a number of interesting aspects of this research project were not explored. Thus, the following recommendations are presented for future study:

1) Much progress was made in molecular identification of fungal strains via ITS2 sequencing analysis. About 9% of all the identified fungal strains had < 97% sequence similarity in the ITS2 rDNA gene region when compared with reference strains from NCBI. Some of these isolates may be potentially new undescribed strains and warrant further investigation.
2) The chemical elicitation study involved the use of two epigenetic modifiers at a single concentration for each of the chemical elicitors. The use of different concentrations of these elicitors in addition to inclusion of a selection of other chemical elicitors could be considered in future studies.
3) Other than the use of chemical inhibitors of epigenetic modification and variation of fermentation media, other approaches for activation of cryptic secondary metabolite

biosynthetic pathways in the studied strains could be considered. This may include the use of fungus–bacterium and fungus–fungus co-culture among other approaches that have been reported to be effective in enhancing the activation of silent fungal gene clusters.

4) This study reported enhanced production of bioactive secondary metabolites from selected fungal strains as a result of chemical elicitation and media diversification (e.g. for strains F6932 and F5912). In-depth studies of the mechanisms involved in the production of bioactive SMs under different growth regime of these fungi would be interesting. This may include but is not limited to transcriptomic analysis.
5) Elucidation of the biosynthetic pathway for the newly discovered compounds muyocopronols and tropicicolide would be interesting. This is so, given the fact that the BGCs for two compounds that are structurally related to these compounds i.e. bionectriol and eushearilide have not been characterized.
6) Regarding CRISPR/Cas9 RNP-mediated genome editing of *Lophiotrema* sp. F6932, homologous recombination frequency of 71.4 % was achieved using 50 bp homology arms and 5µg of the repair template. These two factors among others have been found to have an influence on the CRISPR/Cas9 RNP-mediated deletion efficiency in other filamentous fungi. It would be interesting to assess how variation or the length of homology arms and the repair template concentration would affect the homologous recombination frequency in *Lophiotrema* sp. F6932.
7) Whole genome sequencing was done for numerous fungal strains during this study and analysis of the sequencing results revealed the presence of a large number of SMs gene clusters, majority of whose products remain unknown. Genome-guided studies focusing on

some of the unique clusters identified in such strains may lead to discovery of new fungal natural products.

References

Abdallah, Q., Ge, W., & Fortwendel, J. (2017). A Simple and Universal System for Gene Manipulation in Aspergillus fumigatus: *In vitro*-assembled cas9-guide rna ribonucleoproteins coupled with microhomology repair templates. *MSphere*, *2*(6). https://doi.org/10.1128/msphere.00446-17.

Abdelmohsen, U. R., Grkovic, T., Balasubramanian, S., Kamel, M. S., Quinn, R. J., & Hentschel, U. (2015). Elicitation of secondary metabolism in actinomycetes. *Biotechnology Advances*, *33*(6), 798–811. https://doi.org/10.1016/j.biotechadv.2015.06.003.

Abhijeet Singh, Y. M. (2014). Understanding the Biodiversity and Biological Applications of Endophytic Fungi: A Review. *Journal of Microbial & Biochemical Technology*, *8*(01), 1–11. https://doi.org/10.4172/1948-5948.s8-004.

Ahsan, T., Chen, J., Wu, Y., Irfan, M., & Shafi, J. (2017). Screening, identification, optimization of fermentation conditions, and extraction of secondary metabolites for the biocontrol of *Rhizoctonia Solani* AG-3. *Biotechnology and Biotechnological Equipment*, *31*(1), 91–98. https://doi.org/10.1080/13102818.2016.1259016.

Ai-Leen, G., & Jin Song, R. (2000). The use of gis in ovitrap monitoring for dengue control in singapore. *Dengue Bulletin*, *24*(65), 110–116.

Akone, S. H., Mándi, A., Kurtán, T., Hartmann, R., Lin, W., Daletos, G., & Proksch, P. (2016). Inducing secondary metabolite production by the endophytic fungus *Chaetomium* sp. through fungal–bacterial co-culture and epigenetic modification. *Tetrahedron*, *72*(41), 6340–6347. https://doi.org/10.1016/j.tet.2016.08.022.

Akone, S. H., Pham, C. D., Chen, H., Ola, A. R. B., Ntie-Kang, F., & Proksch, P. (2019). Epigenetic modification, co-culture and genomic methods for natural product discovery. *Physical Sciences Reviews*, *4*(4), 1–13. https://doi.org/10.1515/psr-2018-0118.

Alhakami, H., Mirebrahim, H., & Lonardi, S. (2017). A comparative evaluation of genome assembly reconciliation tools. *Genome biology*, *18*(1), 1-14. https://doi.org/ 10.1186/s13059-017-1213-3.

Alvin, A., Miller, K. I., & Neilan, B. A. (2014). Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiological Research*, *169*(7–8), 483–495. https://doi.org/10.1016/j.micres.2013.12.009.

Aly, A. H., Debbab, A., & Proksch, P. (2011). Fifty years of drug discovery from fungi. *Fungal Diversity*, *50*, 3–19. https://doi.org/10.1007/s13225-011-0116-y.

An, C., Ma, S., Shi, X., Xue, W., Liu, C., & Ding, H. (2020). Isolation, diversity, and antimicrobial activity of fungal endophytes from *Rohdea chinensis* (Baker) N.Tanaka (synonym *Tupistra chinensis* Baker) of Qinling Mountains, China. *PeerJ*, *2020*(6), e9342. https://doi.org/10.7717/peerj.9342.

Ancheeva, E., Daletos, G., & Proksch, P. (2019). Bioactive secondary metabolites from endophytic fungi. *Current Medicinal Chemistry*, *26*. https://doi.org/10.2174/0929867326666190916144709.

Andrioli, W. J., Conti, R., Araújo, M. J., Zanasi, R., Cavalcanti, B. C., Manfrim, V., Toledo, J. S., Tedesco, D., De Moraes, M. O., Pessoa, C., Cruz, A. K., Bertucci, C., Sabino, J., Nanayakkara, D. N. P., Pupo, M. T., & Bastos, J. K. (2014). Mycoleptones A-C and polyketides from the endophyte *Mycoleptodiscus indicus*. *Journal of Natural Products*, *77*(1), 70–78. https://doi.org/10.1021/np4006822.

Ariantari, N. P., Daletos, G., Mándi, A., Kurtán, T., Müller, W. E. G., Lin, W., Ancheeva, E., & Proksch, P. (2019). Expanding the chemical diversity of an endophytic fungus: *Bulgaria inquinans*, an ascomycete associated with mistletoe, through an OSMAC approach. *RSC Advances*, *9*(43), 25119–25132. https://doi.org/10.1039/c9ra03678d.

Armeev, G. A., Gribkova, A. K., Pospelova, I., Komarova, G. A., & Shaytan, A. K. (2019). Linking chromatin composition and structural dynamics at the nucleosome level. *Current Opinion in Structural Biology,* 56, 46–55. https://doi.org/10.1016/j.sbi.2018.11.006.

Arnold, A. E., & Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology*, *88*(3), 541–549. https://doi.org/10.1890/05-1459.

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D., & Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecology Letters*, *3*(4), 267–274. https://doi.org/10.1046/j.1461-0248.2000.00159.x

Atanasov, A. G., Zotchev, S. B., Dirsch, V. M., Orhan, I. E., Banach, M., Rollinger, J. M., Barreca, D., Weckwerth, W., Bauer, R., Bayer, E. A., Majeed, M., Bishayee, A., Bochkov, V., Bonn, G. K., Braidy, N., Bucar, F., Cifuentes, A., D'Onofrio, G., Bodkin, M., … Supuran, C. T. (2021). Natural products in drug discovery: advances and opportunities. *Nature Reviews Drug Discovery, 20*(3), 200–216. https://doi.org/10.1038/s41573-020-00114-z.

Bachmann, B. O., Van Lanen, S. G., & Baltz, R. H. (2014). Microbial genome mining for accelerated natural products discovery: is a renaissance in the making? *Journal of Industrial Microbiology and Biotechnology*, *41*(2), 175-184. https://doi.org/10.1007/s10295-013-1389-9.

Badotti, F., De Oliveira, F. S., Garcia, C. F., Vaz, A. B. M., Fonseca, P. L. C., Nahum, L. A., Oliveira, G., & Góes-Neto, A. (2017). Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). *BMC Microbiology*, *17*(1), 42. https://doi.org/10.1186/s12866-017-0958-x.

Bailey, J. K., Deckert, R., Schweitzer, J. A., Rehill, B. J., Lindroth, R. L., Gehring, C., & Whitham, T. G. (2005). Host plant genetics affect hidden ecological players: Links among Populus, condensed tannins, and fungal endophyte infection. *Canadian Journal of Botany*, *83*(4), 356–361. https://doi.org/10.1139/b05-008.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6,(2) 71–79. https://doi.org/10.1016/j.jpha.2015.11.005.

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., & Wang, L. (2018). Fungal endophytes: Beyond herbivore management. *Frontiers in Microbiology*, *9,* 544. https://doi.org/10.3389/fmicb.2018.00544.

Baral, B., Akhgari, A., & Metsä-Ketelä, M. (2018). Activation of microbial secondary metabolic

pathways: Avenues and challenges. *Synthetic and Systems Biotechnology*, *3*(3), 163–178. https://doi.org/10.1016/j.synbio.2018.09.001.

Barberel, S. I., & Walker, J. R. (2000). The effect of aeration upon the secondary metabolism of microorganisms. *Biotechnology and Genetic Engineering Reviews*, *17*(1), 281-326. https://doi.org/10.1080/02648725.2000.10647996.

Barnes, C. J., van der Gast, C. J., McNamara, N. P., Rowe, R., & Bending, G. D. (2018). Extreme rainfall affects assembly of the root-associated fungal community. *New Phytologist*, *220*(4), 1172–1184. https://doi.org/10.1111/nph.14990.

Bente, H., Mittelsten Scheid, O., & Donà, M. (2020). Versatile in vitro assay to recognize Cas9-induced mutations. *Plant Direct*, *4*(9), e00269. https://doi.org/10.1002/pld3.269.

Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K., & Wolfender, J. L. (2014). Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. *Biotechnology Advances*, *32*(6), 1180–1204. https://doi.org/10.1016/j.biotechadv.2014.03.001.

Bills, G. F., & Gloer, J. B. (2016). Biologically active secondary metabolites from the fungi. *Microbiology spectrum*, *4*(6), 4-6. DOI: https://doi.org/10.1128/microbiolspec.funk-0009-2016.

Bills, G. F., Platas, G., Fillola, A., Jiménez, M. R., Collado, J., Vicente, F., Martín, J., González, A., Bur-Zimmermann, J., Tormo, J. R., & Peláez, F. (2008). Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *Journal of Applied Microbiology*, *104*(6), 1644–1658. https://doi.org/10.1111/j.1365-2672.2008.03735.x.

Bode, H. B., Bethe, B., Höfs, R., & Zeeck, A. (2002). Big effects from small changes: Possible ways to explore nature's chemical diversity. *ChemBioChem*, *3*(7), 619–627. https://doi.org/10.1002/1439-7633(20020703)3:7<619::AID-CBIC619>3.0.CO;2-9.

Bode, H. B., & Zeeck, A. (2000). UV mutagenesis and enzyme inhibitors as tools to elucidate the late biosynthesis of the spirobisnaphthalenes. *Phytochemistry*, *55*(4), 311–316.

https://doi.org/10.1016/S0031-9422(00)00307-1.

Borchardt, J. K. (2002). The Beginnings of drug therapy: ancient mesopotamian medicine. *Drug News & Perspectives*, *15*(3), 187-192. https://doi.org/10.1358/dnp.2002.15.3.840015.

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. *Nature Reviews Microbiology*, *11*(1), 21–32. https://doi.org/10.1038/nrmicro2916.

Brakhage, A. A., Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., & Hertweck, C. (2008). Activation of fungal silent gene clusters: A new avenue to drug discovery. *Natural Compounds as Drugs*, 66, 1–12. https://doi.org/10.1007/978-3-7643-8595-8_1.

Butler, M. S., Yoganathan, K., Buss, A. D., & Ng, S. (2012). Identification of aluminium dioxalate in fungal cultures grown on vermiculite. *The Journal of Antibiotics, 65*(5), 275–276. https://doi.org/10.1038/ja.2012.15.

Calvo, A. M., & Cary, J. W. (2015). Association of fungal secondary metabolism and sclerotial biology. *Frontiers in Microbiology* 6, 62. https://doi.org/10.3389/fmicb.2015.00062.

Cao, Z., Li, S., Lv, J., Gao, H., Chen, G., Awakawa, T., Abe, I., Yao, X., & Hu, D. (2019). Biosynthesis of clinically used antibiotic fusidic acid and identification of two short-chain dehydrogenase/reductases with converse stereoselectivity. *Acta Pharmaceutica Sinica B*, *9*(2), 433–442. https://doi.org/10.1016/j.apsb.2018.10.007.

Carrano, L., & Marinelli, F. (2015). The relevance of chemical dereplication in microbial natural product screening. *Journal of Applied Bioanalysis*, *1*(2), 55–67. https://doi.org/10.17145/jab.15.010.

Caruso, G., Abdelhamid, M. T., Kalisz, A., & Sekara, A. (2020). Linking endophytic fungi to medicinal plants therapeutic activity. A case study on Asteraceae. *Agriculture*, *10*(7), 286. https://doi.org/10.3390/agriculture10070286.

Chadha, N., Mishra, M., Prasad, R., & Varma, A. (2014). Root endophytic fungi: Research update. *Journal of Biology and Life Science*, *5*(2), 135. https://doi.org/10.5296/jbls.v5i2.5960.

Chand, K., Shah, S., Sharma, J., Paudel, M. R., & Pant, B. (2020). Isolation, characterization, and

plant growth-promoting activities of endophytic fungi from a wild orchid Vanda cristata. Plant Signaling & Behavior, 15(5), 1744294. https://doi.org/10.1080/15592324.2020.1744294

Chávez, R., Fierro, F., García-Rico, R. O., & Vaca, I. (2015). Filamentous fungi from extreme environments as a promising source of novel bioactive secondary metabolites. *Frontiers in Microbiology*, *6*, 903. https://doi.org/10.3389/fmicb.2015.00903.

Chen, L., Zhang, Q.-Y., Jia, M., Ming, Q.-L., Yue, W., Rahman, K., Qin, L.-P., & Han, T. (2014). Endophytic fungi with antitumor activities: Their occurrence and anticancer compounds. *Critical Reviews in Microbiology*, *42*(3), 1–20. https://doi.org/10.3109/1040841X.2014.959892. https://doi.org/10.3389/fmicb.2020.01519.

Chen, R., Wong, H., & Burns, B. (2019). New approaches to detect biosynthetic gene clusters in the environment. *Medicines*, *6*(1), 32. https://doi.org/10.3390/medicines6010032.

Chen, J., Akutse, K. S., Saqib, H. S. A., Wu, X., Yang, F., Xia, X., ... & Gurr, G. M. (2020). Fungal endophyte communities of crucifer crops are seasonally dynamic and structured by plant identity, plant tissue and environmental factors. *Frontiers in Microbiology*, *11*, 1519.

Chepkirui, C., & Stadler, M. (2017). The genus *Diaporthe*: A rich source of diverse and bioactive metabolites. *Mycological Progress, 16* (5) 477–494. https://doi.org/10.1007/s11557-017-1288-y.

Chi, W. C., Chen, W., He, C. C., Guo, S. Y., Cha, H. J., Tsang, L. M., Ho, T. W., & Pang, K. L. (2019). A highly diverse fungal community associated with leaves of the mangrove plant *Acanthus ilicifolius* var. *xiamenensis* revealed by isolation and metabarcoding analyses. *PeerJ*, *7*, e7293. https://doi.org/10.7717/peerj.7293.

Chifiriuc, M. C., Filip, R., Constantin, M., Pircalabioru, G. G., Bleotu, C., Burlibasa, L., ... & Mihaescu, G. (2022). Common themes in antimicrobial and anticancer drug resistance. *Frontiers in Microbiology*, 13, 3071. https://doi.org/10.3389/fmicb.2022.960693.

Chomcheon, P., Wiyakrutta, S., Sriubolmas, N., Ngamrojanavanich, N., Mahidol, C., Ruchirawat,

S., & Kittakoop, P. (2009). Metabolites from the endophytic mitosporic *Dothideomycete* sp. LRUB20. *Phytochemistry*, *70*(1), 121–127. https://doi.org/10.1016/J.phytochem.2008.10.007.

Chowdhary, K., Kaushik, N., & Raimundo, M. (2012). Endophytic fungi and their metabolites isolated from Indian medicinal plant. *Phytochemistry Reviews*, *11*(4), 467-485. https://doi.org/10.1007/s11101-012-9264-2.

CLSI. (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts. CLSI document M27-A2. National Committee for Clinical Laboratory Standards. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI. (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard—Second Edition. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI. (2012). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard – Ninth edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

Collemare, J., & Seidl, M. F. (2019). Chromatin-dependent regulation of secondary metabolite biosynthesis in fungi: Is the picture complete? *FEMS Microbiology Reviews*, 43 (6), 591–607). https://doi.org/10.1093/femsre/fuz018.

Corrêa, R. C. G., Rhoden, S. A., Mota, T. R., Azevedo, J. L., Pamphile, J. A., de Souza, C. G. M., Polizeli, M. de L. T. de M., Bracht, A., & Peralta, R. M. (2014). Endophytic fungi: expanding the arsenal of industrial enzyme producers. *Journal of Industrial Microbiology and Biotechnology, 41 (10)*,1467–1478. https://doi.org/10.1007/s10295-014-1496-2.

Da Silva, L. L., Moreno, H. L. A., Correia, H. L. N., Santana, M. F., & de Queiroz, M. V. (2020). *Colletotrichum*: species complexes, lifestyle, and peculiarities of some sources of genetic variability. *Applied Microbiology and Biotechnology*, *104* (5), 1891–1904. https://doi.org/10.1007/s00253-020-10363-y.

Daletos, G., Ebrahim, W., Ancheeva, E., & El-neketi, M. (2017). Microbial coculture and osmac

approach as strategies to induce cryptic fungal biogenetic gene clusters. In *Chemical Biology of Natural Products*, (pp. 233-284). CRC press. https://doi.org/10.1201/9781315117089-8.

Daley, D. K., Brown, K. J., & Badal, S. (2017). Fungal Metabolites. In *Pharmacognosy: Fundamentals, Applications and Strategy* (pp. 413–421). Elsevier Inc. https://doi.org/10.1016/B978-0-12-802104-0.00020-2.

Damm, U., Sato, T., Alizadeh, A., Groenewald, J. Z., & Crous, P. W. (2019). The *Colletotrichum dracaenophilum*, *C. magnum* and *C. orchidearum* species complexes. *Studies in Mycology*, *92*, 1–46. https://doi.org/10.1016/j.simyco.2018.04.001.

Davies, J. (2006). Where have all the antibiotics gone? *The Canadian Journal of Infectious Diseases & Medical Microbiology*, *17*(5), 287. https://doi.org/10.1155/2006/707296.

De Beeck, M., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J., & Colpaert, J. V. (2014). Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PLoS ONE*, *9*(6). https://doi.org/10.1371/journal.pone.0097629.

Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., & Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. In *Molecular Plant Pathology, 13* (4), 414–430. https://doi.org/10.1111/j.1364-3703.2011.00783.x.

Deepika, V. B., Murali, T. S., & Satyamoorthy, K. (2016). Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review. *Microbiological Research*, *182*, 125–140. https://doi.org/10.1016/j.micres.2015.10.009.

Demers, D., Knestrick, M., Fleeman, R., Tawfik, R., Azhari, A., Souza, A., Vesely, B., Netherton, M., Gupta, R., Colon, B., Rice, C., Rodríguez-Pérez, M., Rohde, K., Kyle, D., Shaw, L., & Baker, B. (2018). Exploitation of mangrove endophytic fungi for infectious disease drug discovery. *Marine Drugs*, *16*(10), 376. https://doi.org/10.3390/md16100376.

Deng, H., Gao, R., Liao, X., & Cai, Y. (2017). CRISPR system in filamentous fungi: Current achievements and future directions. *Gene*, *627*, 212–221. https://doi.org/10.1016/j.gene.2017.06.019.

Deshmukh, Kumar, S., Verekar, S. A., & Bhave, S. V. (2014). Endophytic fungi: A reservoir of antibacterials. *Frontiers in Microbiology*, 5, 517. https://doi.org/10.3389/fmicb.2014.00715.

Deshmukh, S. K., Gupta, M. K., Prakash, V., & Saxena, S. (2018). Endophytic fungi: A source of potential antifungal compounds. *Journal of Fungi*, *4*(3), 1–42. https://doi.org/10.3390/jof4030077.

Dias, D. A., Urban, S., & Roessner, U. (2012). A Historical overview of natural products in drug discovery. *Metabolites*, *2*(2), 303–336. https://doi.org/10.3390/metabo2020303.

Ding, G., Zheng, Z., Liu, S., Zhang, H., Guo, L., & Che, Y. (2009). Photinides A-F, cytotoxic benzofuranone-derived γ-lactones from the plant endophytic fungus *Pestalotiopsis photiniae*. *Journal of Natural Products*, *72*(5), 942–945. https://doi.org/10.1021/np900084d.

Dos Reis, J. B. A., Lorenzi, A. S., & do Vale, H. M. M. (2022). Methods used for the study of endophytic fungi: a review on methodologies and challenges, and associated tips. *Archives of Microbiology*, *204*(11), 675. https://doi.org/10.1007/s00203-022-03283-0.

Duan, X., Xu, F., Qin, D., Gao, T., Shen, W., Zuo, S., Yu, B., Xu, J., Peng, Y., & Dong, J. (2019). Diversity and bioactivities of fungal endophytes from *Distylium chinense*, a rare waterlogging tolerant plant endemic to the Three Gorges Reservoir. *BMC Microbiology*, *19*(1), 278. https://doi.org/10.1186/s12866-019-1634-0.

Dutta, D., Puzari, K. C., Gogoi, R., & Dutta, P. (2014). Endophytes: Exploitation as a tool in plant protection. *Brazilian Archives of Biology and Technology*, *57*(5), 621–629. https://doi.org/10.1590/S1516-8913201402043.

Dwibedi, V., Kalia, S., & Saxena, S. (2019). Isolation and enhancement of resveratrol production in *Xylaria psidii* by exploring the phenomenon of epigenetics: using DNA methyltransferases and histone deacetylase as epigenetic modifiers. *Molecular Biology Reports*, *46*(4), 4123–4137. https://doi.org/10.1007/s11033-019-04862-z.

Ebrahim, W., El-Neketi, M., Lewald, L. I., Orfali, R. S., Lin, W., Rehberg, N., Kalscheuer, R., Daletos, G., & Proksch, P. (2016). Metabolites from the fungal endophyte *Aspergillus austroafricanus* in axenic culture and in fungal-bacterial mixed cultures. *Journal of Natural*

Products, *79*(4), 914–922. https://doi.org/10.1021/acs.jnatprod.5b00975.

El-Hawary, S. S., Moawad, A. S., Bahr, H. S., Abdelmohsen, U. R., & Mohammed, R. (2020). Natural product diversity from the endophytic fungi of the genus *Aspergillus*. *RSC Advances*, *10*(37), 22058–22079. https://doi.org/10.1039/d0ra04290k.

Elias, B. C., Said, S., de Albuquerque, S., & Pupo, M. T. (2006). The influence of culture conditions on the biosynthesis of secondary metabolites by *Penicillium verrucosum* Dierck. *Microbiological Research*, *161*(3), 273–280. https://doi.org/10.1016/j.micres.2005.10.003.

Enkler, L., Richer, D., Marchand, A. L., Ferrandon, D., & Jossinet, F. (2016). Genome engineering in the yeast pathogen *Candida glabrata* using the CRISPR-Cas9 system. *Scientific Reports 2016 6:1*, *6*(1), 1–12. https://doi.org/10.1038/srep35766.

Fadiji, A. E., & Babalola, O. O. (2020). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Frontiers in Bioengineering and Biotechnology ,*8, 467. https://doi.org/10.3389/fbioe.2020.00467.

Fang, K., Miao, Y.-F., Chen, L., Zhou, J., Yang, Z.-P., Dong, X.-F., & Zhang, H.-B. (2019). Tissue-specific and geographical variation in endophytic fungi of *Ageratina adenophora* and fungal associations with the environment. *Frontiers in Microbiology*, *10*, 2919. https://doi.org/10.3389/fmicb.2019.02919.

Fernandes, E. G., Pereira, O. L., Silva, C. C. da, Bento, C. B. P., & Queiroz, M. V. de. (2015). Diversity of endophytic fungi in *Glycine max*. *Microbiological Research*, *181*, 84–92. https://doi.org/10.1016/j.micres.2015.05.010

Fernandes, P. (2016). Fusidic acid: A bacterial elongation factor inhibitor for the oral treatment of acute and chronic staphylococcal infections. *Cold Spring Harbor Perspectives in Medicine*, *6*(1), a025437. https://doi.org/10.1101/cshperspect.a025437.

Ferrara, M., Haidukowski, M., Logrieco, A. F., Leslie, J. F., & Mulè, G. (2019). A CRISPR-Cas9 system for genome editing of *Fusarium proliferatum*. *Scientific Reports, 9*(1), 1–9. https://doi.org/10.1038/s41598-019-56270-9.

Fisch, K. M., Gillaspy, A. F., Gipson, M., Henrikson, J. C., Hoover, A. R., Jackson, L., Najar, F.

Z., Wägele, H., & Cichewicz, R. H. (2009). Chemical induction of silent biosynthetic pathway transcription in *Aspergillus niger*. *Journal of Industrial Microbiology and Biotechnology*, *36*(9), 1199–1213. https://doi.org/10.1007/s10295-009-0601-4.

Foster, A. J., Martin-Urdiroz, M., Yan, X., Wright, H. S., Soanes, D. M., & Talbot, N. J. (2018). CRISPR-Cas9 ribonucleoprotein-mediated co-editing and counterselection in the rice blast fungus. *Scientific Reports, 8*(1), 1–12. https://doi.org/10.1038/s41598-018-32702-w.

Fox, E. M., & Howlett, B. J. (2008). Secondary metabolism: regulation and role in fungal biology. *Current Opinion in Microbiology*, *11*(6), 481–487. https://doi.org/10.1016/j.mib.2008.10.007.

Freinkman, E., Oh, D. C., Scott, J. J., Currie, C. R., & Clardy, J. (2009). Bionectriol A, a polyketide glycoside from the fungus *Bionectria* sp. associated with the fungus-growing ant, *Apterostigma dentigerum. Tetrahedron Letters*, *50*(49), 6834–6837. https://doi.org/10.1016/j.tetlet.2009.09.120.

Gakuubi, M. M., Ching, K. C., Munusamy, M., Wibowo, M., Liang, Z.-X., Kanagasundaram, Y., & Ng, S. B. (2022a). Enhancing the discovery of bioactive secondary metabolites from fungal endophytes using chemical elicitation and variation of fermentation media. *Frontiers in Microbiology*, *13*, 898976. https://doi.org/10.3389/FMICB.2022.898976.

Gakuubi, M. M., Ching, K. C., Munusamy, M., Wibowo, M., Lim, C. T., Ma, G. L., ... & Ng, S. B. (2022b). CRISPR/Cas9 RNP-assisted validation of palmarumycin biosynthetic gene cluster in Lophiotrema sp. F6932. *Frontiers in Microbiology*, *13*, 1012115. https://doi.org/10.3389/fmicb.2022.1012115.

Gakuubi, M. M., Munusamy, M., Liang, Z.-X., & Ng, S. B. (2021). Fungal endophytes: A promising frontier for discovery of novel bioactive compounds. *Journal of Fungi, 7*(10), 786. https://doi.org/10.3390/JOF7100786.

Gamboa, M. A., Laureano, S., & Bayman, P. (2003). Measuring diversity of endophytic fungi in leaf fragments: Does size matter? *Mycopathologia*, *156*(1), 41–45. https://doi.org/10.1023/A:1021362217723.

Gao, Y., Shang, Q., Li, W., Guo, W., Stojadinovic, A., Mannion, C., ... & Chen, T. (2020).

Antibiotics for cancer treatment: A double-edged sword. *Journal of Cancer*, *11*(17), 5135. https://doi.org/10.7150/jca.47470

García-Estrada, C., Domínguez-Santos, R., Kosalková, K., & Martín, J.-F. (2018). Transcription factors controlling primary and secondary metabolism in filamentous fungi: the β-lactam paradigm. *Fermentation*, *4*(2), 47. https://doi.org/10.3390/fermentation4020047.

García-Kirchner, O., Segura-Granados, M., & Rodríguez-Pascual, P. (2005). Effect of media composition and growth conditions on production of β-glucosidase by *Aspergillus niger* C-6. *Applied Biochemistry and Biotechnology*, *121,* 347–359. https://doi.org/10.1007/978-1-59259-991-2_30.

García-Pajón, C. M., & Collado, I. G. (2003). Secondary metabolites isolated from *Colletotrichum* species. *Natural Product Reports*, 20 (4), 426–431. https://doi.org/10.1039/b302183c.

Garnica, S., Schön, M. E., Abarenkov, K., Riess, K., Liimatainen, K., Niskanen, T., Dima, B., Soop, K., Frøslev, T. G., Jeppesen, T. S., Peintner, U., Kuhnert-Finkernagel, R., Brandrud, T. E., Saar, G., Oertel, B., & Ammirati, J. F. (2016). Determining threshold values for barcoding fungi: Lessons from *Cortinarius* (Basidiomycota), a highly diverse and widespread ectomycorrhizal genus. *FEMS Microbiology Ecology*, *92*(4). https://doi.org/10.1093/femsec/fiw045.

Girão, M., Ribeiro, I., Ribeiro, T., Azevedo, I. C., Pereira, F., Urbatzka, R., Leão, P. N., & Carvalho, M. F. (2019). Actinobacteria isolated from laminaria ochroleuca: a source of new bioactive compounds. *Frontiers in Microbiology*, *10*, 683. https://doi.org/10.3389/fmicb.2019.00683.

Gong, A., Zhou, T., Xiao, C., Jiang, W., Zhou, Y., Zhang, J., ... & Zhang, C. (2019). Association between dipsacus saponin VI level and diversity of endophytic fungi in roots of Dipsacus asperoides. *World Journal of Microbiology and Biotechnology*, *35*, 1-14. https://doi.org/10.1007/s11274-019-2616-y.

González-Menéndez, V., Pérez-Bonilla, M., Pérez-Victoria, I., Martín, J., Muñoz, F., Reyes, F., & Genilloud, O. (2016). Multicomponent analysis of the differential induction of secondary

metabolite profiles in fungal endophytes. *Molecules*, *21*(2), 234. https://doi.org/10.3390/molecules21020234.

González-Menéndez, V., Crespo, G., De Pedro, N., Diaz, C., Martín, J., Serrano, R., Mackenzie, T. A., Justicia, C., Reyes González-Tejero, M., Casares, M., Vicente, F., Reyes, F., Tormo, J. R., & Genilloud, O. (2018). Fungal endophytes from arid areas of Andalusia: high potential sources for antifungal and antitumoral agents. *Scientific Reports*, *8*(1), 1-13. https://doi.org/10.1038/s41598-018-28192-5.

González-Menéndez, V., Crespo, G., Toro, C., Martín, J., De Pedro, N., Tormo, J. R., & Genilloud, O. (2019). Extending the metabolite diversity of the endophyte *Dimorphosporicola tragani*. *Metabolites*, *9*(10), 197. https://doi.org/10.3390/metabo9100197.

Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016). Endophytes: A treasure house of bioactive compounds of medicinal importance. *Frontiers in Microbiology*, *7*, 1538. https://doi.org/10.3389/fmicb.2016.01538.

Grigoletto, D. F., Trivella, D. B. B., Tempone, A. G., Rodrigues, A., Correia, A. M. L., & Lira, S. P. (2020). Antifungal compounds with anticancer potential from *Trichoderma* sp. P8BDA1F1, an endophytic fungus from *Begonia venosa*. *Brazilian Journal of Microbiology*, *51*(3), 989–997. https://doi.org/10.1007/S42770-020-00270-9/FIGURES/3.

Gunatilaka, A. A. L. (2006). Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactivity, and implications of their occurrence. *Journal of Natural Products*, 69 (3), 509–526. https://doi.org/10.1021/np058128n.

Gupta, M., & Shukla, K. K. (2020). Endophytic fungi: A treasure trove of novel bioactive compounds. In *Bioactive Natural Products in Drug Discovery* (pp. 427–449). Springer Singapore. https://doi.org/10.1007/978-981-15-1394-7_14.

Hamzah, T. N. T., Lee, S. Y., Hidayat, A., Terhem, R., Faridah-Hanum, I., & Mohamed, R. (2018). Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *Rhizophora mucronata*, and identification of potential antagonists against the soil-borne fungus, *Fusarium solani. Frontiers in Microbiology*, *9*, 1707.

https://doi.org/10.3389/fmicb.2018.01707.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., ... & Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews*, *79*(3), 293-320. https://doi.org/10.1128/mmbr.00050-14.

Harwoko, H., Lee, J., Hartmann, R., Mándi, A., Kurtán, T., Müller, W. E. G., Feldbrügge, M., Kalscheuer, R., Ancheeva, E., Daletos, G., Frank, M., Liu, Z., & Proksch, P. (2020). Azacoccones F-H, new flavipin-derived alkaloids from an endophytic fungus *Epicoccum nigrum* MK214079. *Fitoterapia*, *146*, 104698. https://doi.org/10.1016/J.fitote.2020.104698.

Hashem, A. H., Attia, M. S., Kandil, E. K., Fawzi, M. M., Abdelrahman, A. S., Khader, M. S., ... & Abdelaziz, A. M. (2023). Bioactive compounds and biomedical applications of endophytic fungi: a recent review. *Microbial Cell Factories*, *22*(1), 1-23. https://doi.org/10.1186/s12934-023-02118-x.

Hautbergue, T., Jamin, E. L., Debrauwer, L., Puel, O., & Oswald, I. P. (2018). From genomics to metabolomics, moving toward an integrated strategy for the discovery of fungal secondary metabolites. *Natural Product Reports*, *35*(2), 147–173. https://doi.org/10.1039/c7np00032d.

Henrich, C. J., & Beutler, J. A. (2013). Matching the power of high throughput screening to the chemical diversity of natural products. *Natural Product Reports*, *30*(10), 1284–1298. https://doi.org/10.1039/c3np70052f.

Hewage, R. T., Aree, T., Mahidol, C., Ruchirawat, S., & Kittakoop, P. (2014). One strain-many compounds (OSMAC) method for production of polyketides, azaphilones, and an isochromanone using the endophytic fungus *Dothideomycete* sp. *Phytochemistry*, *108*, 87–94. https://doi.org/10.1016/j.phytochem.2014.09.013.

Hill, G. T., Mitkowski, N. A., Aldrich-Wolfe, L., Emele, L. R., Jurkonie, D. D., Ficke, A., Maldonado-Ramirez, S., Lynch, S. T., & Nelson, E. B. (2000). Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology*, *15*(1), 25–36. https://doi.org/10.1016/S0929-1393(00)00069-X.

Ho, B. C., Lua, H. K., Ibrahim, B., Yeo, R. S. W., Athen, P., Leong, P. K. F., Ibrahim, A., Koh, S. L., Ibrahim, H., Lindsay, S., Chin, L. L., Seah, W. W., & Middleton, D. J. (2019). The plant diversity in Bukit Timah Nature Reserve, Singapore. *Gardens' Bulletin Singapore*, *71*(suppl.1), 41–144. https://doi.org/10.26492/gbs71(suppl.1).2019-04.

Hoang, M. T. V., Irinyi, L., Chen, S. C. A., Sorrell, T. C., Meyer, W., Arabatzis, M., Arthur, I., Cano-Lira, J. F., Cardinali, G., Castañón, L. R., Chen, S., Chen, W., Chindamporn, A., Colombo, A. L., Desnos-Ollivier, M., De Beer, W., De Hoog, S., Fungal, W., Dromer, F., … Zancopé-Oliveira, R. M. (2019). Dual DNA barcoding for the molecular identification of the agents of invasive fungal infections. *Frontiers in Microbiology*, *10*, 1647. https://doi.org/10.3389/fmicb.2019.01647.

Hofstetter, V., Buyck, B., Eyssartier, G., Schnee, S., & Gindro, K. (2019). The unbearable lightness of sequenced-based identification. *Fungal Diversity*, *96*(1), 243–284. https://doi.org/10.1007/s13225-019-00428-3.

Hosoe, T., Fukushima, K., Takizawa, K., Itabashi, T., Kawahara, N., Vidotto, V., & Kawai, K. (2006). A new antifungal macrolide, eushearilide, isolated from *Eupenicillium shearii*. *The Journal of Antibiotics, 59*(9), 597–600. https://doi.org/10.1038/ja.2006.80.

Huang, Y. L. (2020). Effect of host, environment and fungal growth on fungal leaf endophyte communities in Taiwan. *Journal of Fungi*, *6*(4), 1–13. https://doi.org/10.3390/jof6040244.

Hyde, K. D., Xu, J., Rapior, S., Jeewon, R., Lumyong, S., Niego, A. G. T., Abeywickrama, P. D., Aluthmuhandiram, J. V. S., Brahamanage, R. S., Brooks, S., Chaiyasen, A., Chethana, K. W. T., Chomnunti, P., Chepkirui, C., Chuankid, B., de Silva, N. I., Doilom, M., Faulds, C., Gentekaki, E., & Stadler, M. (2019). The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Diversity*, *97*(1), 1–136. https://doi.org/10.1007/s13225-019-00430-9.

Ibragimova, S., Szebenyi, C., Sinka, R., Alzyoud, E. I., Homa, M., Vágvölgyi, C., Nagy, G., & Papp, T. (2020). CRISPR-Cas9-based mutagenesis of the mucormycosis-causing fungus Lichtheimia corymbifera. *International Journal of Molecular Sciences*, *21*(10), 3727. https://doi.org/10.3390/ijms21103727.

Ibrahim, M., Oyebanji, E., Fowora, M., Aiyeolemi, A., Orabuchi, C., Akinnawo, B., & Adekunle, A. A. (2021). Extracts of endophytic fungi from leaves of selected Nigerian ethnomedicinal plants exhibited antioxidant activity. *BMC Complementary Medicine and Therapies*, *21*(1), 1-13. https://doi.org/10.1186/s12906-021-03269-3.

Isaka, M., Palasarn, S., Sommai, S., Laksanacharoen, P., & Srichomthong, K. (2018). Maleic anhydride and chromone derivatives from the endophytic fungus BCC 54265 (*Botryosphaeriaceae*). *Natural Product Research*, *32*(13), 1506–1511. https://doi.org/10.1080/14786419.2017.1385010.

Ishikawa, F. H., Barcelos, Q. de L., de Souza, E. A., & Dias, E. S. (2010). Factors affecting the production and regeneration of protoplasts from *Colletotrichum lindemuthianum*. *Ciência e Agrotecnologia*, *34*(1), 74–79. https://doi.org/10.1590/S1413-70542010000100009.

Jacobs, J. Z., Ciccaglione, K. M., Tournier, V., & Zaratiegui, M. (2014). Implementation of the CRISPR-Cas9 system in fission yeast. *Nature Communications*, *5*, 5344. https://doi.org/10.1038/ncomms6344.

Jia, M., Chen, L., Xin, H. L., Zheng, C. J., Rahman, K., Han, T., & Qin, L. P. (2016). A friendly relationship between endophytic fungi and medicinal plants: A systematic review. *Frontiers in Microbiology*, *7*, 906. https://doi.org/10.3389/fmicb.2016.00906.

Jia, Q., Qu, J., Mu, H., Sun, H., & Wu, C. (2020). Foliar endophytic fungi: diversity in species and functions in forest ecosystems. *Symbiosis*, *80*(2), 103-132. https://doi.org/10.1007/s13199-019-00663-x.

Jiang, C., Lv, G., Tu, Y., Cheng, X., Duan, Y., Zeng, B., & He, B. (2021). Applications of CRISPR/Cas9 in the synthesis of secondary metabolites in filamentous fungi. *Frontiers in Microbiology*, *12*, 638096. https://doi.org/10.3389/fmicb.2021.638096.

Jin, F. J., Hu, S., Wang, B. T., & Jin, L. (2021). Advances in genetic engineering technology and its application in the industrial fungus *Aspergillus oryzae*. *Frontiers in Microbiology*, *12*, 353. https://doi.org/10.3389/fmicb.2021.644404.

Jin, L.-Q., Xu, Z. W., Men, X. H., Bo-Zhang, Liu, Z. Q., & Zheng, Y. G. (2020). Enhancement of

protoplast preparation and regeneration of *Hirsutella sinensis* based on process optimization. *Biotechnology Letters, 42*(11), 2357–2366. https://doi.org/10.1007/S10529-020-02958-2.

Jones, R. N., Mendes, R. E., Sader, H. S., & Castanheira, M. (2011). In vitro antimicrobial findings for fusidic acid tested against contemporary (2008-2009) gram-positive organisms collected in the United States. *Clinical Infectious Diseases*. *52*(suppl_7), S477-S486. https://doi.org/10.1093/cid/cir163.

Ju, Y. M., Juang, S. H., Chen, K. J., & Lee, T. H. (2007). TMC-151 a monoacetate, a new polyketide from *Bionectria ochroleuca*. *Zeitschrift für Naturforschung B*, *62*(4), 561-564. https://doi.org/10.1515/ZNB-2007-0412/machinereadablecitation/RIS.

Kanehara, R., Hayasaka, A., Inose, K., Okamura, T., Miura, S., Tanaka, K., & Hashimoto, M. (2021). Decaspirones and palmarumycins from *Phaeoseptum* sp. KT4106: Chirality reinvestigation of palmarumycins CP4a and CP5. *Tetrahedron*, *92*, 132251. https://doi.org/10.1016/J.tet.2021.132251.

Kasai, Y., Komatsu, K., Shigemori, H., Tsuda, M., Mikami, Y., & Kobayashi, J. (2005). Cladionol A, a polyketide glycoside from marine-derived fungus *Gliocladium* Species. *Journal of Natural Products*, *68*(5), 777–779. https://doi.org/10.1021/np050046b.

Kaul, S., Gupta, S., Ahmed, M., & Dhar, M. K. (2012). Endophytic fungi from medicinal plants: A treasure hunt for bioactive metabolites. *Phytochemistry Reviews,* 11, (4), 487–505. https://doi.org/10.1007/s11101-012-9260-6.

Kaushik, N., Díaz, C. E., Chhipa, H., Julio, L. F., Andrés, M. F., & González-Coloma, A. (2020). Chemical composition of an aphid antifeedant extract from an endophytic fungus, *Trichoderma* sp. EFI671. *Microorganisms*, *8*(3), 420. https://doi.org/10.3390/microorganisms8030420.

Keller, N. P. (2019). Fungal secondary metabolism: regulation, function and drug discovery. *Nature Reviews Microbiology*, *17*(3), 167–180. https://doi.org/10.1038/s41579-018-0121-1.

Keller, N. P., Turner, G., & Bennett, J. W. (2005). Fungal secondary metabolism - From biochemistry to genomics. *Nature Reviews Microbiology*, *3*(12), 937–947.

https://doi.org/10.1038/nrmicro1286.

Kemami Wangun, H. V., Dahse, H. M., & Hertweck, C. (2007). Epicoccamides B-D, glycosylated tetramic acid derivatives from an *Epicoccum* sp. associated with the tree fungus *Pholiota squarrosa. Journal of Natural Products*, *70*(11), 1800–1803. https://doi.org/10.1021/np070245q.

Khaldi, N., Seifuddin, F. T., Turner, G., Haft, D., Nierman, W. C., Wolfe, K. H., & Fedorova, N. D. (2010). SMURF: genomic mapping of fungal secondary metabolite clusters. *47*(9), 736–741. https://doi.org/10.1016/j.fgb.2010.06.003.

Khalil, A. M. A., Hassan, S. E. D., Alsharif, S. M., Eid, A. M., Ewais, E. E. D., Azab, E., ... & Fouda, A. (2021). Isolation and characterization of fungal endophytes isolated from medicinal plant Ephedra pachyclada as plant growth-promoting. *Biomolecules*, *11*(2), 140. https://doi.org/10.3390/biom11020140.

Khan, A. L., Shahzad, R., Al-Harrasi, A., & Lee, I. J. (2017). Endophytic microbes: a resource for producing extracellular enzymes. In *Endophytes: Crop productivity and protection* (pp. 95-110). Springer, Cham. https://doi.org/10.1007/978-3-319-66544-3_5.

Khan, A. L., Al-Harrasi, A., Al-Rawahi, A., Al-Farsi, Z., Al-Mamari, A., Waqas, M., ... & Lee, I. J. (2016). Endophytic fungi from frankincense tree improves host growth and produces extracellular enzymes and indole acetic acid. *PloS one*, *11*(6), e0158207. https://doi.org/10.1371/journal.pone.0158207.

Khare, E., Mishra, J., & Arora, N. K. (2018). Multifaceted interactions between endophytes and plant: Developments and prospects. *Frontiers in Microbiology 9*, 2732. https://doi.org/10.3389/fmicb.2018.02732.

Kharwar, R. N., Mishra, A., Gond, S. K., Stierle, A., & Stierle, D. (2011). Anticancer compounds derived from fungal endophytes: Their importance and future challenges. *Natural Product Reports*, *28*(7), 1208–1228. https://doi.org/10.1039/c1np00008j.

Khiralla, A., Spina, R., Yagi, S., Mohamed, I., & Laurain-Mattar, D. (2016). Endophytic fungi: Occurrence, classification, function and natural products. In *Endophytic Fungi: Diversity,*

Characterization and Biocontrol. Nova Publishers: New York, NY, USA, pp. 1–19.

Kim, H., Son, H., & Lee, Y. W. (2014). Effects of light on secondary metabolism and fungal development of Fusarium graminearum. *Journal of Applied Microbiology*, *116*(2), 380-389. https://doi.org/10.1111/jam.12381.

Kim, J. W., & Shim, S. H. (2019). The fungus *Colletotrichum* as a source for bioactive secondary metabolites. *Archives of Pharmacal Research, 42*(9), 735-753. https://doi.org/10.1007/s12272-019-01142-z.

Kjærbølling, I., Mortensen, U. H., Vesth, T., & Andersen, M. R. (2019). Strategies to establish the link between biosynthetic gene clusters and secondary metabolites. *Fungal Genetics and Biology*, *130*, 107–121. https://doi.org/10.1016/j.fgb.2019.06.001.

Kloepper, J. W., McInroy, J. A., Liu, K., & Hu, C. H. (2013). Symptoms of fern distortion syndrome resulting from inoculation with opportunistic endophytic fluorescent *Pseudomonas* spp. *PLoS One*, *8*(3), e58531. https://doi.org/10.1371/journal.pone.0058531.

Ko Ko, T. W., Stephenson, S. L., Bahkali, A. H., & Hyde, K. D. (2011). From morphology to molecular biology: can we use sequence data to identify fungal endophytes?. *Fungal Diversity*, *50*, 113-120. https://doi.org/10.1007/s13225-011-0130-0.

Krappmann, S. (2017). CRISPR-Cas9, the new kid on the block of fungal molecular biology. *Medical Mycology*, *55*(1), 16–23. https://doi.org/10.1093/mmy/MYW097.

Krohn, K., Michel, A., Flörke, U., Aust, H. -J, Draeger, S., & Schulz, B. (1994). Biologically active metabolites from fungi, 5. palmarumycins C1–C16 from *Coniothyrium* sp.: isolation, structure elucidation, and biological activity. *Liebigs Annalen Der Chemie*, *1994*(11), 1099–1108. https://doi.org/10.1002/JLAC.199419941108.

Kuivanen, J., Korja, V., Holmström, S., & Richard, P. (2019). Development of microtiter plate scale CRISPR/Cas9 transformation method for *Aspergillus niger* based on in vitro assembled ribonucleoprotein complexes. *Fungal Biology and Biotechnology, 6*(1), 1–12. https://doi.org/10.1186/S40694-019-0066-9.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis

version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870-1874. https://doi.org/10.1093/molbev/msw054.

Kumara, P. M., Shweta, S., Vasanthakumari, M. M., Sachin, N., Manjunatha, B. L., Jadhav, S. S., Ravikanth, G., Ganeshaiah, K. N., & Shaanker, R. U. (2014). Endophytes and plant secondary metabolite synthesis: Molecular and evolutionary perspective. In *Advances in Endophytic Research* (pp. 177–190). Springer, New Delhi. https://doi.org/10.1007/978-81-322-1575-2_9.

Kusari P., Spiteller M., Kayser O., Kusari S. *Microbial Diversity and Biotechnology in Food Security*. Springer; Berlin/Heidelberg, Germany: 2014. Recent advances in research on *Cannabis sativa* L. endophytes and their prospect for the pharmaceutical industry; pp. 3–15

Lewis, K. (2013). Platforms for antibiotic discovery. *Nature Reviews Drug Discovery*, *12*(5), 371–387. https://doi.org/10.1038/nrd3975.

Li, C., Sarotti, A. M., Yang, B., Turkson, J., & Cao, S. (2017). A New N-methoxypyridone from the Co-cultivation of hawaiian endophytic fungi *Camporesia sambuci* FT1061 and *Epicoccum sorghinum* FT1062. *Molecules,* *22*(7), 1166. https://doi.org/10.3390/molecules22071166.

Li, D., Tang, Y., Lin, J., & Cai, W. (2017). Methods for genetic transformation of filamentous fungi. *Microbial Cell Factories*, *16*(1), 1 -13. https://doi.org/10.1186/S12934-017-0785-7.

Li, H. T., Zhou, H., Duan, R. T., Li, H. Y., Tang, L. H., Yang, X. Q., Yang, Y. Bin, & Ding, Z. T. (2019). Inducing secondary metabolite production by co-culture of the endophytic fungus phoma sp. and the symbiotic Fungus *Armillaria* sp. *Journal of Natural Products*, *82*(4), 1009–1013. https://doi.org/10.1021/acs.jnatprod.8b00685.

Li, P., Wu, Z., Liu, T., & Wang, Y. (2016). Biodiversity, phylogeny, and antifungal functions of endophytic fungi associated with *Zanthoxylum bungeanum*. *International Journal of Molecular Sciences*, *17*(9), 1541. https://doi.org/10.3390/ijms17091541.

Li, Y., Shan, T., Mou, Y., Li, P., Zhao, J., Zhao, W., Peng, Y., Zhou, L., & Ding, C. (2012). Enhancement of palmarumycin C 12 and C 13 production in liquid culture of the endophytic fungus *Berkleasmium* sp. Dzf12 by oligosaccharides from its host plant *Dioscorea*

zingiberensis. *Molecules*, *17*(4), 3761–3773. https://doi.org/10.3390/molecules17043761.

Liu, Y., Liu, N., Yin, Y., Chen, Y., Jiang, J., & Ma, Z. (2015). Histone H3K4 methylation regulates hyphal growth, secondary metabolism and multiple stress responses in F usarium graminearum. *Environmental Microbiology*, *17*(11), 4615-4630. https://doi.org/10.1111/1462-2920.12993.

Lücking, R., Aime, M. C., Robbertse, B., Miller, A. N., Ariyawansa, H. A., Aoki, T., Cardinali, G., Crous, P. W., Druzhinina, I. S., Geiser, D. M., Hawksworth, D. L., Hyde, K. D., Irinyi, L., Jeewon, R., Johnston, P. R., Kirk, P. M., Malosso, E., May, T. W., Meyer, W., … Schoch, C. L. (2020). Unambiguous identification of fungi: Where do we stand and how accurate and precise is fungal DNA barcoding? *IMA Fungus*, *11*(1), 1 -32. https://doi.org/10.1186/s43008-020-00033-z.

Lugtenberg, B. J. J., Caradus, J. R., & Johnson, L. J. (2016). Fungal endophytes for sustainable crop production. *FEMS Microbiology Ecology*, *92*(12). https://doi.org/10.1093/femsec/fiw194.

Ma, X., Nontachaiyapoom, S., Jayawardena, R. S., Hyde, K. D., Gentekaki, E., Zhou, S., Qian, Y., Wen, T., & Kang, J. (2018). Endophytic *Colletotrichum* species from *Dendrobium* spp. in China and Northern Thailand. *MycoKeys*, *43*(43), 23–57. https://doi.org/10.3897/mycokeys.43.25081.

Ma, Y. J., Lu, C. S., & Wang, J. W. (2018). Effect of 5-azacytidine on growth and hypocrellin production of *Shiraia bambusicola*. *Frontiers in Microbiology*, *9*, 2508. https://doi.org/10.3389/fmicb.2018.02508.

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V., & Brakhage, A. A. (2016). Regulation and role of fungal secondary metabolites. *Annual Review of Genetics*, *50*(1), 371–392. https://doi.org/10.1146/annurev-genet-120215-035203.

Mafezoli, J., Xu, Y. ming, Hilário, F., Freidhof, B., Espinosa-Artiles, P., dos Santos, L. C., de Oliveira, M. C. F., & Gunatilaka, A. A. L. (2018). Modulation of polyketide biosynthetic pathway of the endophytic fungus, *Anteaglonium* sp. FL0768, by copper (II) and anacardic

acid. *Phytochemistry Letters*, *28*, 157–163. https://doi.org/10.1016/j.phytol.2018.10.011.

Magotra, A., Kumar, M., Kushwaha, M., Awasthi, P., Raina, C., Gupta, A. P., Shah, B. A., Gandhi, S. G., & Chaubey, A. (2017). Epigenetic modifier induced enhancement of fumiquinazoline C production in *Aspergillus fumigatus* (GA-L7): an endophytic fungus from *Grewia asiatica* L. *AMB Express*, *7*(1), 1 -10. https://doi.org/10.1186/s13568-017-0343-z.

Mahapatra, S., & Banerjee, D. (2010). Diversity and screening for antimicrobial activity of endophytic fungi from *Alstonia scholaris*. *Acta Microbiologica et Immunologica Hungarica*, *57*(3), 215–223. https://doi.org/10.1556/AMicr.57.2010.3.6.

Manganyi, M. C., & Ateba, C. N. (2020). Untapped potentials of endophytic fungi: A review of novel bioactive compounds with biological applications. *Microorganisms*, *8*(12), 1–25. https://doi.org/10.3390/microorganisms8121934.

Marcellano, J. P., Collanto, A. S., & Fuentes, R. G. (2017). Antibacterial activity of endophytic fungi isolated from the bark of *Cinnamomum mercadoi*. *Pharmacognosy Journal*, *9*(3), 405–409. https://doi.org/10.5530/pj.2017.3.69.

Martinez-Klimova, E., Rodríguez-Peña, K., & Sánchez, S. (2017). Endophytes as sources of antibiotics. *Biochemical Pharmacology,* 134, 1–17. https://doi.org/10.1016/j.bcp.2016.10.010.

Masumoto, H., & Degawa, Y. (2019). The effect of surface sterilization and the type of sterilizer on the genus composition of lichen-inhabiting fungi with notes on some frequently isolated genera. *Mycoscience*, *60*(6), 331-342. https://doi.org/10.1016/j.myc.2019.07.004

McCluskey, K., & Baker, S. E. (2017). Diverse data supports the transition of filamentous fungal model organisms into the post-genomics era. *Mycology*, *8*(2), 67–83. https://doi.org/10.1080/21501203.2017.1281849.

Medema, M. H., Blin, K., Cimermancic, P., De Jager, V., Zakrzewski, P., Fischbach, M. A., Weber, T., Takano, E., & Breitling, R. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Research*, *39*(suppl_2), W339-W346.

https://doi.org/10.1093/NAR/GKR466.

Mehravar, M., Shirazi, A., Mehrazar, M. M., & Nazari, M. (2019). In vitro pre-validation of gene editing by CRISPR/Cas9 ribonucleoprotein. *Avicenna Journal of Medical Biotechnology*, *11*(3), 259.

Meshram, V., & Gupta, M. (2019). Endophytic fungi: A quintessential source of potential bioactive compounds. In *Endophytes for a Growing World* (pp. 277–309). Cambridge University Press: Cambridge, UK. https://doi.org/10.1017/9781108607667.014.

Mishra, K. P., Ganju, L., Sairam, M., Banerjee, P. K., & Sawhney, R. C. (2008). A review of high throughput technology for the screening of natural products. *Biomedicine and Pharmacotherapy,* 62, (2) 94–98. https://doi.org/10.1016/j.biopha.2007.06.012.

Monciardini, P., Iorio, M., Maffioli, S., Sosio, M., & Donadio, S. (2014). Discovering new bioactive molecules from microbial sources. *Microbial biotechnology*, *7*(3), 209-220. https://doi.org/10.1111/1751-7915.12123.

Moody, S. C. (2014). Microbial co-culture: Harnessing intermicrobial signaling for the production of novel antimicrobials. *Future Microbiology*, 9(5), 575–578). https://doi.org/10.2217/fmb.14.25.

Moriwaki, A., Kihara, J., Kobayashi, T., Tokunaga, T., Arase, S., & Honda, Y. (2004). Insertional mutagenesis and characterization of a polyketide synthase gene (PKS1) required for melanin biosynthesis in *Bipolaris oryzae*. *FEMS Microbiology Letters*, *238*(1), 1–8. https://doi.org/10.1016/J.femsle.2004.07.007.

Motoyama, T., Yun, C.-S., & Osada, H. (2021). Biosynthesis and biological function of secondary metabolites of the rice blast fungus *Pyricularia oryzae*. *Journal of Industrial Microbiology and Biotechnology*, 48, 9-108. https://doi.org/10.1093/JIMB/KUAB058.

Mou, Y., Xu, D., Mao, Z., Dong, X., Lin, F., Wang, A., Lai, D., Zhou, L., & Xie, B. (2015). Enhancement of palmarumycin c12 and c13 production by the endophytic fungus *Berkleasmium* sp. Dzf12 in an aqueous-organic solvent system. *Molecules*, *20*(11), 20320–20333. https://doi.org/10.3390/molecules201119700.

Mourad, E. F., Sarhan, M. S., Daanaa, H. S. A., Abdou, M., Morsi, A. T., Abdelfadeel, M. R., ... & Hegazi, N. A. (2018). Plant materials are sustainable substrates supporting new technologies of plant-only-based culture media for in vitro culturing of the plant microbiota. *Microbes and Environments*, *33*(1), 40-49. https://doi.org/10.1264/jsme2.ME17135.

Ng, S. B., Kanagasundaram, Y., Fan, H., Arumugam, P., Eisenhaber, B., & Eisenhaber, F. (2018). The 160K natural organism library, a unique resource for natural products research. *Nature Biotechnology*, *36*(7), 570–573. https://doi.org/10.1038/nbt.4187.

Nielsen, K. F., & Larsen, T. O. (2015). The importance of mass spectrometric dereplication in fungal secondary metabolite analysis. *Frontiers in Microbiology*, *6*, 71. https://doi.org/10.3389/fmicb.2015.00071.

Ning, Y., Hu, B., Yu, H., Liu, X., Jiao, B., & Lu, X. (2022). Optimization of protoplast preparation and establishment of genetic transformation system of an arctic-derived fungus *Eutypella* sp. *Frontiers in Microbiology*, 13:769008. https://doi.org/10.3389/fmicb.2022.769008.

Nisa, H., Kamili, A. N., Nawchoo, I. A., Shafi, S., Shameem, N., & Bandh, S. A. (2015). Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review. *Microbial Pathogenesis*, *82*, 50–59. https://doi.org/10.1016/j.micpath.2015.04.001.

Nødvig, C. S., Nielsen, J. B., Kogle, M. E., & Mortensen, U. H. (2015). A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLOS ONE*, *10*(7), e0133085. https://doi.org/10.1371/journal.pone.0133085.

Ola, A. R. B., Thomy, D., Lai, D., Brötz-Oesterhelt, H., & Proksch, P. (2013). Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through coculture with *Bacillus subtilis*. *Journal of Natural Products*, *76*(11), 2094–2099. https://doi.org/10.1021/np400589h.

Ortega, H. E., Torres-Mendoza, D., E., Z. C., & Cubilla-Rios, L. (2021). Structurally uncommon secondary metabolites derived from endophytic fungi. *Journal of Fungi 2021, 7*(7), 570. https://doi.org/10.3390/jof7070570.

Ouedraogo, J. P., & Tsang, A. (2020). CRISPR_Cas systems for fungal research. *Fungal Biology*

Reviews, *34*(4), 189–201. https://doi.org/10.1016/J.fbr.2020.10.002.

Paranagama, P. A., Wijeratne, E. M. K., & Gunatilaka, A. A. L. (2007). Uncovering biosynthetic potential of plant-associated fungi: Effect of culture conditions on metabolite production by *Paraphaeosphaeria quadriseptata* and *Chaetomium chiversii*. *Journal of Natural Products*, *70*(12), 1939–1945. https://doi.org/10.1021/np070504b.

Patil, R. H., Patil, M. P., & Maheshwari, V. L. (2016). Bioactive secondary metabolites from endophytic fungi: a review of biotechnological production and their potential applications. *Studies in Natural Products Chemistry*, *49*, 189–205. https://doi.org/10.1016/B978-0-444-63601-0.00005-3

Petrini, O. (1991). Fungal endophytes of tree leaves. In *Microbial ecology of leaves* (pp. 179-197). Springer New York.

Pillay, L. C., Nekati, L., Makhwitine, P. J., & Ndlovu, S. I. (2022). Epigenetic activation of silent biosynthetic gene clusters in endophytic fungi using small molecular modifiers. *Frontiers in Microbiology*, *13*, 231. https://doi.org/10.3389/fmicb.2022.815008.

Pollastro, F., & Minassi, A. (2021). Exploring the universe of natural products: Recent advances in synthesis, isolation and structural elucidation. *Plants*, *10*(11), 2368. https://doi.org/10.3390/plants10112368.

Pommier, Y., Leo, E., Zhang, H., & Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chemistry & Biology*, *17*(5), 421-433. https://doi.org/10.1016/j.chembiol.2010.04.012.

Qiao, Y. M., Yu, R. L., & Zhu, P. (2019). Advances in targeting and heterologous expression of genes involved in the synthesis of fungal secondary metabolites. *RSC advances*, *9*(60), 35124-35134. https://doi.org/10.1039/c9ra06908a.

Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. *Journal of Natural Products*, *80*(3), 756–770. https://doi.org/10.1021/acs.jnatprod.6b01085.

Ramesha, K. P., Chandra Mohana, N., Chandra Nayaka, S., & Satish, S. (2021). Epigenetic

modifiers revamp secondary metabolite production in endophytic *Nigrospora sphaerica*. *Frontiers in Microbiology*, *12*, 3485. https://doi.org/10.3389/fmicb.2021.730355.

Rastogi, A., Murik, O., Bowler, C., & Tirichine, L. (2016). PhytoCRISP-Ex: a web-based and stand-alone application to find specific target sequences for CRISPR/CAS editing. *BMC Bioinformatics, 17*(1), 1–4. https://doi.org/10.1186/S12859-016-1143-1.

Rather, R. A., Srinivasan, V., & Anwar, M. (2018). Seasonal deviation effects foliar endophyte assemblage and diversity in *Asparagus racemosus* and *Hemidesmus indicus*. *BMC Ecology*, *18*(1), 1–11. https://doi.org/10.1186/s12898-018-0211-y.

Reen, F. J., Romano, S., Dobson, A. D. W., & O'Gara, F. (2015). The sound of silence: Activating silent biosynthetic gene clusters in marine microorganisms. *Marine Drugs*, *13*(8), 4754–4783. https://doi.org/10.3390/md13084754.

Ren, N., Liu, J., Yang, D., Liu, X., Zhou, J., & Peng, Y. (2018). Preparation and regeneration of protoplasts from the ethyl vincamine producing fungus CH1 (*Geomyces* sp.). *Natural Product Communications*, *13*(2), 145–148. https://doi.org/10.1177/1934578x1801300209.

Rim, S. O., Roy, M., Jeon, J., Montecillo, J. A. V., Park, S. C., & Bae, H. (2021). Diversity and communities of fungal endophytes from four Pinus species in Korea. *Forests*, *12*(3), 302. https://doi.org/10.3390/f12030302.

Robbins, T., Kapilivsky, J., Cane, D. E., & Khosla, C. (2016). Roles of conserved active site residues in the ketosynthase domain of an assembly line polyketide synthase. *Biochemistry*, *55*(32), 4476. https://doi.org/10.1021/ACS.biochem.6B00639.

Rodriguez, R. J., White, J. F., Arnold, A. E., & Redman, R. S. (2009). Fungal endophytes: Diversity and functional roles. *New Phytologist*, *182*(2), 314–330. https://doi.org/10.1111/j.1469-8137.2009.02773.x.

Rojas-Aedo, J. F., Gil-Durán, C., Goity, A., Vaca, I., Levicán, G., Larrondo, L. F., & Chávez, R. (2018). The developmental regulator Pcz1 affects the production of secondary metabolites in the filamentous fungus *Penicillium roqueforti*. *Microbiological Research*, *212*, 67–74. https://doi.org/10.1016/j.micres.2018.05.005.

Roopa, G., Madhusudhan, M. C., Sunil, K. C. R., Lisa, N., Calvin, R., Poornima, R., Zeinab, N., Kini, K. R., Prakash, H. S., & Geetha, N. (2015). Identification of Taxol-producing endophytic fungi isolated from Salacia oblonga through genomic mining approach. *Journal of Genetic Engineering and Biotechnology*, *13*(2), 119–127. https://doi.org/10.1016/j.jgeb.2015.09.002.

Rossman, A. Y., Adams, G. C., Cannon, P. F., Castlebury, L. A., Crous, P. W., Gryzenhout, M., Jaklitsch, W. M., Mejia, L. C., Stoykov, D., Udayanga, D., Voglmayr, H., & Walker, D. M. (2015). Recommendations of generic names in *Diaporthales* competing for protection or use. *IMA Fungus*, *6*(1), 145–154. https://doi.org/10.5598/imafungus.2015.06.01.09.

Roth, M. G., & Chilvers, M. I. (2019). A protoplast generation and transformation method for soybean sudden death syndrome causal agents *Fusarium virguliforme* and *F. brasiliense*. *Fungal Biology and Biotechnology*, *6*(1), 1–8. https://doi.org/10.1186/s40694-019-0070-0.

Said, G., & Ahmad, F. (2022). Effects of salt concentration on the production of cytotoxic geodin from marine-derived fungus *Aspergillus* sp. *Turkish Journal of Biochemistry*, *47*(4), 399-402. https://doi.org/10.1515/tjb-2022-0058.

Sahu, P. K., Tilgam, J., Mishra, S., Hamid, S., Gupta, A., Verma, S. K., & Kharwar, R. N. (2022). Surface sterilization for isolation of endophytes: Ensuring what (not) to grow. *Journal of Basic Microbiology*, *62*(6), 647-668. https://doi.org/10.1002/jobm.202100462.

Sayed, A. M., Hassan, M. H. A., Alhadrami, H. A., Hassan, H. M., Goodfellow, M., & Rateb, M. E. (2020). Extreme environments: microbiology leading to specialized metabolites. *Journal of Applied Microbiology*, *128*(3), 630–657. https://doi.org/10.1111/jam.14386.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., & Consortium, F. B. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241–6246. https://doi.org/10.1073/pnas.1117018109.

Schüller, A., Wolansky, L., Berger, H., Studt, L., Gacek-Matthews, A., Sulyok, M., & Strauss, J. (2020). A novel fungal gene regulation system based on inducible VPR-dCas9 and

nucleosome map-guided sgRNA positioning. *Applied Microbiology and Biotechnology*, *104*(22), 9801–9822. https://doi.org/10.1007/s00253-020-10900-9.

Schulz, B., Römmert, A. K., Dammann, U., Aust, H. J., & Strack, D. (1999). The endophyte-host interaction: A balanced antagonism? *Mycological Research*, *103*(10), 1275–1283. https://doi.org/10.1017/S0953756299008540.

Schuster, M., & Kahmann, R. (2019). CRISPR-Cas9 genome editing approaches in filamentous fungi and oomycetes. *Fungal Genetics and Biology*, *130*, 43–53. https://doi.org/10.1016/j.fgb.2019.04.016.

Shan, T., Tian, J., Wang, X., Mou, Y., Mao, Z., Lai, D., Dai, J., Peng, Y., Zhou, L., & Wang, M. (2014). Bioactive spirobisnaphthalenes from the endophytic fungus *Berkleasmium* sp. *Journal of Natural Products*, *77*(10), 2151–2160. https://doi.org/10.1021/np400988a.

Shearer, C. A., Descals, E., Kohlmeyer, B., Kohlmeyer, J., Marvanová, L., Padgett, D., Porter, D., Raja, H. A., Schmit, J. P., Thorton, H. A., & Voglymayr, H. (2007). Fungal biodiversity in aquatic habitats. *Biodiversity and Conservation*, *16*(1), 49–67. https://doi.org/10.1007/s10531-006-9120-z.

Shwab, E. K., Jin, W. B., Tribus, M., Galehr, J., Graessle, S., & Keller, N. P. (2007). Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryotic Cell*, *6*(9), 1656–1664. https://doi.org/10.1128/ec.00186-07.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210-3212. https://doi.org/10.1093/bioinformatics/btv351.

Sirota, F. L., Goh, F., Low, K.-N., Yang, L.-K., Crasta, S. C., Eisenhaber, B., Eisenhaber, F., Kanagasundaram, Y., & Ng, S. B. (2018). Isolation and identification of an anthracimycin analogue from nocardiopsis kunsanensis , a halophile from a saltern, by genomic mining strategy . *Journal of Genomics*, *6*, 63–73. https://doi.org/10.7150/jgen.24368.

Skellam, E. (2019). Strategies for engineering natural product biosynthesis in fungi. *Trends in Biotechnology*, *37*(4), 416-427. https://doi.org/10.1016/j.tibtech.2018.09.003.

Soldatou, S., Eldjarn, G. H., Huerta-Uribe, A., Rogers, S., & Duncan, K. R. (2019). Linking biosynthetic and chemical space to accelerate microbial secondary metabolite discovery. *FEMS Microbiology Letters*, 366 (13), 142. https://doi.org/10.1093/femsle/fnz142.

Strobel, G. A. (2003). Endophytes as sources of bioactive products. *Microbes and Infection*, 5 (6), 535–544). https://doi.org/10.1016/s1286-4579(03)00073-x.

Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*, *67*(4), 491–502. https://doi.org/10.1128/mmbr.67.4.491-502.2003.

Stroe, M., Netzker, T., Scherlach, K., Krüger, T., Hertweck, C., Valiante, V., & Brakhage, A. A. (2020). Targeted induction of a silent fungal gene cluster encoding the bacteria-specific germination inhibitor fumigermin. *ELife*, *9*, e52541. https://doi.org/10.7554/eLife.52541.

Sun, X., & Guo, L. D. (2012). Endophytic fungal diversity: Review of traditional and molecular techniques. *Mycology*, *3*(1), 65–76. https://doi.org/10.1080/21501203.2012.656724.

Supratman, U., Suzuki, T., Nakamura, T., Yokoyama, Y., Harneti, D., Maharani, R., Salam, S., Abdullah, F. F., Koseki, T., & Shiono, Y. (2019). New metabolites produced by endophyte *Clonostachys rosea* B5 − 2. *Natural Product Research*, *35*(9), 1525-1531. https://doi.org/10.1080/14786419.2019.1656629.

Tamura, M., Mochizuki, N., Nagatomi, Y., Toriba, A., & Hayakawa, K. (2014). Characterization of fumonisin a-series by high-resolution liquid chromatography-orbitrap mass spectrometry. *Toxins* *6*(8), 2580–2593. https://doi.org/10.3390/toxins6082580.

Tanaka, A., Takemoto, D., Chujo, T., & Scott, B. (2012). Fungal endophytes of grasses. *Current Opinion in Plant Biology*, *15*(4), 462–468. https://doi.org/10.1016/j.pbi.2012.03.007.

Thi Minh Le, T., Thi Hong Hoang, A., Thi Bich Le, T., Thi Bich Vo, T., Van Quyen, D., & Hoang Chu, H. (2019). Isolation of endophytic fungi and screening of Huperzine A–producing fungus from Huperzia serrata in Vietnam. *Scientific Reports*, *9*(1), 16152. https://doi.org/10.1038/s41598-019-52481-2.

Toghueo, K. M., & Boyom, F. F. (2019). Endophytes from ethno-pharmacological plants: Sources

of novel antioxidants- A systematic review. *Biocatalysis and Agricultural Biotechnology*, *22*, 101430. https://doi.org/10.1016/j.bcab.2019.101430.

Toghueo, R. M., Sahal, D., & Boyom, F. F. (2020). Recent advances in inducing endophytic fungal specialized metabolites using small molecule elicitors including epigenetic modifiers. *Phytochemistry*, *174*, 112338. https://doi.org/10.1016/j.phytochem.2020.112338.

Tonoi, T., Inohana, T., Sato, T., Noda, Y., Ikeda, M., Akutsu, M., Murata, T., Maekawa, Y., Tanaka, A., Seki, R., Ohkusu, M., Kamei, K., Ishiwada, N., & Shiina, I. (2019). Total synthesis and antimicrobial evaluation of 23-demethyleushearilide and extensive antimicrobial evaluation of all synthetic stereoisomers of (16Z,20E)-eushearilide and (16E,20E)-eushearilide. *Molecules*, *24*(19), 3437. https://doi.org/10.3390/molecules24193437.

Tonoi, T., Kawahara, R., Inohana, T., & Shiina, I. (2016). Enantioselective total synthesis of naturally occurring eushearilide and evaluation of its antifungal activity. *Journal of Antibiotics*, *69*(9), 697–701. https://doi.org/10.1038/JA.2015.146.

Triastuti, A., Vansteelandt, M., Barakat, F., Trinel, M., Jargeat, P., Fabre, N., Amasifuen Guerra, C. A., Mejia, K., Valentin, A., & Haddad, M. (2019). How histone deacetylase inhibitors alter the secondary metabolites of *Botryosphaeria mamane*, an endophytic fungus isolated from *Bixa orellana*. *Chemistry and Biodiversity*, *16*(4), e1800485. https://doi.org/10.1002/cbdv.201800485.

Tudor, D., Robinson, S. C., & Cooper, P. A. (2013). The influence of pH on pigment formation by lignicolous fungi. *International Biodeterioration & Biodegradation*, *80*, 22-28. https://doi.org/10.1016/j.ibiod.2012.09.013.

Turenne, C. Y., Sanche, S. E., Hoban, D. J., Karlowsky, J. A., & Kabani, A. M. (1999). Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *Journal of Clinical Microbiology*, *37*(6), 1846–1851. https://doi.org/10.1128/jcm.37.6.1846-1851.1999.

Tychanowicz, G. K., De Souza, D. F., Souza, C. G. M., Kadowaki, M. K., & Peralta, R. M. (2006).

Copper improves the production of laccase by the white-rot fungus *Pleurotus pulmonarius* in solid state fermentation. *Brazilian Archives of Biology and Technology*, *49*(5), 699–704. https://doi.org/10.1590/S1516-89132006000600002.

Udayanga, D., Liu, X., McKenzie, E. H. C., Chukeatirote, E., Bahkali, A. H. A., & Hyde, K. D. (2011). The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. *Fungal Diversity*, *50*(1), 189–225. https://doi.org/10.1007/s13225-011-0126-9.

Uzma, F., Mohan, C. D., Hashem, A., Konappa, N. M., Rangappa, S., Kamath, P. V., Singh, B. P., Mudili, V., Gupta, V. K., Siddaiah, C. N., Chowdappa, S., Alqarawi, A. A., & Abd-Allah, E. F. (2018). Endophytic fungi-alternative sources of cytotoxic compounds: A review. *Frontiers in Pharmacology*, *9*, 309. https://doi.org/10.3389/fphar.2018.00309.

Valente, S., Piombo, E., Schroeckh, V., Meloni, G. R., Heinekamp, T., Brakhage, A. A., & Spadaro, D. (2021). CRISPR-Cas9-Based Discovery of the verrucosidin biosynthesis gene cluster in *Penicillium polonicum*. *Frontiers in Microbiology*, *12*, 1114. https://doi.org/10.3389/fmicb.2021.660871.

Vancov, T., & Keen, B. (2009). Amplification of soil fungal community DNA using the ITS86F and ITS4 primers. *FEMS Microbiology Letters*, *296*(1), 91–96. https://doi.org/10.1111/j.1574-6968.2009.01621.x.

VanderMolen, K. M., Raja, H. A., El-Elimat, T., & Oberlies, N. H. (2013). Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *AMB Express*, *3*, (1), 71. https://doi.org/10.1186/2191-0855-3-71.

Vasundhara, M., Kumar, A., & Reddy, M. S. (2016). Molecular approaches to screen bioactive compounds from endophytic fungi. *Frontiers in Microbiology* 7, 1774. https://doi.org/10.3389/fmicb.2016.01774.

Venieraki, A., Dimou, M., & Katinakis, P. (2017). Endophytic fungi residing in medicinal plants have the ability to produce the same or similar pharmacologically active secondary metabolites as their hosts. *Hellenic Plant Protection Journal*, *10*(2), 51-66.

https://doi.org/10.1515/hppj-2017-0006.

Venkatachalam, M., Gérard, L., Milhau, C., Vinale, F., Dufossé, L., & Fouillaud, M. (2019). Salinity and temperature influence growth and pigment production in the marine-derived fungal strain Talaromyces albobiverticillius 30548. *Microorganisms*, *7*(1), 10. https://doi.org/10.3390/microorganisms7010010.

Verma, V. C., Gond, S. K., Kumar, A., Kharwar, R. N., Boulanger, L. A., & Strobel, G. A. (2011). Endophytic fungal flora from roots and fruits of an Indian neem plant Azadirachta indica A. Juss., and impact of culture media on their isolation. *Indian Journal of Microbiology*, *51*, 469-476. https://doi.org/10.1007/s12088-011-0121-6.

Voigt, O., Knabe, N., Nitsche, S., Erdmann, E. A., Schumacher, J., & Gorbushina, A. A. (2020). An advanced genetic toolkit for exploring the biology of the rock-inhabiting black fungus *Knufia petricola*. *Scientific Reports, 10*(1), 1–14. https://doi.org/10.1038/s41598-020-79120-5.

Von Bubnoff, A. (2006). Seeking new antibiotics in nature's backyard. *Cell*, *127*(5), 867-869. https://doi.org/10.1016/j.cell.2006.11.021

Wang, J, P., Lin, W., Wray, V., Lai, D., & Proksch, P. (2013). Induced production of depsipeptides by co-culturing *Fusarium tricinctum* and *Fusarium begoniae*. *Tetrahedron Letters*, *54*(20), 2492–2496. https://doi.org/10.1016/j.tetlet.2013.03.005.

Wang, B., You, J., King, J. B., Cai, S., Park, E., Powell, D. R., & Cichewicz, R. H. (2014). Polyketide glycosides from bionectria ochroleuca inhibit *Candida albicans* biofilm formation. *Journal of Natural Products*, *77*(10), 2273–2279. https://doi.org/10.1021/np500531j.

Wang, Q., Zhong, C., & Xiao, H. (2020). Genetic engineering of filamentous fungi for efficient protein expression and secretion. *Frontiers in Bioengineering and Biotechnology*, *8*, 293. https://doi.org/10.3389/fbioe.2020.00293.

Wei, Y., Zhou, X., Liu, L., Lu, J., Wang, Z., Yu, G., Hu, L., Lin, J., Sun, X., & Tang, K. (2012). An efficient transformation system of taxol-producing endophytic fungus EFY-21 (*Ozonium sp.*). *African Journal of Biotechnology*, *9*(12), 1726–1733. https://doi.org/10.4314/ajb.v9i12.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols A guide to methods and applications. San Diego, California. Academic Press. pp. 315–322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1.

Wicklow, D. T., Jordan, A. M., & Gloer, J. B. (2009). Antifungal metabolites (monorden, monocillins I, II, III) from *Colletotrichum graminicola*, a systemic vascular pathogen of maize. *Mycological Research*, *113*(12), 1433-1442. https://doi.org/10.1016/J.mycres.2009.10.001.

Wiemann, P., & Keller, N. P. (2014). Strategies for mining fungal natural products. *Journal of Industrial Microbiology and Biotechnology, 41*(2), 301-313.https://doi.org/10.1007/s10295-013-1366-3.

Wohlleben, W., Mast, Y., Stegmann, E., & Ziemert, N. (2016). Antibiotic drug discovery. *Microbial Biotechnology*, *9*(5), 541–548. https://doi.org/10.1111/1751-7915.12388.

Wolfender, J. L., Marti, G., Thomas, A., & Bertrand, S. (2015). Current approaches and challenges for the metabolite profiling of complex natural extracts. *Journal of Chromatography A*, *1382*, 136–164. https://doi.org/10.1016/J.chroma.2014.10.091.

Wright, A. D., Osterhage, C., & König, G. M. (2003). Epicoccamide, a novel secondary metabolite from a jellyfish-derived culture *of Epicoccum purpurascens*. *Organic & Biomolecular Chemistry*, *1*(3), 507–510. https://doi.org/10.1039/b208588g.

Wu, J. De, & Chou, J. C. (2019). Optimization of protoplast preparation and regeneration of a medicinal fungus *Antrodia cinnamomea*. *Mycobiology*, *47*(4), 483. https://doi.org/10.1080/12298093.2019.1687252.

Wu, J. S., Yao, G. S., Shi, X. H., Rehman, S. U., Xu, Y., Fu, X. M., Zhang, X. L., Liu, Y., & Wang, C. Y. (2020). Epigenetic agents trigger the production of bioactive nucleoside derivatives and bisabolane sesquiterpenes from the marine-derived fungus *Aspergillus versicolor*. *Frontiers in Microbiology*, *11*, 85. https://doi.org/10.3389/fmicb.2020.00085.

Wu, Z., Su, Q., Cui, Y., He, H., Wang, J., Zhang, Y., Zhao, Y., Abul, H., Yang, Y., & Long, Y.

(2020). Temporal and spatial pattern of endophytic fungi diversity of *Camellia sinensis* (cv. Shu Cha Zao). *BMC Microbiology*, *20*(1), 270. https://doi.org/10.1186/s12866-020-01941-1.

Xiong, Z. Q., Yang, Y. Y., Zhao, N., & Wang, Y. (2013). Diversity of endophytic fungi and screening of fungal paclitaxel producer from Anglojap yew, *Taxus x media*. *BMC Microbiology*, *13*(1), 71. https://doi.org/10.1186/1471-2180-13-71.

Xu, J. (2016). Fungal DNA barcoding. *Genome*, 59 (11), 913–932. https://doi.org/10.1139/gen-2016-0046.

Xu, L., Wu, P., Xue, J., Molnar, I., & Wei, X. (2017). Antifungal and cytotoxic β-resorcylic acid lactones from a *Paecilomyces* species. *Journal of Natural Products*, *80*(8), 2215–2253. https://doi.org/10.1021/acs.jnatprod.7b00066.

Xu, T.-C., Lu, Y.-H., Wang, J.-F., Song, Z.-Q., Hou, Y.-G., Liu, S.-S., Liu, C.-S., & Wu, S.-H. (2021). Bioactive secondary metabolites of the genus *Diaporthe* and anamorph *Phomopsis* from terrestrial and marine habitats and endophytes: 2010–2019. *Microorganisms*, *9*(2), 217. https://doi.org/10.3390/microorganisms9020217. https://doi.org/10.3390/jof9020172.

Xue, M., Hou, X., Fu, J., Zhang, J., Wang, J., Zhao, Z., ... & Zhou, L. (2023). Recent advances in search of bioactive secondary metabolites from fungi triggered by chemical epigenetic modifiers. *Journal of Fungi*, *9*(2), 172.

Yamamoto, N., & Bibby, K. (2014). Clustering of fungal community internal transcribed spacer sequence data obscures taxonomic diversity. *Environmental Microbiology*, *16*(8), 2491–2500. https://doi.org/10.1111/1462-2920.12390.

Yang, X. L., Huang, L., & Ruan, X. L. (2014). Epigenetic modifiers alter the secondary metabolite composition of a plant endophytic fungus, *Pestalotiopsis crassiuscula* obtained from the leaves of *Fragaria chiloensis*. *Journal of Asian Natural Products Research*, *16*(4), 412–417. https://doi.org/10.1080/10286020.2014.881356.

Yip, K. M., Xu, J., Zhou, S. S., Lau, Y. M., Chen, Q. L., Tang, Y. C., Yang, Z. J., Yao, Z. P., Ding, P., Chen, H. B., & Zhao, Z. Z. (2019). Antibacterial radicicol analogues from pochonia chlamydosporia and their biosynthetic gene cluster. *Journal of Agricultural and Food*

Chemistry, *67*(26), 7266–7273. https://doi.org/10.1021/acs.jafc.9B01977.

Yogabaanu, U., Weber, J. F. F., Convey, P., Rizman-Idid, M., & Alias, S. A. (2017). Antimicrobial properties and the influence of temperature on secondary metabolite production in cold environment soil fungi. *Polar Science*, *14*, 60–67. https://doi.org/10.1016/j.polar.2017.09.005.

You, Y. H., Park, J. M., Seo, Y. G., Lee, W., Kang, M. S., & Kim, J. G. (2017). Distribution, characterization, and diversity of the endophytic fungal communities on Korean seacoasts showing contrasting geographic conditions. *Mycobiology*, *45*(3), 150–159. https://doi.org/10.5941/myco.2017.45.3.150.

Yu, Y., Chen, Z., Xie, H., Feng, X., Wang, Y., & Xu, P. (2022). Overhauling the effect of surface sterilization on analysis of endophytes in tea plants. *Frontiers in Plant Science*, 13: 849658. https://doi.org/10.3389/fpls.2022.849658.

Yun, C. S., Nishimoto, K., Motoyama, T., Shimizu, T., Hino, T., Dohmae, N., Nagano, S., & Osada, H. (2020). Unique features of the ketosynthase domain in a nonribosomal peptide synthetase-polyketide synthase hybrid enzyme, tenuazonic acid synthetase 1. *The Journal of Biological Chemistry*, *295*(33), 11602–11612. https://doi.org/10.1074/jbc.ra120.013105.

Zabala, A. O., Xu, W., Chooi, Y. H., & Tang, Y. (2012). Characterization of a silent azaphilone gene cluster from *Aspergillus niger* ATCC 1015 reveals a hydroxylation-mediated pyran-ring formation. *Chemistry and Biology*, *19*(8), 1049–1059. https://doi.org/10.1016/j.chembiol.2012.07.004.

Zeilinger, S., García-Estrada, C., & Martín, J. F. (2015). Fungal secondary metabolites in the "OMICS" Era. In *Biosynthesis and Molecular Genetics of Fungal Secondary Metabolites, Volume 2* (pp. 1-12). Springer, New York, NY. https://doi.org/10.1007/978-1-4939-2531-5_1.

Zhang, Y., Ouyang, L., Nan, Y., & Chu, J. (2019). Efficient gene deletion and replacement in *Aspergillus niger* by modified in vivo CRISPR/Cas9 systems. *Bioresources and Bioprocessing*, *6*(1), 1–8. https://doi.org/10.1186/s40643-019-0239-7.

Zhao, J, Wang, X., Sun, W., Mou, Y., Peng, Y., & Zhou, L. (2013). Medium optimization for

palmarumycin C13 production in liquid culture of endophytic fungus *Berkleasmium* sp. Dzf12 using response surface methodology. *Electronic Journal of Biotechnology*, *16*(6), 16–16. https://doi.org/10.2225/vol16-issue6-fulltext-10.

Zimmerman, N. B., & Vitousek, P. M. (2012). Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(32), 13022–13027. https://doi.org/10.1073/pnas.1209872109.

Zou, G., Xiao, M., Chai, S., Zhu, Z., Wang, Y., & Zhou, Z. (2020). Efficient genome editing in filamentous fungi via an improved CRISPR-Cas9 ribonucleoprotein method facilitated by chemical reagents. *Microbial biotechnology*, *14*(6), 2343-2355. https://doi.org/10.1111/1751-7915.13652.

Appendices

Appendix I. Identified endophytic fungal isolates from the four habitats in Singapore:

Habitat 1 (Upper Seletar, Upper Pierce and MacRitchie reservoirs), Habitat 2 (Cluny Road), Habitat 3 (Pulau Ubin and St. John's islands), Habitat 4 (Bukit Timah Nature Reserve and Kent Ridge park).

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
1	F2681	Habitat 1	Flowers	*Lasiodiplodia theobromae* (MT644474.1)	100.0	OM791857	*Ascomycota, Dothideomycetes, Botryosphaeriales*
2	F4434	Habitat 1	Leaves	*Aspergillus assiutensis* (MT640286.1)	99.7	OM791858	*Ascomycota, Eurotiomycetes, Eurotiales*
3	F4440	Habitat 1	Leaves	*Neopestalotiopsis saprophytica* (MT576586.1)	99.7	OM791859	*Ascomycota, Sordariomycetes, Xylariales*
4	F2682	Habitat 1	Flowers	*Aspergillus flavus* (MT497451.1)	99.7	OM791860	*Ascomycota, Eurotiomycetes, Eurotiales*
5	F4448	Habitat 1	Leaves	*Aspergillus assiutensis* (MT640286.1)	99.7	OM791861	*Ascomycota, Eurotiomycetes, Eurotiales*
6	F4451	Habitat 1	Leaves	*Phomopsis* sp. (DQ780437.1)	99.7	OM791862	*Ascomycota, Sordariomycetes, Diaporthales*
7	F4452	Habitat 1	Leaves	*Trichoderma* sp. (MT557551.1)	100.0	OM791863	*Ascomycota, Sordariomycetes, Hypocreales*
8	F4437	Habitat 1	Leaves	*Coniella heterospora* (LT800501.1)	96.8	OM791864	*Ascomycota, Sordariomycetes, Diaporthales*
9	F10318	Habitat 1	Leaves	*Bartalinia* sp. (MH482848.1)	100.0	OM791865	*Ascomycota, Sordariomycetes, Xylariales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
10	F10339	Habitat 1	Leaves	*Chaetomium* sp. (MF495440.1)	99.6	OM791866	*Ascomycota, Sordariomycetes, Sordariales*
11	F12134	Habitat 1	Plant samples	*Aspergillus assiutensis* (MT640286.1)	99.7	OM791867	*Ascomycota, Eurotiomycetes, Eurotiales*
12	F10670	Habitat 1	Leaves	*Chrysoporthe* sp. (CP064907.1)	99.7	OM791868	*Ascomycota, Sordariomycetes, Diaporthales*
13	F4447	Habitat 1	Leaves	*Neopestalotiopsis saprophytica* (MT576586.1)	99.7	OM791869	*Ascomycota, Sordariomycetes, Xylariales*
14	F4445	Habitat 1	Leaves	*Phomopsis* sp. (GU066685.1)	100.0	OM791870	*Ascomycota, Sordariomycetes, Diaporthales*
15	F4442	Habitat 1	Leaves	*Pestalotiopsis* sp. (MT075872.1)	99.3	OM791871	*Ascomycota, Sordariomycetes, Xylariales*
16	F10429	Habitat 1	Leaves	*Aspergillus assiutensis* (MT640286.1)	99.7	OM791872	*Ascomycota, Eurotiomycetes, Eurotiales*
17	F4446	Habitat 1	Leaves	*Pestalotiopsis* sp. (KX960814.1)	100.0	OM791873	*Ascomycota, Sordariomycetes, Xylariales*
18	F12124	Habitat 1	Plant samples	*Humicola fuscoatra* (MT557193.1)	99.7	OM791874	*Ascomycota, Sordariomycetes, Sordariales*
19	F4431	Habitat 1	Leaves	*Neopestalotiopsis clavispora* (MG386209.1)	100.0	OM791875	*Ascomycota, Sordariomycetes, Xylariales*
20	F10338	Habitat 1	Leaves	*Aspergillus flavus* (MT626059.1)	99.3	OM791876	*Ascomycota, Eurotiomycetes, Eurotiales*
21	F11599	Habitat 1	Basidiomycetes	*Aspergillus assiutensis* (MT640286.1)	99.0	OM791877	*Ascomycota, Eurotiomycetes, Eurotiales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
22	F3084	Habitat 1	Stems	*Talaromyces* sp. (MN543991.1)	100.0	OM791878	*Ascomycota, Eurotiomycetes, Eurotiales*
23	F10764	Habitat 1	Leaves	*Corynespora cassiicola* (MN393243.1)	100.0	OM791879	*Ascomycota, Dothideomycetes, Pleosporales*
24	F10132	Habitat 2	Flowers	*Neopestalotiopsis clavispora* (MN519192.1)	100.0	OM791914	*Ascomycota, Sordariomycetes, Xylariales*
25	F10133	Habitat 2	Flowers	*Diaporthe phaseolorum* (MT043765.1)	100.0	OM791915	*Ascomycota, Sordariomycetes, Diaporthales*
26	F10134	Habitat 2	Flowers	*Fusarium petroliphilum* (KY436196.1)	99.3	OM791916	*Ascomycota, Sordariomycetes, Hypocreales*
27	F10136	Habitat 2	Flowers	*Fusarium proliferatum* (MT476359.1)	100.0	OM791917	*Ascomycota, Sordariomycetes, Hypocreales*
28	F10137	Habitat 2	Flowers	*Rhizomucor variabilis* (HM639971.1)	99.4	OM791918	*Mucoromycota, Zygomycetes, Mucorales*
29	F10144	Habitat 2	Flowers	*Colletotrichum asianum* (MT350261.1)	100.0	OM791919	*Ascomycota, Sordariomycetes, Glomerellales*
30	F10145	Habitat 2	Leaves	*Phaeoacremonium* sp. (MK120896.1)	99.3	OM791920	*Ascomycota, Sordariomycetes, Xylariales*
31	F10146	Habitat 2	Flowers	*Colletotrichum* sp. (KM510463.1)	100.0	OM791921	*Ascomycota, Sordariomycetes, Glomerellales*
32	F10148	Habitat 2	Flowers	*Gliocephalotrichum bulbilium* (MN326476.1)	100.0	OM791922	*Ascomycota, Sordariomycetes, Hypocreales*
33	F10149	Habitat 2	Leaves	*Cytospora rhizophorae* (JN083839.1)	99.3	OM791923	*Ascomycota, Sordariomycetes, Diaporthales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
34	F10153	Habitat 2	Leaves	*Colletotrichum gloeosporioides* (MT043801.1)	100.0	OM791924	*Ascomycota, Sordariomycetes, Glomerellales*
35	F10154	Habitat 2	Leaves	*Colletotrichum tropicicola* (MN826680.1)	99.7	OM791925	*Ascomycota, Sordariomycetes, Glomerellales*
36	F10156	Habitat 2	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM791926	*Ascomycota, Sordariomycetes, Glomerellales*
37	F10163	Habitat 2	Leaves	*Phialemoniopsis curvata* (MN511348.1)	100.0	OM791927	*Ascomycota, Sordariomycetes, Xylariales*
38	F5912	Habitat 2	Leaves	*Muyocopron laterale* (NR_164055.1)	99.7	OM791928	*Ascomycota, Dothideomycetes, Muyocopronales*
39	F5924	Habitat 2	Leaves	*Daldinia eschscholtzii* (MT065673.1)	100.0	OM791929	*Ascomycota, Sordariomycetes, Xylariales*
40	F5990	Habitat 2	Leaves	*Colletotrichum fructicola* (MT424894.1)	99.3	OM791930	*Ascomycota, Sordariomycetes, Glomerellales*
41	F6133	Habitat 2	Stems	*Phomopsis* sp. (MK587755.1)	100.0	OM791931	*Ascomycota, Sordariomycetes, Diaporthales*
42	F5920	Habitat 2	Leaves	*Hypoxylon fendleri* (KY173350.1)	99.3	OM791933	*Ascomycota, Sordariomycetes, Xylariales*
43	F4873	Habitat 2	Leaves	*Arthrinium hydei* (NR_121557.1)	100.0	OM791934	*Ascomycota, Sordariomycetes, Xylariales*
44	F5838	Habitat 2	Leaves	*Phomopsis* sp. (MK587755.1)	100.0	OM791935	*Ascomycota, Sordariomycetes, Diaporthales*
45	F4872	Habitat 2	Leaves	*Nigrospora sphaerica* (KX778679.1)	99.7	OM791936	*Ascomycota, Sordariomycetes, Xylariales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
46	F6006	Habitat 2	Leaves	*Colletotrichum karsti* (MN273072.1)	99.3	OM791937	*Ascomycota, Sordariomycetes, Glomerellales*
47	F5914	Habitat 2	Leaves	*Diaporthe phaseolorum* (MH930433.1)	94.1	OM791938	*Ascomycota, Sordariomycetes, Diaporthales*
48	F6062	Habitat 2	Stems	*Phomopsis* sp. (AB505410.1)	99.3	OM791939	*Ascomycota, Sordariomycetes, Diaporthales*
49	F6068	Habitat 2	Leaves	*Diaporthe* sp. (MT355680.1)	100.0	OM791940	*Ascomycota, Sordariomycetes, Diaporthales*
50	F6069	Habitat 2	Leaves	*Phomopsis* sp. (MK587755.1)	100.0	OM791941	*Ascomycota, Sordariomycetes, Diaporthales*
51	F6125	Habitat 2	Leaves	*Diaporthe searlei* (NR_168241.1)	100.0	OM791942	*Ascomycota, Sordariomycetes, Diaporthales*
52	F10032	Habitat 2	Leaves	*Graphium* sp. (FJ946489.1)	100.0	OM791943	*Ascomycota, Sordariomycetes, Hypocreales*
53	F9801	Habitat 2	Stems	*Talaromyces wortmannii* (KF984828.1)	99.4	OM791944	*Ascomycota, Eurotiomycetes, Eurotiales*
54	F9798	Habitat 2	Fruits	*Melanoctona tectonae* (MK952330.1)	91.7	OM791945	*Ascomycota, Eurotiomycetes, Chaetothyriales*
55	F9803	Habitat 2	Stems	*Colletotrichum dracaenophilum* (DQ286208.1)	99.7	OM791946	*Ascomycota, Sordariomycetes, Glomerellales*
56	F9805	Habitat 2	Fruits	*Colletotrichum simmondsii* (MK541034.1)	93.8	OM791947	*Ascomycota, Sordariomycetes, Glomerellales*
57	F9916	Habitat 2	Leaves	*Cladosporium dominicanum* (KY827344.1)	99.7	OM791948	*Ascomycota, Sordariomycetes, Glomerellales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
58	F10164	Habitat 2	Leaves	*Muyocopron laterale* (NR_164055.1)	97.6	OM791949	*Ascomycota, Dothideomycetes, Muyocopronales*
59	F9779	Habitat 2	Stems	*Phialemonium curvatum* (GU219470.1)	99.7	OM791950	*Ascomycota, Sordariomycetes, Xylariales*
60	F9792	Habitat 2	Fruits	*Cylindromonium eugeniicola* (NR_166338.1)	93.1	OM791951	*Ascomycota, Sordariomycetes, Hypocreales*
61	F6122	Habitat 2	Leaves	*Daldinia eschscholtzii* (MT065673.1)	100.0	OM791953	*Ascomycota, Sordariomycetes, Xylariales*
62	F5919	Habitat 2	Leaves	*Xylaria* sp. (MN701035.1)	100.0	OM791954	*Ascomycota, Sordariomycetes, Xylariales*
63	F6361	Habitat 2	Leaves	*Metarhizium anisopliae* (MK955481.1)	100.0	OM791956	*Ascomycota, Sordariomycetes, Hypocreales*
64	F10130	Habitat 2	Flowers	*Sclerotinia trifoliorum* (KT970795.1)	97.8	OM791958	*Ascomycota, Leotiomycetes, Helotiales*
65	F9917	Habitat 2	Leaves	*Lecanicillium testudineum* (MT512660.1)	100.0	OM791959	*Ascomycota, Sordariomycetes, Hypocreales*
66	F10161	Habitat 2	Leaves	*Myriangium* sp. (EF464585.1)	90.2	OM791960	*Ascomycota, Dothideomycetes, Myriangiales*
67	F5906	Habitat 2	Leaves	*Colletotrichum asianum* (MT350261.1)	96.5	OM791961	*Ascomycota, Sordariomycetes, Glomerellales*
68	F5899	Habitat 2	Leaves	*Phomopsis* sp. (MN856266.1)	98.3	OM791962	*Ascomycota, Sordariomycetes, Diaporthales*
69	F5857	Habitat 2	Leaves	*Hypoxylon monticulosum* (MN427954.1)	99.7	OM791963	*Ascomycota, Sordariomycetes, Xylariales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
70	F5861	Habitat 2	Leaves	*Daldinia eschscholtzii* (MT065673.1)	100.0	OM791964	*Ascomycota, Sordariomycetes, Xylariales*
71	F4655	Habitat 2	Leaves	*Colletotrichum gloeosporioides* (MT043801.1)	100.0	OM791965	*Ascomycota, Sordariomycetes, Glomerellales*
72	F5845	Habitat 2	Leaves	*Pestalotiopsis microspora* (MT075872.1)	100.0	OM791966	*Ascomycota, Sordariomycetes, Xylariales*
73	F5852	Habitat 2	Leaves	*Colletotrichum siamense* (KY646464.1)	100.0	OM791967	*Ascomycota, Sordariomycetes, Glomerellales*
74	F5915	Habitat 2	Leaves	*Colletotrichum fructicola* (MT424894.1)	100.0	OM791968	*Ascomycota, Sordariomycetes, Glomerellales*
75	F5935	Habitat 2	Leaves	*Colletotrichum gloeosporioides* (KC010546.1)	99.3	OM791969	*Ascomycota, Sordariomycetes, Glomerellales*
76	F6142	Habitat 2	Leaves	*Colletotrichum gloeosporioides* (MT416209.1)	99.3	OM791970	*Ascomycota, Sordariomycetes, Glomerellales*
77	F6358	Habitat 2	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM791971	*Ascomycota, Sordariomycetes, Glomerellales*
78	F4657	Habitat 2	Leaves	*Aspergillus nomius* (MK192017.1)	98.7	OM791974	*Ascomycota, Eurotiomycetes, Eurotiales*
79	F4876	Habitat 2	Leaves	*Neopestalotiopsis saprophytica* (MT576586.1)	100.0	OM791975	*Ascomycota, Sordariomycetes, Xylariales*
80	F6139	Habitat 2	Leaves	*Paratubakia subglobosa* (NR_161043.2)	91.7	OM791976	*Ascomycota, Sordariomycetes, Diaporthales*
81	F9794	Habitat 2	Fruits	*Soloacrosporiella acaciae* (NR_137986.1)	95.0	OM791977	*Ascomycota, Dothideomycetes, incertae sedis*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
82	F5910	Habitat 2	Leaves	*Diaporth*e sp. (MT495430.1)	100.0	OM791978	*Ascomycota, Sordariomycetes, Diaporthales*
83	F5850	Habitat 2	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM791979	*Ascomycota, Sordariomycetes, Glomerellales*
84	F4659	Habitat 2	Leaves	*Lecanicillium testudineum* (MT512660.1)	100.0	OM791980	*Ascomycota, Sordariomycetes, Hypocreales*
85	F5864	Habitat 2	Leaves	*Lecanicillium testudineum* (MT512660.1)	100.0	OM791981	*Ascomycota, Sordariomycetes, Hypocreales*
86	F5926	Habitat 2	Stems	*Cophinforma atrovirens* (MF436146.1)	99.0	OM791982	*Ascomycota, Dothideomycetes, Botryosphaeriales*
87	F5930	Habitat 2	Stems	*Hypoxylon* sp. (KU683912.1)	97.9	OM791983	*Ascomycota, Sordariomycetes, Xylariales*
88	F5931	Habitat 2	Stems	*Fusarium verticillioides* (MT598827.1)	100.0	OM791984	*Ascomycota, Sordariomycetes, Hypocreales*
89	F5998	Habitat 2	Leaves	*Diaporthe phaseolorum* (MT043770.1)	100.0	OM791985	*Ascomycota, Sordariomycetes, Diaporthales*
90	F6003	Habitat 2	Stems	*Diaporthe phaseolorum* (MT043765.1)	100.0	OM791986	*Ascomycota, Sordariomycetes, Diaporthales*
91	F6053	Habitat 2	Leaves	*Phyllosticta capitalensis* (MK243489.1)	94.2	OM791987	*Ascomycota, Dothideomycetes, Botryosphaeriales*
92	F6055	Habitat 2	Stems	*Phialemoniopsis ocularis* (MH045585.1)	100.0	OM791988	*Ascomycota, Sordariomycetes, Xylariales*
93	F6059	Habitat 2	Stems	*Phomopsis* sp. (KX655604.1)	100.0	OM791989	*Ascomycota, Sordariomycetes, Diaporthales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
94	F6066	Habitat 2	Leaves	*Phomopsis* sp. (DQ780437.1)	99.7	OM791990	*Ascomycota, Sordariomycetes, Diaporthales*
95	F6071	Habitat 2	Stems	*Hypoxylon investiens* (KY828945.1)	99.3	OM791991	*Ascomycota, Sordariomycetes, Xylariales*
96	F6076	Habitat 2	Leaves	*Phialemoniopsis ocularis* (MH045585.1)	100.0	OM791992	*Ascomycota, Sordariomycetes, Xylariales*
97	F6120	Habitat 2	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM791993	*Ascomycota, Sordariomycetes, Glomerellales*
98	F6121	Habitat 2	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM791994	*Ascomycota, Sordariomycetes, Glomerellales*
99	F6123	Habitat 2	Leaves	*Phomopsis phyllanthicola* (MH930424.1)	99.7	OM791995	*Ascomycota, Sordariomycetes, Diaporthales*
100	F6124	Habitat 2	Leaves	*Phomopsis phyllanthicola* (MH930424.1)	99.0	OM791996	*Ascomycota, Sordariomycetes, Diaporthales*
101	F6127	Habitat 2	Leaves	*Lecythophora* sp. (MG250451.1)	90.7	OM791997	*Ascomycota, Sordariomycetes, Coniochaetales*
102	F6129	Habitat 2	Stems	*Phomopsis* sp. (AB505410.1)	99.7	OM791998	*Ascomycota, Sordariomycetes, Diaporthales*
103	F6131	Habitat 2	Leaves	*Phomopsis* sp. (AB505410.1)	99.3	OM791999	*Ascomycota, Sordariomycetes, Diaporthales*
104	F6132	Habitat 2	Leaves	*Diaporthe* sp. (MT355680.1)	100.0	OM792000	*Ascomycota, Sordariomycetes, Diaporthales*
105	F6136	Habitat 2	Leaves	*Phyllosticta* sp. (MH393348.1)	99.3	OM792001	*Ascomycota, Dothideomycetes, Botryosphaeriales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
106	F6140	Habitat 2	Leaves	*Paratubakia subglobosa* (NR_161043.1)	93.9	OM792002	*Ascomycota, Sordariomycetes, Diaporthales*
107	F6141	Habitat 2	Leaves	*Paratubakia subglobosa* (NR_161043.1)	93.9	OM792003	*Ascomycota, Sordariomycetes, Diaporthales*
108	F6143	Habitat 2	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM792004	*Ascomycota, Sordariomycetes, Glomerellales*
109	F6144	Habitat 2	Stems	*Phomopsis* sp. (MK587755.1)	100.0	OM792005	*Ascomycota, Sordariomycetes, Diaporthales*
110	F6360	Habitat 2	Stems	*Diaporthe searlei* (NR_168241.1)	100.0	OM792006	*Ascomycota, Sordariomycetes, Diaporthales*
111	F6341	Habitat 2	Leaves	*Phomopsis* sp. (AB505410.1)	99.3	OM792007	*Ascomycota, Sordariomycetes, Diaporthales*
112	F10129	Habitat 2	Flowers	*Phomopsis asparagi* (MT459267.1)	97.6	OM791913	*Ascomycota, Sordariomycetes, Diaporthales*
113	F6702	Habitat 3	Stems	*Colletotrichum fructicola* (MT424894.1)	99.0	OM791880	*Ascomycota, Sordariomycetes, Glomerellales*
114	F6703	Habitat 3	Stems	*Colletotrichum asianum* (MT350261.1)	100.0	OM791881	*Ascomycota, Sordariomycetes, Glomerellales*
115	F6704	Habitat 3	Fruits	*Colletotrichum fructicola* (MT424894.1)	99.0	OM791882	*Ascomycota, Sordariomycetes, Glomerellales*
116	F6709	Habitat 3	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM791883	*Ascomycota, Sordariomycetes, Glomerellales*
117	F6712	Habitat 3	Stems	*Colletotrichum fructicola* (MT424894.1)	99.3	OM791884	*Ascomycota, Sordariomycetes, Glomerellales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
118	F6713	Habitat 3	Stems	*Phomopsis* sp. (MN856266.1)	98.3	OM791885	*Ascomycota, Sordariomycetes, Diaporthales*
119	F6714	Habitat 3	Leaves	*Phomopsis* sp. (DQ780437.1)	99.3	OM791886	*Ascomycota, Sordariomycetes, Diaporthales*
120	F6715	Habitat 3	Leaves	*Pestalotiopsis cocculi* (MT000051.1)	99.7	OM791887	*Ascomycota, Sordariomycetes, Xylariales*
121	F6716	Habitat 3	Leaves	*Phomopsi*s sp. (MN099444.1)	100.0	OM791888	*Ascomycota, Sordariomycetes, Diaporthales*
122	F6720	Habitat 3	Leaves	*Colletotrichum asianum* (MT350261.1)	99.3	OM791889	*Ascomycota, Sordariomycetes, Glomerellales*
123	F6728	Habitat 3	Stems	*Phomopsis* sp. (MH472614.1)	99.6	OM791890	*Ascomycota, Sordariomycetes, Diaporthales*
124	F6730	Habitat 3	Stems	*Diaporthe* sp. (MT495430.1)	99.7	OM791891	*Ascomycota, Sordariomycetes, Diaporthales*
125	F6731	Habitat 3	Stems	*Phomopsis* sp. (KX655604.1)	99.6	OM791892	*Ascomycota, Sordariomycetes, Diaporthales*
126	F6733	Habitat 3	Leaves	*Mycosphaerella* sp. (KX258811.1)	99.3	OM791893	*Ascomycota, Dothideomycetes, Mycosphaerellales*
127	F6734	Habitat 3	Leaves	*Colletotrichum* sp. (MT570096.1)	99.0	OM791894	*Ascomycota, Sordariomycetes, Glomerellales*
128	F6738	Habitat 3	Stems	*Colletotrichum fructicola* (KC845282.1)	98.7	OM791895	*Ascomycota, Sordariomycetes, Glomerellales*
129	F6743	Habitat 3	Stems	*Diaporthe searlei* (NR_168241.1)	99.7	OM791896	*Ascomycota, Sordariomycetes, Diaporthales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
130	F6910	Habitat 3	Leaves	*Mycosphaerella* sp. (KX258811.1)	99.3	OM791897	*Ascomycota, Dothideomycetes, Mycosphaerellales*
131	F6914	Habitat 3	Leaves	*Mycosphaerella* sp. (KX258811.1)	99.3	OM791898	*Ascomycota, Dothideomycetes, Mycosphaerellales*
132	F6923	Habitat 3	Stems	*Colletotrichum* sp. (MT570096.1)	99.0	OM791899	*Ascomycota, Sordariomycetes, Glomerellales*
133	F6929	Habitat 3	Stems	*Fusarium solani* (MN637841.1)	100.0	OM791900	*Ascomycota, Sordariomycetes, Hypocreales*
134	F6930	Habitat 3	Leaves	*Mycosphaerella* sp. (KX258811.1)	99.3	OM791901	*Ascomycota, Dothideomycetes, Mycosphaerellales*
135	F6931	Habitat 3	Leaves	*Mycosphaerella* sp. (KX258811.1)	100.0	OM791902	*Ascomycota, Dothideomycetes, Mycosphaerellales*
136	F6928	Habitat 3	Leaves	*Xylaria* sp. (LC424444.1)	97.2	OM791903	*Ascomycota, Sordariomycetes, Xylariales*
137	F6932	Habitat 3	Stems	*Lophiotrema* sp. (MK587671.1)	99.0	OM791904	*Ascomycota, Dothideomycetes, Pleosporales*
138	F6732	Habitat 3	Leaves	*Leptosphaerulina* sp. (MF405203.1)	96.6	OM791905	*Ascomycota, Dothideomycetes, Pleosporales*
139	F6913	Habitat 3	Stems	*Leptosphaerulina* sp. (KY827352.1)	99.7	OM791906	*Ascomycota, Dothideomycetes, Pleosporales*
140	F6915	Habitat 3	Leaves	*Mycosphaerella* sp. (KX258811.1)	99.6	OM791907	*Ascomycota, Dothideomycetes, Mycosphaerellales*
141	F6916	Habitat 3	Leaves	*Acremonium hennebertii* (MN637805.1)	98.2	OM791908	*Ascomycota, Sordariomycetes, Hypocreales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
142	F6917	Habitat 3	Leaves	*Leptosphaerulina* sp. (JN850998.1)	96.3	OM791909	*Ascomycota, Dothideomycetes, Pleosporales*
143	F6717	Habitat 3	Leaves	*Phyllosticta capitalensis* (MT568601.1)	99.3	OM791910	*Ascomycota, Dothideomycetes, Botryosphaeriales*
144	F6705	Habitat 3	Leaves	*Phyllosticta* sp. (MG545069.1)	99.3	OM791911	*Ascomycota, Dothideomycetes, Botryosphaeriales*
145	F6724	Habitat 3	Leaves	*Phyllosticta* sp. (MK120855.1)	98.7	OM791912	*Ascomycota, Dothideomycetes, Botryosphaeriales*
146	F4327	Habitat 3	Leaves	*Lasiodiplodia theobromae* (MT644474.1)	99.3	OM791932	*Ascomycota, Dothideomycetes, Botryosphaeriales*
147	F4331	Habitat 3	Leaves	*Colletotrichum gloeosporioides* (MT043801.1)	100.0	OM791952	*Ascomycota, Sordariomycetes, Glomerellales*
148	F4428	Habitat 3	Stems	*Pestalotiopsis* sp. (MT102590.1)	100.0	OM791955	*Ascomycota, Sordariomycetes, Xylariales*
149	F4430	Habitat 3	Leaves	*Colletotrichum scovillei* (LC488852.1)	100.0	OM791957	*Ascomycota, Sordariomycetes, Glomerellales*
150	F4320	Habitat 3	Leaves	*Colletotrichum* sp. (MT570096.1)	99.3	OM791972	*Ascomycota, Sordariomycetes, Glomerellales*
151	F4324	Habitat 3	Leaves	*Nigrospora* sp. (MT645657.1)	100.0	OM791973	*Ascomycota, Sordariomycetes, Xylariales*
152	F4925	Habitat 4	Leaves	*Trichoderma longibrachiatum* (MT316380.1)	100.0	OM792008	*Ascomycota, Sordariomycetes, Hypocreales*
153	F4926	Habitat 4	Frond from fish tail palm	*Arthrinium garethjonesii* (MW481715.1)	98.5	OM792009	*Ascomycota, Sordariomycetes, Xylariales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
154	F4930	Habitat 4	Frond from fish tail palm	*Trichoderma longibrachiatum* (MT316380.1)	99.0	OM792010	*Ascomycota, Sordariomycetes, Hypocreales*
155	F6550	Habitat 4	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM792011	*Ascomycota, Sordariomycetes, Glomerellales*
156	F6552	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	99.3	OM792012	*Ascomycota, Sordariomycetes, Glomerellales*
157	F6554	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	99.3	OM792013	*Ascomycota, Sordariomycetes, Glomerellales*
158	F6559	Habitat 4	Stems	*Phomopsis* sp. (MK587755.1)	100.0	OM792014	*Ascomycota, Sordariomycetes, Diaporthales*
159	F6586	Habitat 4	Leaves	*Colletotrichum gloeosporioides* (MT416209.1)	99.3	OM792015	*Ascomycota, Sordariomycetes, Glomerellales*
160	F6595	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM792016	*Ascomycota, Sordariomycetes, Glomerellales*
161	F6600	Habitat 4	Leaves	*Colletotrichum magnisporum* (MH151055.1)	97.9	OM792017	*Ascomycota, Sordariomycetes, Glomerellales*
162	F6601	Habitat 4	Leaves	*Phyllosticta capitalensis* (MT568601.1)	99.3	OM792018	*Ascomycota, Dothideomycetes, Botryosphaeriales*
163	F6603	Habitat 4	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM792019	*Ascomycota, Sordariomycetes, Glomerellales*
164	F6608	Habitat 4	Stems	*Phomopsis* sp. (MK120615.1)	100.0	OM792020	*Ascomycota, Sordariomycetes, Diaporthales*
165	F6613	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	99.3	OM792021	*Ascomycota, Sordariomycetes, Glomerellales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
166	F6614	Habitat 4	Leaves	*Colletotrichum siamense* (MT219898.1)	100.0	OM792022	*Ascomycota, Sordariomycetes, Glomerellales*
167	F6690	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	99.3	OM792023	*Ascomycota, Sordariomycetes, Glomerellales*
168	F6691	Habitat 4	Leaves	*Hypoxylon* sp. (KU683912.1)	98.3	OM792024	*Ascomycota, Sordariomycetes, Xylariales*
169	F6545	Habitat 4	Flowers	*Cosmospora vilior* (JN541223.1)	100.0	OM792025	*Ascomycota, Sordariomycetes, Hypocreales*
170	F6547	Habitat 4	Flowers	*Diaporthe searlei* (NR_168241.1)	100.0	OM792026	*Ascomycota, Sordariomycetes, Diaporthales*
171	F6553	Habitat 4	Leaves	*Phomopsis* sp. (GU066672.1)	100.0	OM792027	*Ascomycota, Sordariomycetes, Diaporthales*
172	F6555	Habitat 4	Stems	*Diaporthe* sp. (KY790594.1)	97.9	OM792028	*Ascomycota, Sordariomycetes, Diaporthales*
173	F6557	Habitat 4	Stems	*Phomopsis* sp. (MK587755.1)	100.0	OM792029	*Ascomycota, Sordariomycetes, Diaporthales*
174	F6561	Habitat 4	Leaves	*Aspergillus flavus* (MT497451.1)	99.0	OM792030	*Ascomycota, Eurotiomycetes, Eurotiales*
175	F6567	Habitat 4	Leaves	*Endomelanconiopsis* sp. (MF579571.1)	100.0	OM792031	*Ascomycota, Dothideomycetes, Botryosphaeriales*
176	F6569	Habitat 4	Leaves	*Neofusicoccum parvum* (MT093349.1)	99.7	OM792032	*Ascomycota, Dothideomycetes, Botryosphaeriales*
177	F6575	Habitat 4	Flowers	*Phomopsis* sp. (KX655604.1)	100.0	OM792033	*Ascomycota, Sordariomycetes, Diaporthales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
178	F6578	Habitat 4	Stems	*Hypoxylon* sp. (KU683912.1)	98.6	OM792034	*Ascomycota, Sordariomycetes, Xylariales*
179	F6580	Habitat 4	Stems	*Diaporthe ambigua* (KU935706.1)	95.2	OM792035	*Ascomycota, Sordariomycetes, Diaporthales*
180	F6581	Habitat 4	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM792036	*Ascomycota, Sordariomycetes, Glomerellales*
181	F6582	Habitat 4	Leaves	*Diaporthe longicolla* (MK119224.1)	99.7	OM792037	*Ascomycota, Sordariomycetes, Diaporthales*
182	F6584	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	99.3	OM792038	*Ascomycota, Sordariomycetes, Glomerellales*
183	F6585	Habitat 4	Leaves	*Colletotrichum siamense* (MW186174.1)	100.0	OM792039	*Ascomycota, Sordariomycetes, Glomerellales*
184	F6686	Habitat 4	Leaves	*Helminthosporium genistae* (NR_155195.1)	94.8	OM792040	*Ascomycota, Dothideomycetes, Pleosporales*
185	F6587	Habitat 4	Leaves	*Colletotrichum horii* (KR995727.1)	100.0	OM792041	*Ascomycota, Sordariomycetes, Glomerellales*
186	F6591	Habitat 4	Stems	*Colletotrichum asianum* (MT350261.1)	100.0	OM792042	*Ascomycota, Sordariomycetes, Glomerellales*
187	F6592	Habitat 4	Stems	*Phomopsis* sp. (MK311340.1)	99.7	OM792043	*Ascomycota, Sordariomycetes, Diaporthales*
188	F6548	Habitat 4	Flowers	*Cylindrocladiella* sp. (JX243750.1)	100.0	OM792044	*Ascomycota, Sordariomycetes, Hypocreales*
189	F6549	Habitat 4	Flowers	*Cylindrocladiella* sp. (JX243750.1)	100.0	OM792045	*Ascomycota, Sordariomycetes, Hypocreales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
190	F6597	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM792046	*Ascomycota, Sordariomycetes, Glomerellales*
191	F6598	Habitat 4	Leaves	*Colletotrichum gloeosporioides* (KJ617392.1)	99.7	OM792047	*Ascomycota, Sordariomycetes, Glomerellales*
192	F6599	Habitat 4	Leaves	*Colletotrichum siamense* (MW186174.1)	100.0	OM792048	*Ascomycota, Sordariomycetes, Glomerellales*
193	F6602	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM792049	*Ascomycota, Sordariomycetes, Glomerellales*
194	F6606	Habitat 4	Stems	*Colletotrichum magnisporum* (MH151055.1)	99.0	OM792050	*Ascomycota, Sordariomycetes, Glomerellales*
195	F6609	Habitat 4	Stems	*Phomopsis phyllanthicola* (MH930424.1)	99.3	OM792051	*Ascomycota, Sordariomycetes, Diaporthales*
196	F6611	Habitat 4	Leaves	*Colletotrichum fructicola* (MT424894.1)	99.3	OM792052	*Ascomycota, Sordariomycetes, Glomerellales*
197	F6615	Habitat 4	Leaves	*Diaporthe* sp. (MT568516.1)	100.0	OM792053	*Ascomycota, Sordariomycetes, Diaporthales*
198	F6617	Habitat 4	Leaves	*Simplicillium* sp. (AB378536.1)	100.0	OM792054	*Ascomycota, Sordariomycetes, Hypocreales*
199	F6688	Habitat 4	Leaves	*Colletotrichum asianum* (MT350261.1)	100.0	OM792055	*Ascomycota, Sordariomycetes, Glomerellales*
200	F6693	Habitat 4	Leaves	*Cosmospora vilior* (JN541223.1)	100.0	OM792056	*Ascomycota, Sordariomycetes, Hypocreales*
201	F10400	Habitat 4	Leaves	*Sarocladium* sp. (MN128515.1)	98.3	OM792057	*Ascomycota, Sordariomycetes, Hypocreales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
202	F10390	Habitat 4	Leaves	*Coprinopsis cinerea* (MH443753.1)	100.0	OM792058	*Basidiomycota, Agaricomycetes, Agaricales*
203	F6610	Habitat 4	Stems	*Phomopsis asparagi* (MT459267.1)	97.6	OM792059	*Ascomycota, Sordariomycetes, Diaporthales*
204	F6616	Habitat 4	Leaves	*Cosmospora vilior* (JN541223.1)	100.0	OM792060	*Ascomycota, Sordariomycetes, Hypocreales*
205	F11602	Habitat 4	Basidiome	*Neonectria* sp. (KY413710.1)	100.0	OM792061	*Ascomycota, Sordariomycetes, Hypocreales*
206	F6620	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM792062	*Ascomycota, Sordariomycetes, Glomerellales*
207	F11652	Habitat 4	Fern	*Pseudocercospora humuli* (GU214676.1)	98.9	OM792063	*Ascomycota, Dothideomycetes, Mycosphaerellales*
208	F11610	Habitat 4	Fern/Frond	*Colletotrichum asianum* (MT350261.1)	100.0	OM792064	*Ascomycota, Sordariomycetes, Glomerellales*
209	F11616	Habitat 4	Leaves	*Diaporthe* sp. (MK229151.1)	98.0	OM792065	*Ascomycota, Sordariomycetes, Diaporthales*
210	F11608	Habitat 4	Fern	*Colletotrichum gloeosporioides* (MT557555.1)	99.3	OM792066	*Ascomycota, Sordariomycetes, Glomerellales*
211	F6612	Habitat 4	Leaves	*Colletotrichum fructicola* (MT424894.1)	100.0	OM792067	*Ascomycota, Sordariomycetes, Glomerellales*
212	F4923	Habitat 4	Leaves	*Daldinia eschscholtzii* (MT065673.1)	100.0	OM792068	*Ascomycota, Sordariomycetes, Xylariales*
213	F6692	Habitat 4	Leaves	*Colletotrichum* sp. (MT577046.1)	100.0	OM792069	*Ascomycota, Sordariomycetes, Glomerellales*

#	Fungal isolate*	Habitat	Substrate/ plant tissue	Closest Relative in NCBI (Accession No.)	Percent Identity	Accession Number	Phylum, Class, Order
214	F6697	Habitat 4	Leaves	*Daldinia eschscholtzii* (KY440188.1)	100.0	OM792070	*Ascomycota, Sordariomycetes, Xylariales*
215	F11601	Habitat 4	Basidiome	*Rhodofomitopsis feei* (MG437308.1)	100.0	OM792071	*Basidiomycota, Agaricomycetes, Polyporales*
216	F11607	Habitat 4	Fern/frond	*Hypoxylon polyporoideum* (JQ009311.1)	99.7	OM792072	*Ascomycota, Sordariomycetes, Xylariales*
217	F11609	Habitat 4	Fern	*Malaysiasca phaii* (NR_154193.1)	95.0	OM792073	*Ascomycota, Sordariomycetes, Glomerellales*
218	F11615	Habitat 4	Leaves	*Hansfordia* sp. (MW133921.1)	98.9	OM792074	*Ascomycota, Sordariomycetes, Xylariales*
219	F11618	Habitat 4	Leaves	*Fumiglobus pieridicola* (NR_153985.1)	93.3	OM792075	*Ascomycota, Dothideomycetes, Capnodiales*
220	F11651	Habitat 4	Fern	*Muyocopron chromolaenicola* (NR_168858.1)	95.3	OM792076	*Ascomycota, Dothideomycetes, Muyocopronales*
221	F11653	Habitat 4	Fern	*Paramicrothyrium* sp. (KU747847.1)	100.0	OM792077	*Ascomycota, Dothideomycetes, incertae sedis*
222	F11663	Habitat 4	Leaves	*Scolecoxyphium blechni* (NR_168838.1)	96.8	OM792078	*Ascomycota, Dothideomycetes, Capnodiales*

Appendix II. Anaysis of Variance (ANOVA): Chemical elicitation

S. aureus	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3162	2	1581	3.552	.076
Within Groups	21809	12	1817		
Total	24971	14			

C. albicans	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1439	2	719.3	0.788	.431
Within Groups	18023	12	1502		
Total	19462	14			

A. fumigatus	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1795	2	897.6	0.8	.423
Within Groups	36906	12	3075		
Total	38701	14			

A549	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	313.3	2	156.6	0.151	.811
Within Groups	50603	12	4217		
Total	50916.3	14			

MIA PaCa-2	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1999	2	999.6	0.595	.509
Within Groups	39181	12	3265		
Total	41180	14			

PANC-1	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	31208	2	15604	0.922	.362
Within Groups	306835	12	22570		
Total	338043	14			

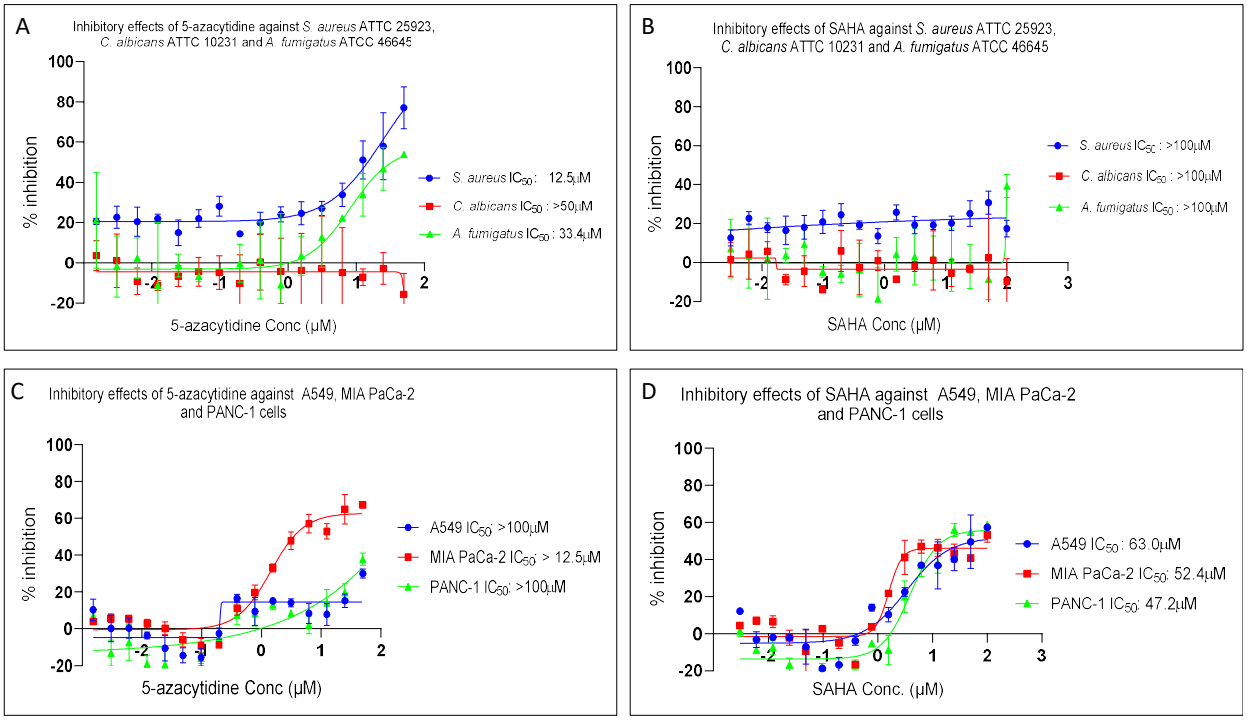
Appendix III. Anaysis of Variance (ANOVA): Different growth media

A549	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13164	4	3291	4.422	.006
Within Groups	164617	46	3579		
Total	177781	50			

MIA PaCa-2	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14619	4	3655	3.258	.02
Within Groups	189036	46	4109		
Total	203655	50			

PANC-1	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2179	4	544.7	0.733	.53
Within Groups	112932	46	2455		
Total	115111	50			

Appendix IV. Inhibitory activity of the two chemical elicitors against the study microbial pathogens (A and B) and cancer cell lines (C and D).



Appendix V. NMR spectral data of palmarumycin CP_{30} (1) and palmarumycin C_8 (2).

	1[a]		2[b]	
Position	^{13}C, type	^{1}H, mult. (J = Hz)	^{13}C, type	^{1}H, mult. (J = Hz)
1	105.5, C		95.6, C	
2	64.5, CH	4.20, d (5.8)	135.4, CH	6.77, s
3	124.9, CH	5.89, dd (5.8, 9.9)	131.4, C	
4	124.8, CH	7.02, d (9.9)	185.4, C	
4a	121.8, C		63.0, C	
5	151.4, C		61.5, CH	5.12, t (3.0)
6	120.1, CH	6.84, d (8.9)	23.3, CH_2	2.09, m
7	119.7, CH	6.71, d (8.9)	32.8, CH_2	2.51, m, 2.79, m
8	148.0, C		194.3, C	
8a	116.2, C		61.3, C	
1’	147.8, C		144.6, C	
2’	111.3, CH	7.16, dd (0.7, 7.5)	110.4, CH	7.10, d (7.5)
3’	128.7, CH	7.51, m	127.7, CH	7.49, t (7.7)
4’	122.6, CH	7.60, dd (0.6, 8.4)	121.6, CH	7.58, d (8.8)
4a’	135.5, C		134.2, C	
5’	121.6, CH	7.53, dd (0.6, 8.1)	121.6, CH	7.56, d (8.7)
6’	128.5, CH	7.44, m	127.4, CH	7.43, t (7.9)
7’	110.2, CH	6.87, dd (0.6, 7.5)	109.5, CH	6.93, d (7.5)
8’	149.0, C		145.2, C	
8a’	114.9, C		112.4, C	

[a]^{1}H (400 MHz) and ^{13}C (100 MHz) in methanol-d_4. [b]^{1}H (400 MHz) and ^{13}C (100 MHz) in chloroform-*d*. Assignments based on COSY, HSQC and HMBC and comparison with literature compounds. Chemical shifts (δ) in ppm. s, singlet; d, doublet; dd, doublet of doublet; t, triplet; and m, multiplet. One proton unless otherwise stated.

Appendix VI. The NMR spectral data of muyocopronol A (3), B (4) and C (5**)**

Position	3 [a]		**4** [a]		**5** [a]	
	^{13}C, type	^{1}H, mult. (*J* in Hz)	^{13}C, type	^{1}H, mult. (*J* in Hz)	^{13}C, type	^{1}H, mult. (*J* in Hz)
1	177.8, C		177.2, C		182.7, C	
2	44.7, CH	2.65, m	47.6, CH	2.55, m	48.4, CH	2.33, m
3	82.2, CH	4.08, d (10.1)	76.9, CH	4.13, dd (8.2, 8.2)	76.8, CH	4.05, dd (7.5, 7.5)
4	136.0, C		131.5, CH	5.44, dd (15.2, 8.2)	132.8, CH	5.49, dd (15.3, 7.5)
5	135.3, CH	5.32, d (8.4)	139.6, CH	5.71, dd (15.2, 8.2)	137.2, CH	5.70, dd (15.3, 7.5)
6	36.61, CH	2.65, m	41.1, CH	2.33, m	41.1, CH	2.33, m
7	84.4, CH	3.70, m	84.0, CH	3.67, m	83.9, CH	3.66, m
8	137.6, C		137.3, C		137.6, C	
9	133.8, CH	5.29, d (9.6)	134.0, CH	5.25, d (9.4)	133.60, CH	5.26, d (8.2)
10	36.65, CH	2.65, m	36.3, CH	2.64, m	36.65, CH	2.65, m
11	84.6, CH	3.70, m	84.8, CH	3.67, m	88.4, CH	3.69, d (8.9)
12	138.8, C		137.6, C		137.7, C	
13	132.6, CH	5.09, d (10.1)	133.9, CH	5.25, d (9.4)	133.64, CH	5.28, d (8.2)
14	44.7, CH	2.65, m	36.4, CH	2.64, m	36.70, CH	2.65, m
15	39.7, CH_2	1.22, m	84.7, CH	3.67, m	88.6, CH	3.69, d (8.9)
16	33.4, CH	1.32, m	138.9, C		138.8, C	
17	31.8, CH_2	1.33, m	132.8, CH	5.07, d (9.8)	132.4, CH	5.09, d (9.1)
18	11.5, CH_3	0.87, m	39.7, CH	2.64, m	39.7, CH	2.65, m
19	14.7, CH_3	0.98, d (7.0)	39.6, CH_2	1.20, m	39.8, CH_2	1.20, m
20	10.7, CH_3	1.64, d (1.1)	33.4, CH	1.32, m	33.4, CH	1.33, m

Position	3 [a]		**4** [a]		**5** [a]	
	^{13}C, type	^{1}H, mult. (*J* in Hz)	^{13}C, type	^{1}H, mult. (*J* in Hz)	^{13}C, type	^{1}H, mult. (*J* in Hz)
21	17.7, CH_3	0.79, d (6.7)	31.8, CH_2	1.21, m	31.8, CH_2	1.29, m
22	11.5, CH_3	1.68, d (0.9)	11.8, CH_3	0.86, m	11.8, CH_3	0.86, m
23	17.6, CH_3	0.79, d (6.7)	14.5, CH_3	1.09, d (7.0)	15.2, CH_3	1.10, d (7.2)
24	11.8, CH_3	1.70, d (1.0)	17.7, CH_3	0.84, m	17.58, CH_3	0.84, m
25	67.5, CH_2	3.37, m 3.45, m	11.4, CH_3	1.64, d (0.9)	11.3, CH_3	1.65, d (1.0)
26	19.3, CH_3	0.87, m	17.57, CH_3	0.78, d (4.8)	17.73, CH_3	0.78, d (5.7)
27			11.2, CH_3	1.69, d (1.0)	11.4, CH_3	1.68, d (1.0)
28			17.56, CH_3	0.76, d (4.8)	17.67, CH_3	0.79, d (5.7)
29			11.1, CH_3	1.67, d (0.9)	11.6, CH_3	1.69, d (1.0)
30			67.6, CH_2	3.33, m 3.47, m	67.5, CH_3	3.37, m 3.44, m
31			19.3, CH_3	0.86, m	19.3, CH_3	0.86, m
1'	68.0, CH_2	4.20, dd (11.4, 6.2) 4.47, dd (11.4, 2.7)	64.5, CH_2	3.54, m 3.64, m		
2'	70.4, CH	3.91, m	71.7, CH	3.93, m		
3'	70.1, CH	3.81, m	73.9, CH	5.20, dd (7.9, 1.4)		
4'	70.0, CH	3.81, m	71.0, CH	3.90, m		
5'	73.0, CH	3.70, m	72.0, CH	3.53, m		
6'	65.2, CH_2	3.65, m 3.82, m	65.0, CH_2	3.64, m 3.81, m		

[a]^{1}H (400 MHz) and ^{13}C (100 MHz) in methanol-d_4. Assignments based on COSY, HSQC and HMBC and comparison with literature compounds. Chemical shifts (δ) in ppm. d, doublet; dd,

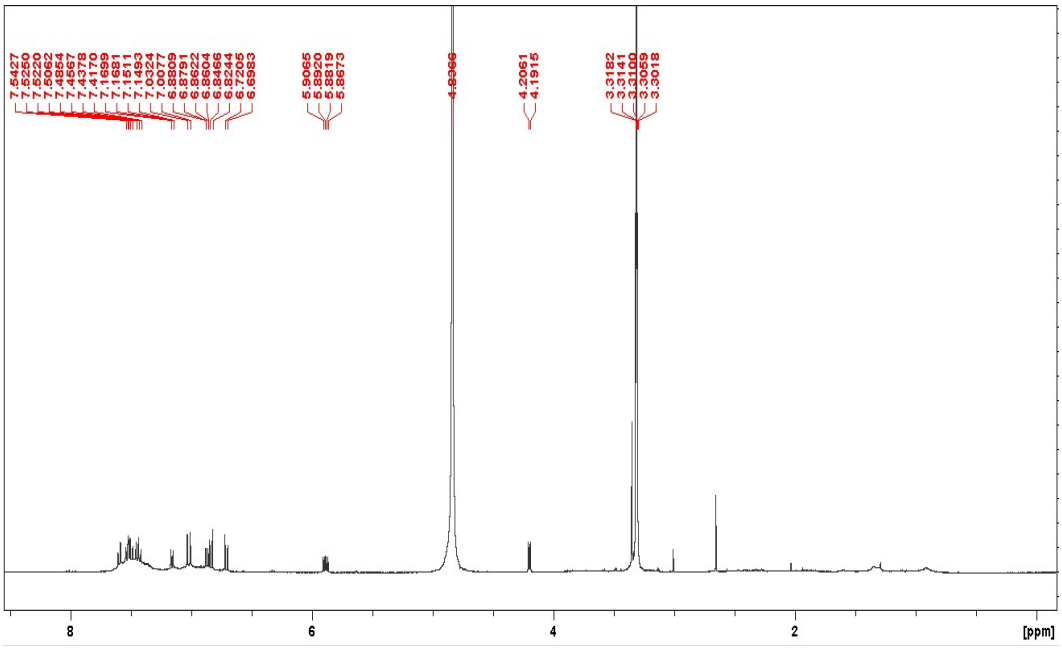
Appendix VII. NMR spectral data of tropicicolide

Position	6[a]	
	^{13}C, mult. (J = Hz), type	^{1}H, mult. (J = Hz)
1	171.9, C	
2	42.1, d (3.5), CH_2	2.52, m, 2.81, m
3	74.1, d (5.9), CH	4.54, m
4	36.3, d, (5.4), CH_2	1.65, m
5	25.4, CH_2	1.40, m, 1.48, m
6	30.7, CH_2	1.30, m, 1.39, m
7	29.5, CH_2	1.36, m
8	29.6, CH_2	1.36, m
9	33.1, CH_2	1.99, m
10	132.0, CH	5.39, m
11	130.7, CH	5.39, m
12	33.3, CH_2	1.99, m
13	33.4, CH_2	1.99, m
14	131.9, CH	5.39, m
15	131.0, CH	5.39, m
16	33.5, CH_2	2.06, m
17	33.5, CH_2	2.06, m
18	131.9, CH	5.35, m
19	131.2, CH	5.37, m
20	33.6, CH_2	2.06, m
21	33.8, CH_2	2.06, m

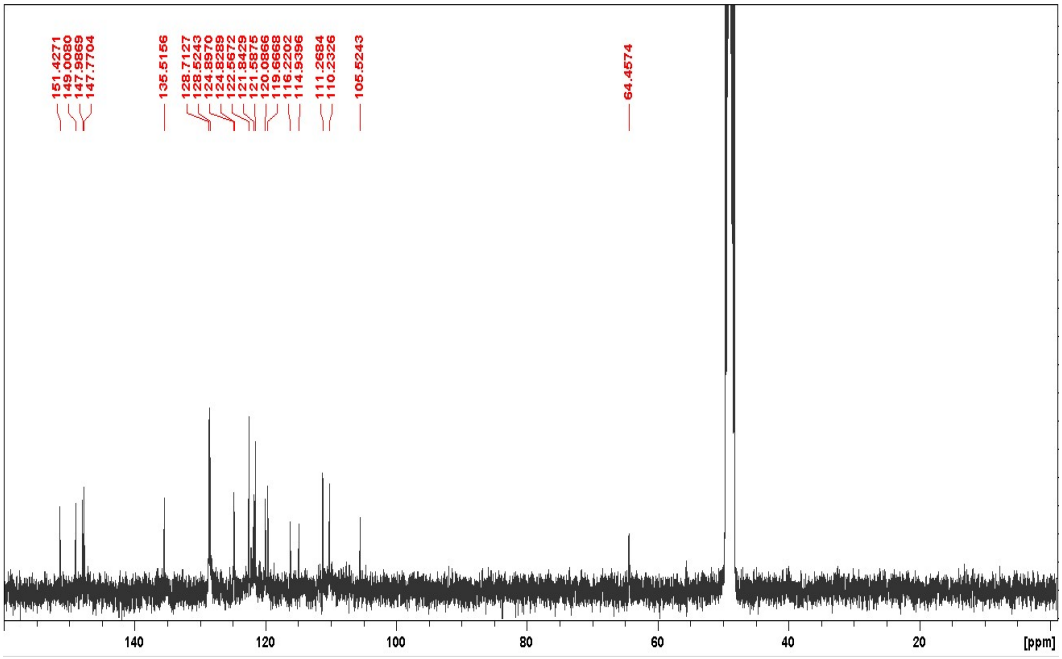
Position	6[a]	
	^{13}C, mult. (J = Hz), type	^{1}H, mult. (J = Hz)
22	134.5, CH	5.51, m
23	126.6, CH	5.39, m
24	39.9, CH_2	2.25, m
25	72.4, CH	4.89, m
26	19.9, CH_3	1.21, d (6.2)
27	60.3, d (5.1), CH_2	4.27, m
28	67.6, CH_2	3.63, m
N-$(CH_3)_3$	54.7, t (3.7), CH_3	3.22, s

[a]^{1}H (400 MHz) and ^{13}C (100 MHz) in methanol-d_4. Assignments based on COSY, HSQC and HMBC and comparison with literature compound, eushearilide. Chemical shifts (δ) in ppm. s, singlet; d, doublet; t, triplet; and m, multiplet. One proton unless otherwise stated.

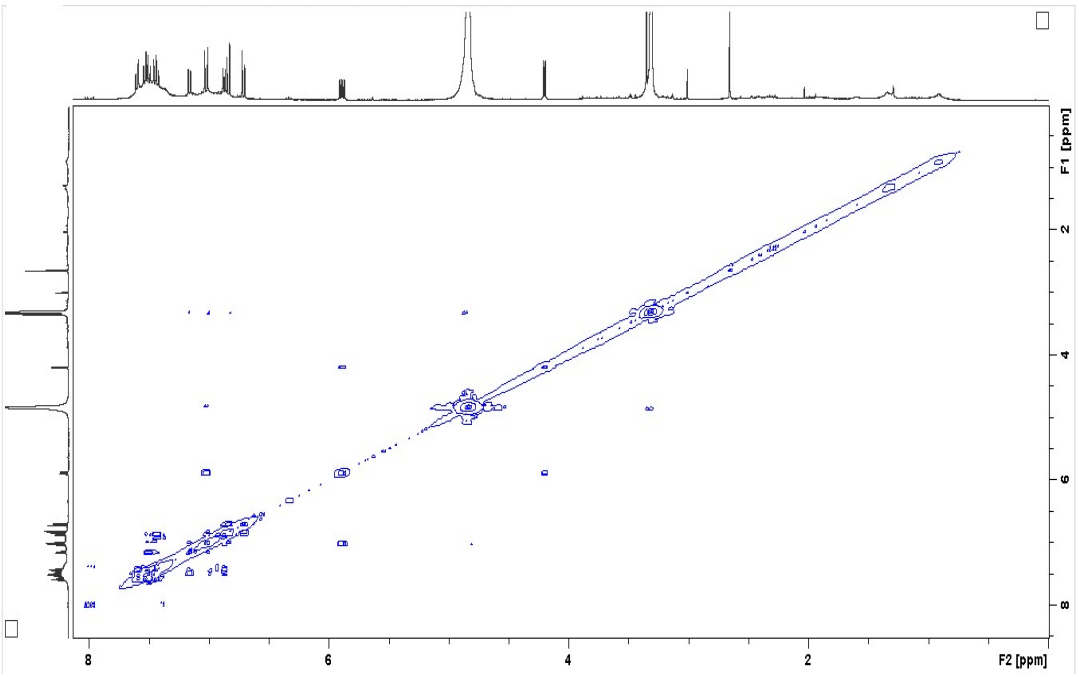
Appendix VIII. ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC spectra for the 6 compounds.



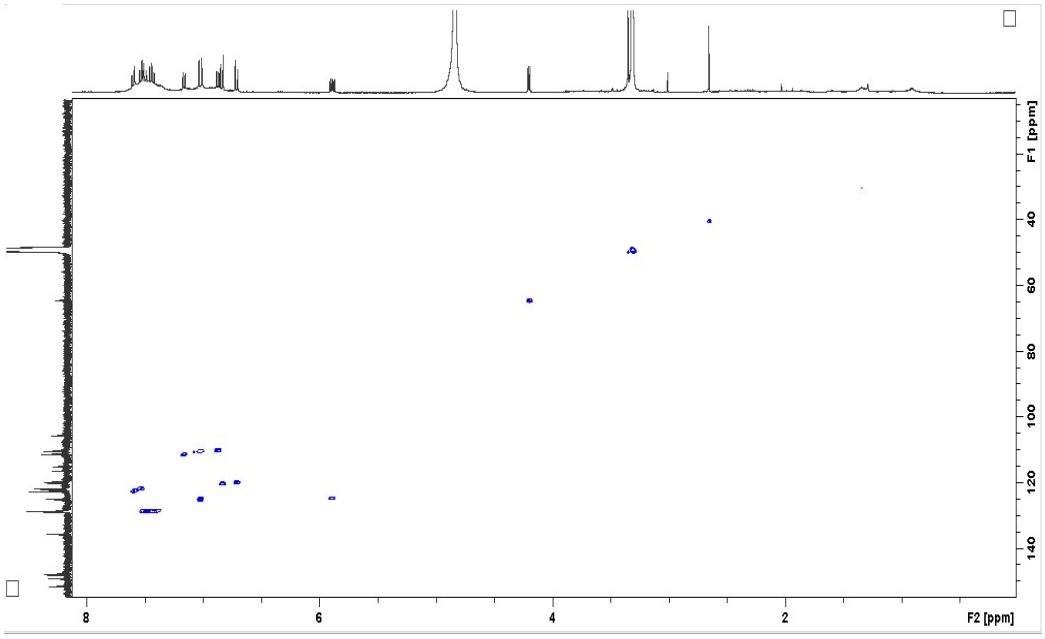
^{1}H NMR spectrum (CD_3OD, 400 MHz) of palmarumycin CP_{30} (**1**).



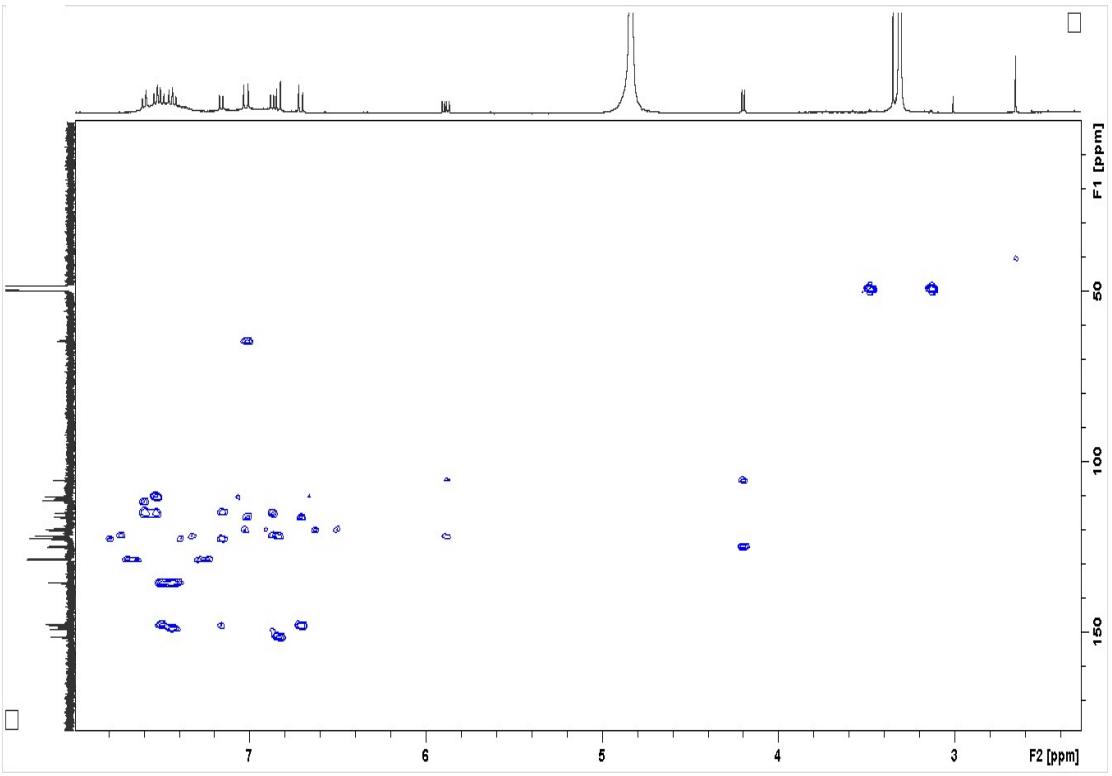
^{13}C NMR spectrum (CD_3OD, 100 MHz) of palmarumycin CP_{30} (**1**).



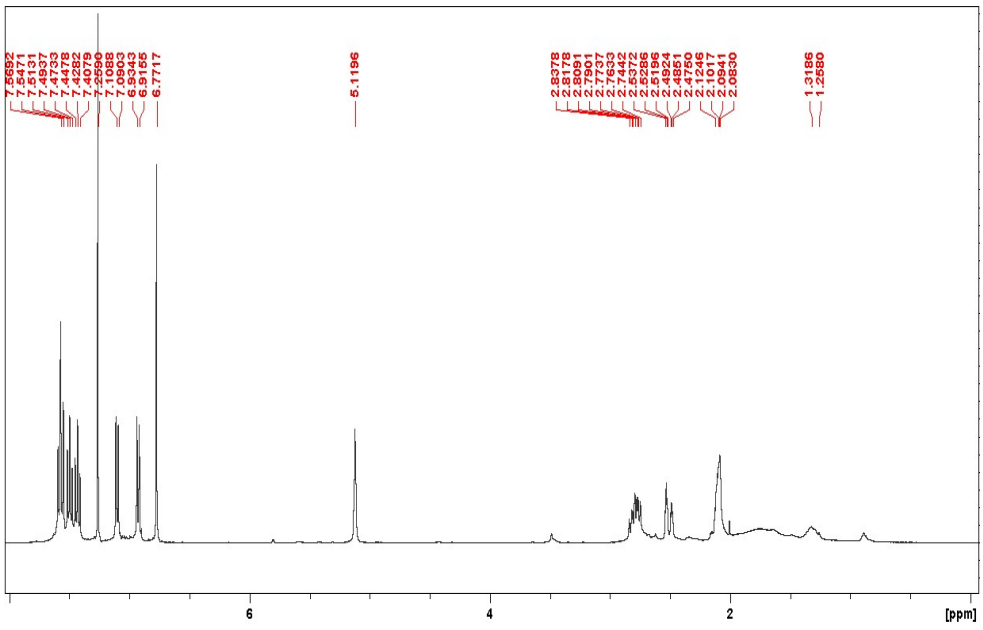
COSY spectrum (CD_3OD) of palmarumycin CP_{30} (**1**).



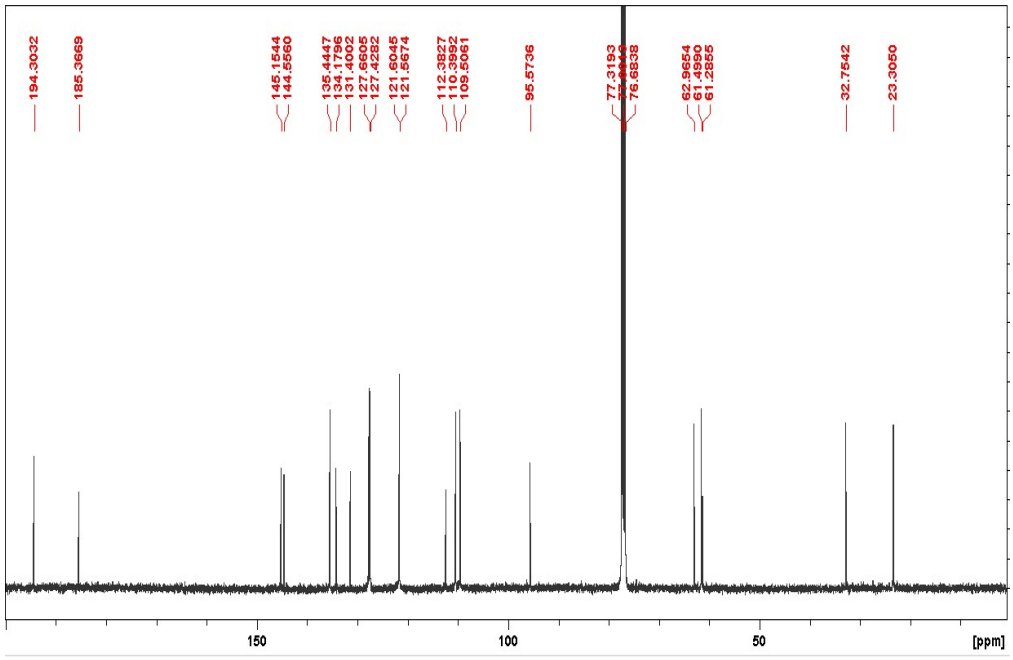
HSQC spectrum (CD_3OD) of palmarumycin CP_{30} (**1**).



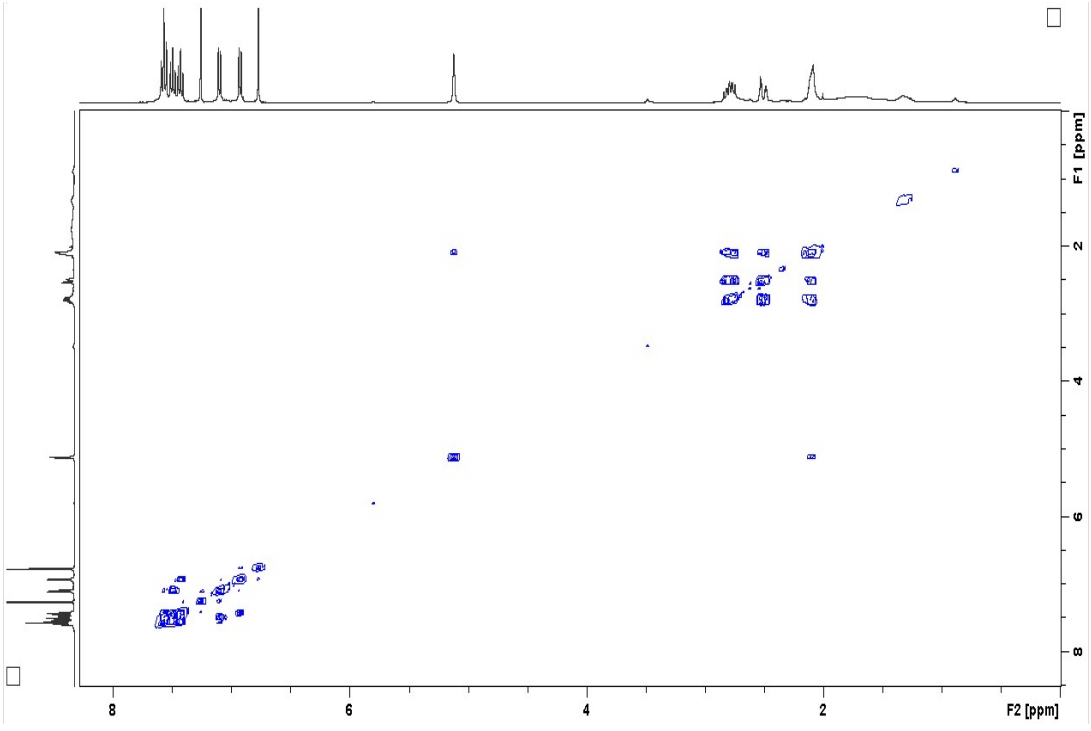
HMBC spectrum (CD_3OD) of palmarumycin CP_{30} (**1**).



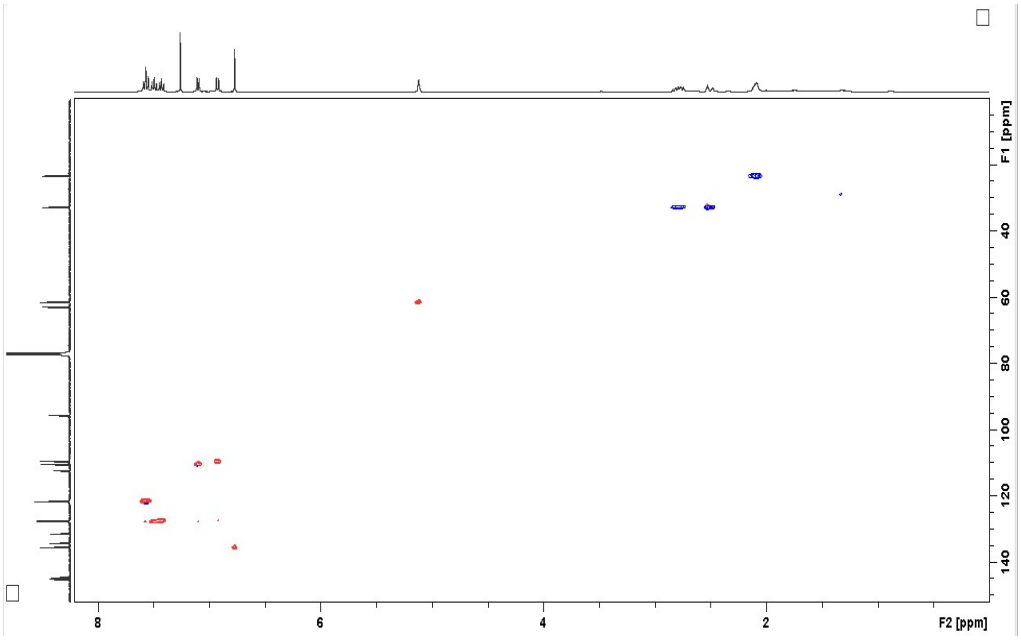
^1H NMR spectrum (CD_3OD, 400 MHz) of palmarumycin C_8 (**2**).



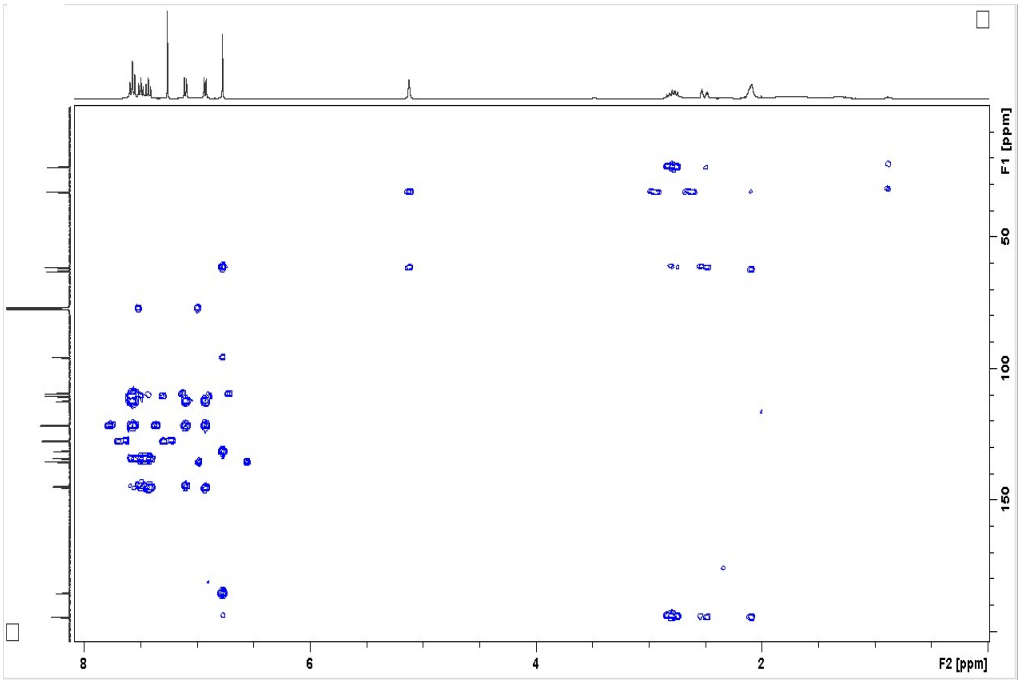
^{13}C NMR spectrum (CD_3OD, 100 MHz) of palmarumycin C_8 (**2**).



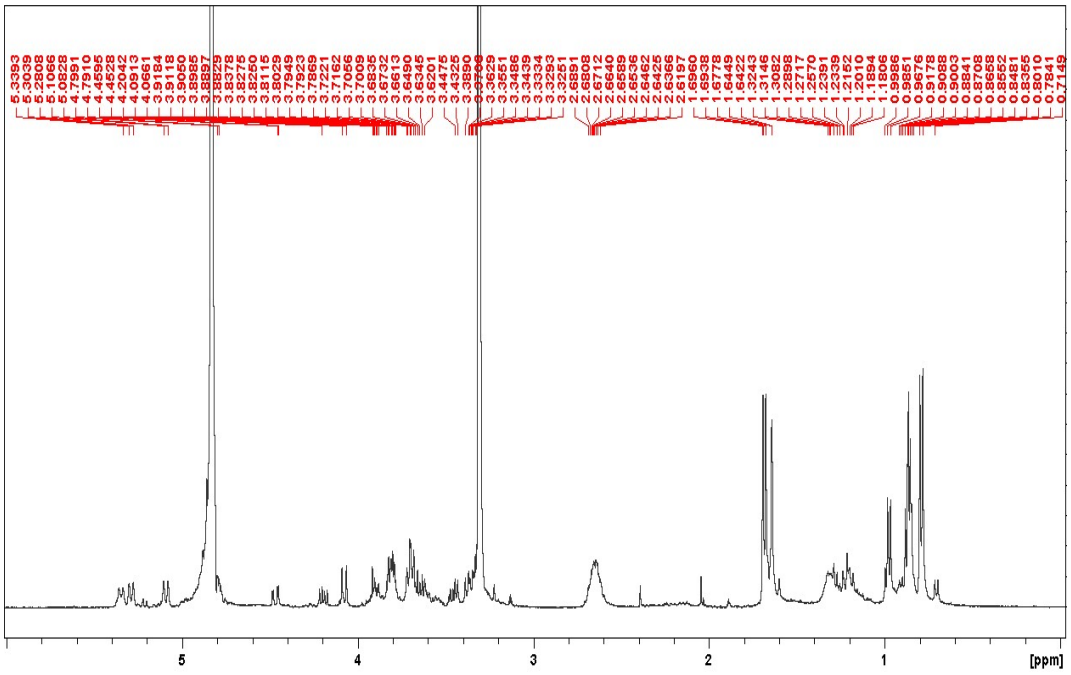
COSY spectrum (CD_3OD) of palmarumycin C_8 (**2**).



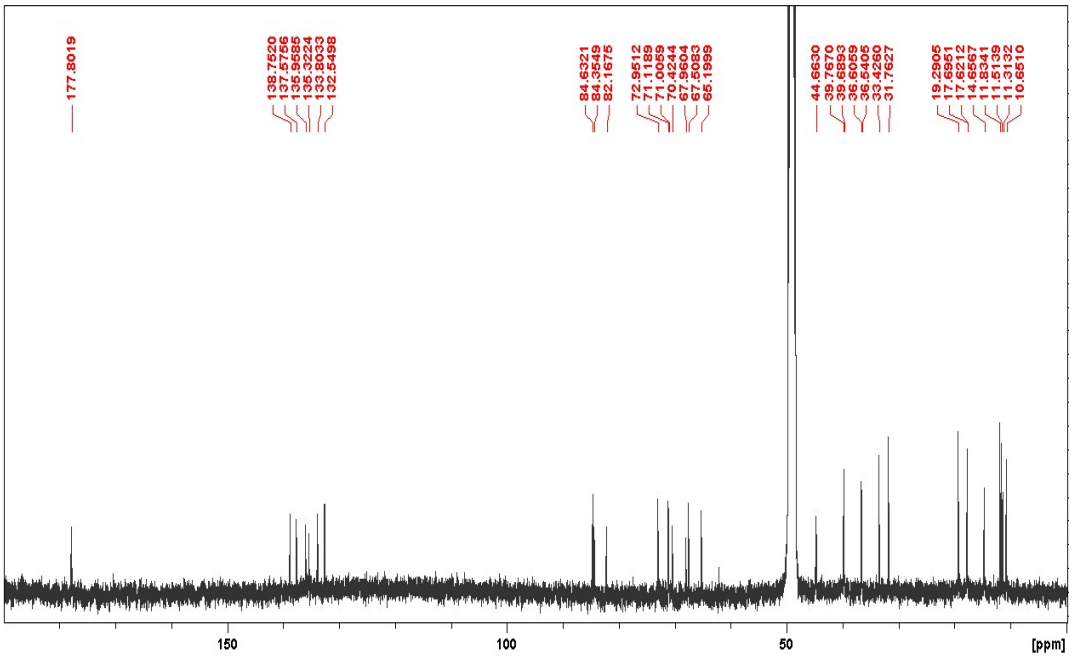
HSQC spectrum (CD_3OD) of palmarumycin C_8 (**2**).



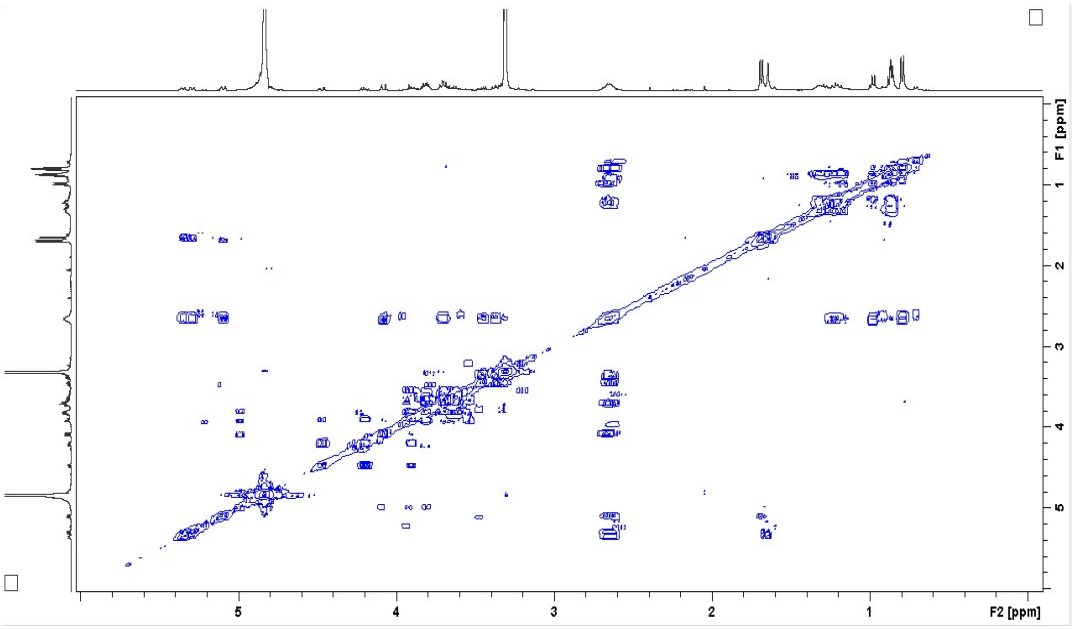
HMBC spectrum (CD_3OD) of palmarumycin C_8 (**2**).



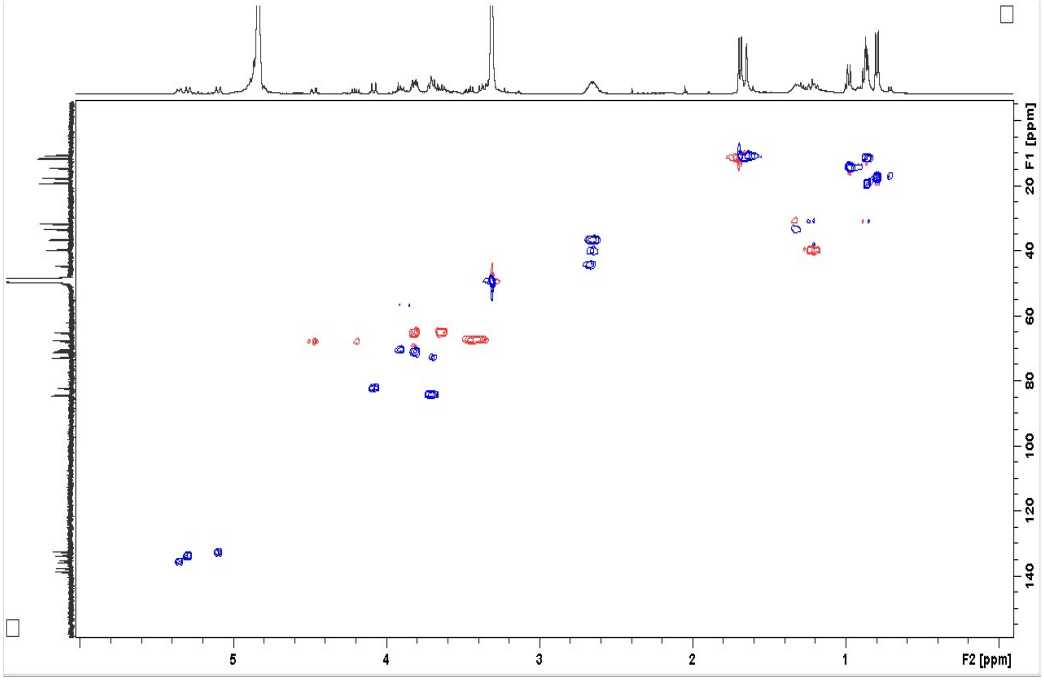
^{1}H NMR spectrum (CD_3OD, 400 MHz) of muyocopronol A (**3**).



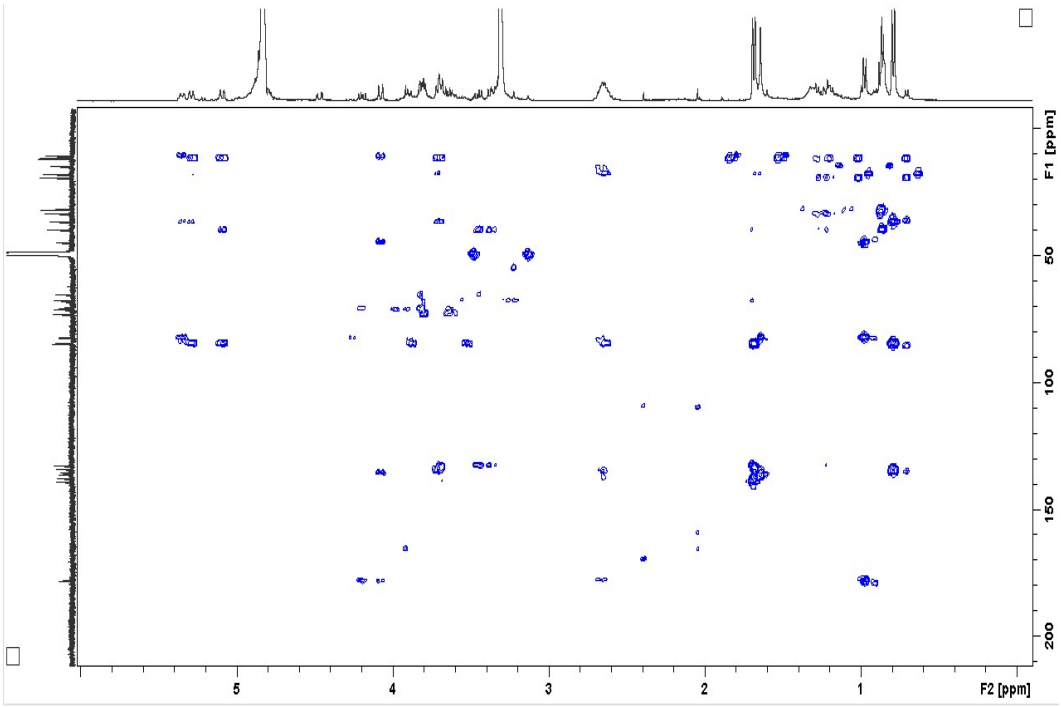
^{13}C NMR spectrum (CD_3OD 100 MHz) of muyocopronol A (**3**).



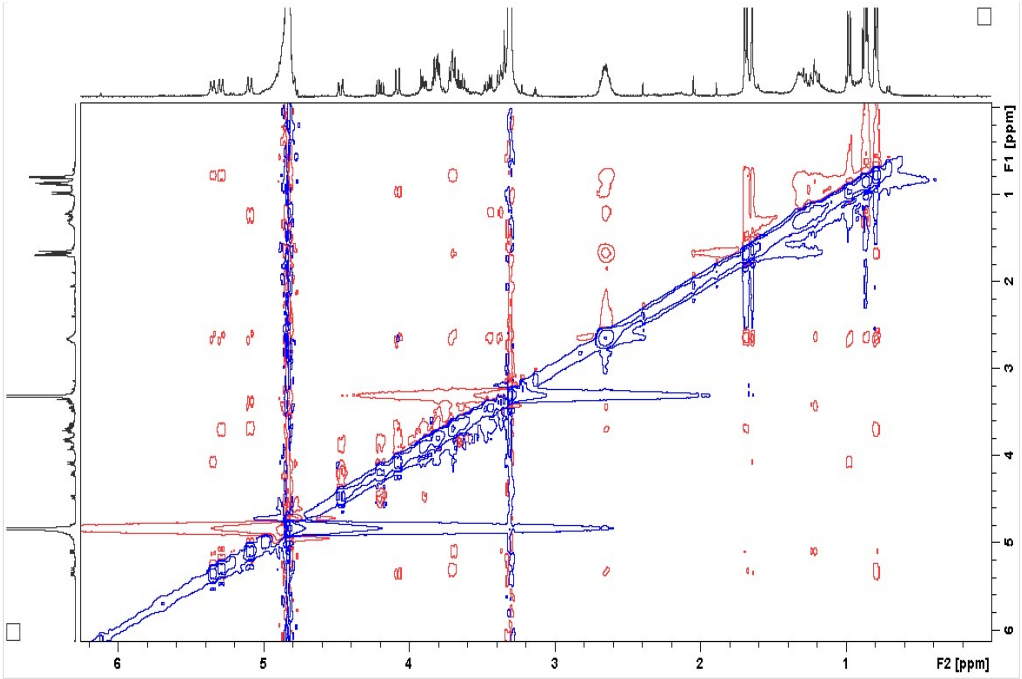
COSY spectrum (CD_3OD) of muyocopronol A (**3**).



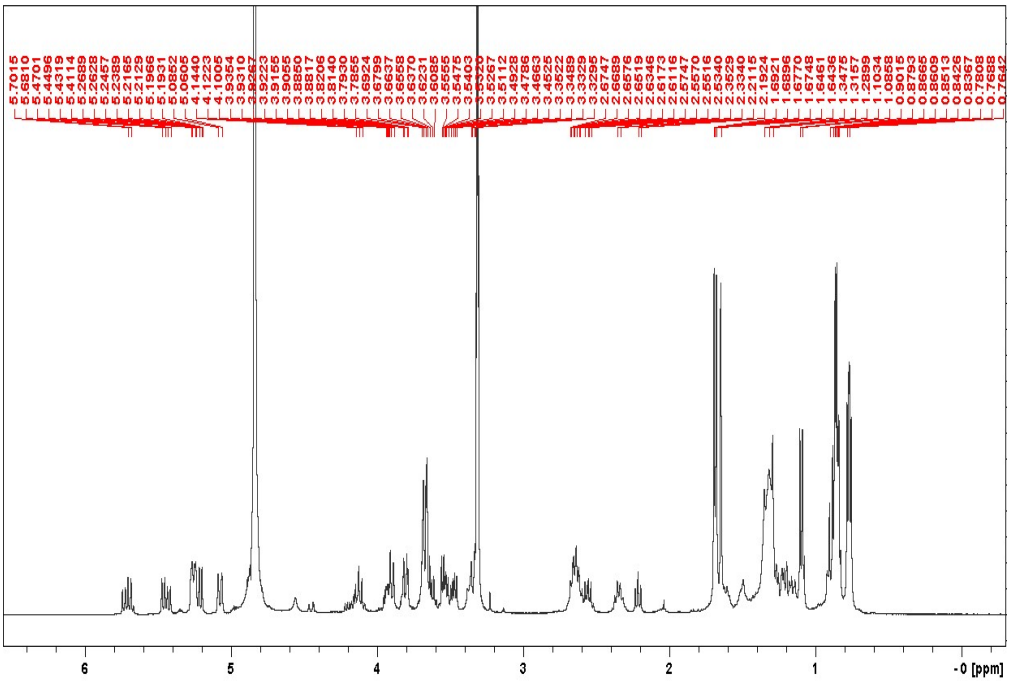
HSQC spectrum (CD_3OD) of muyocopronol A (**3**).



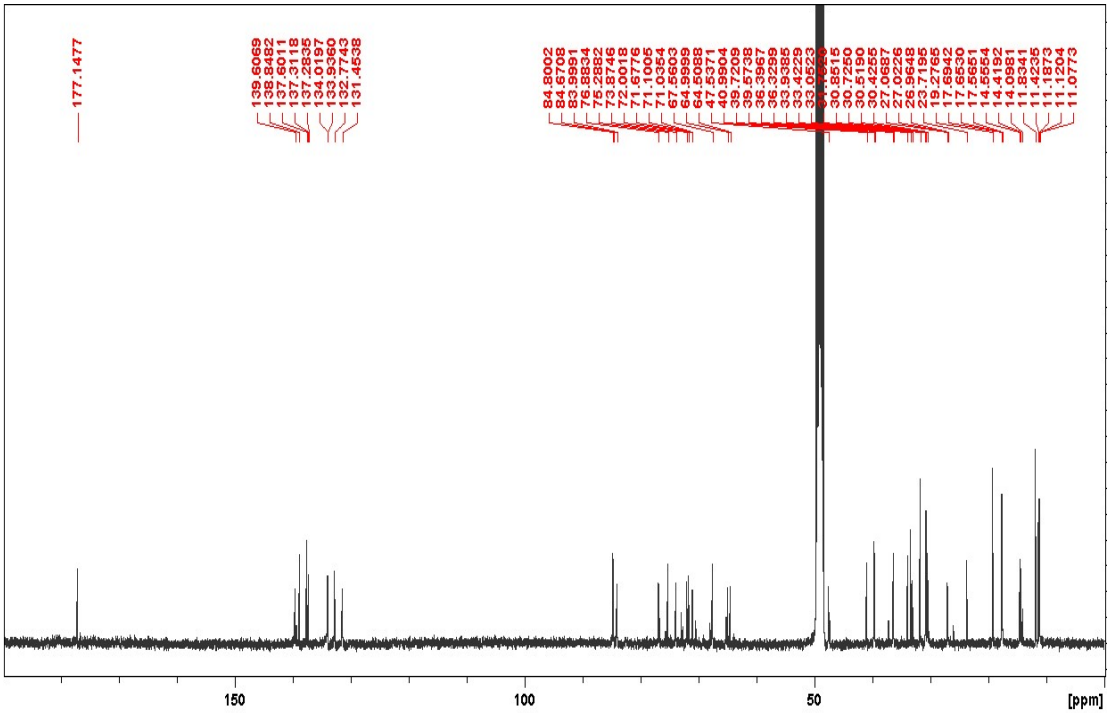
HMBC spectrum (CD_3OD) of muyocopronol A (**3**).



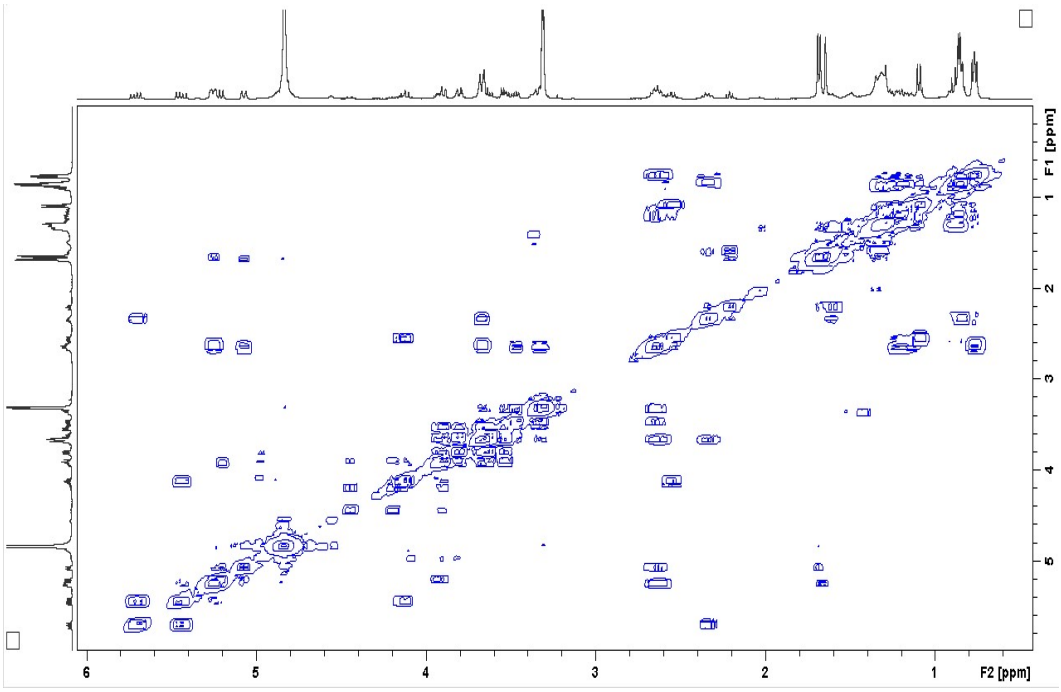
NOESY spectrum (CD_3OD) of muyocopronol A (**3**).



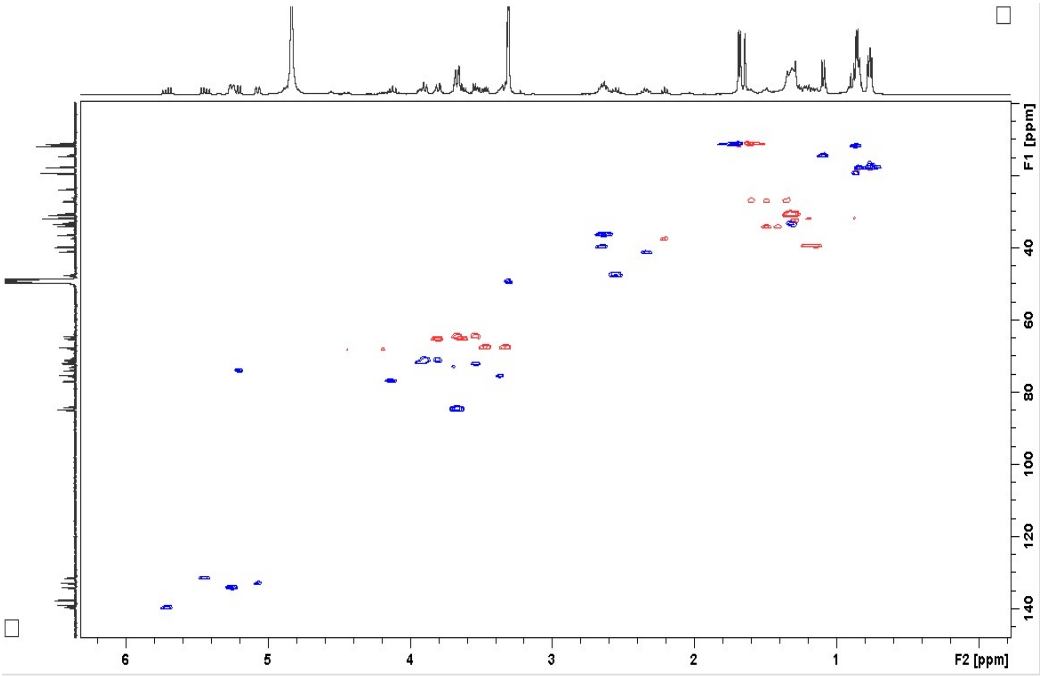
^{1}H NMR spectrum (CD_3OD, 400 MHz) of muyocopronol B (**4**).



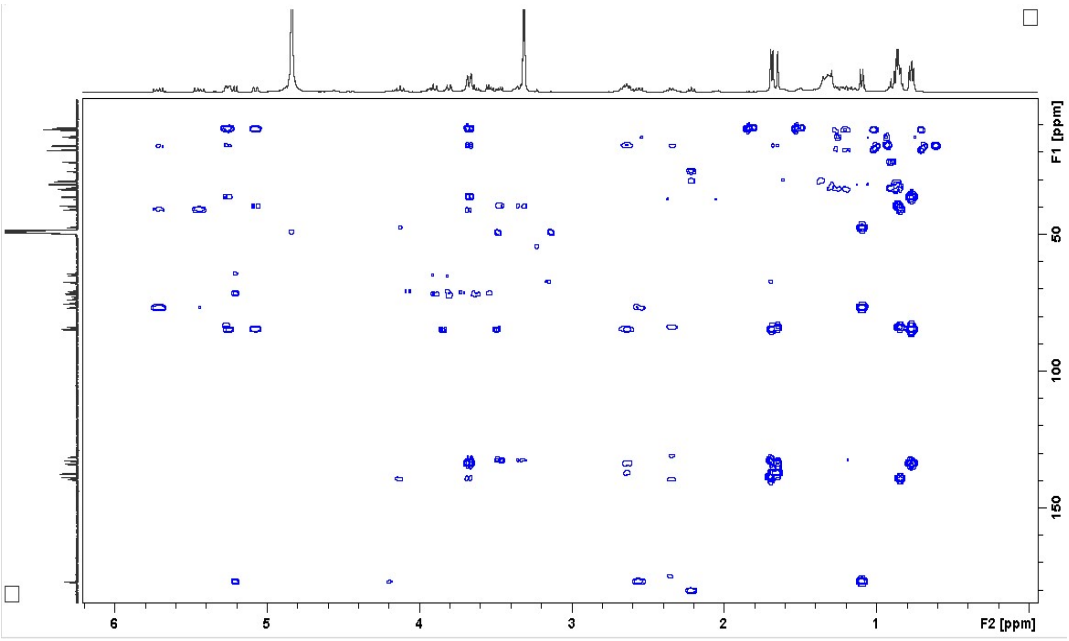
^{13}C NMR spectrum (CD_3OD 100 MHz) muyocopronol B (**4**).



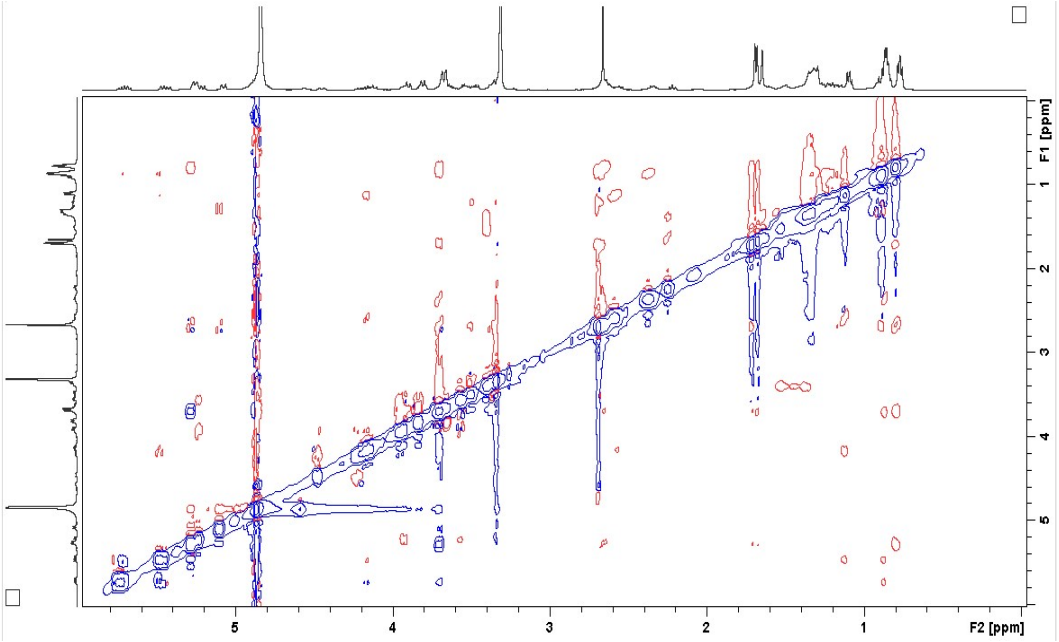
COSY spectrum (CD_3OD) of muyocopronol B (**4**).



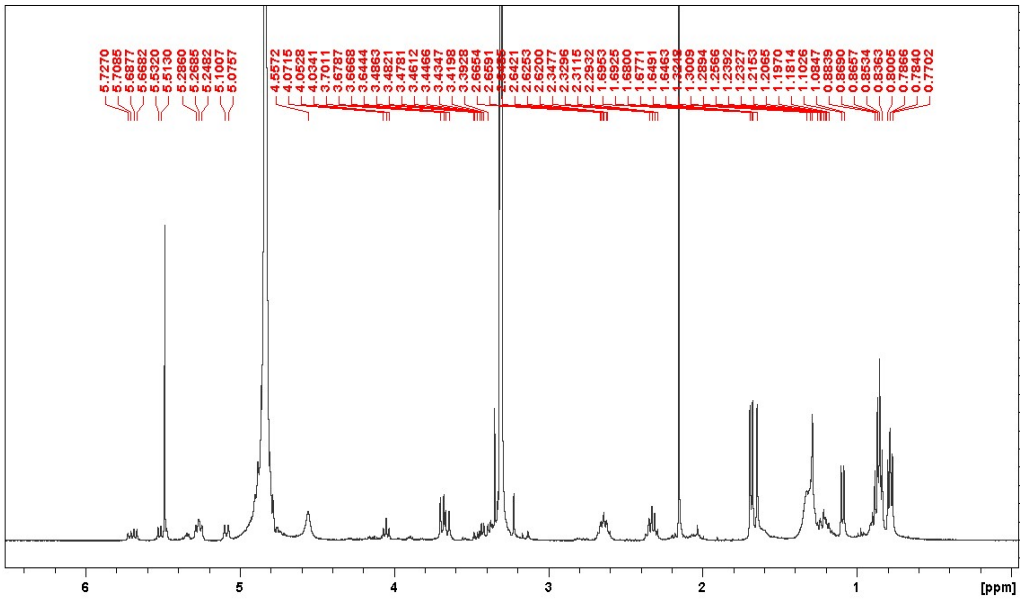
HSQC spectrum (CD_3OD) of muyocopronol B (**4**).



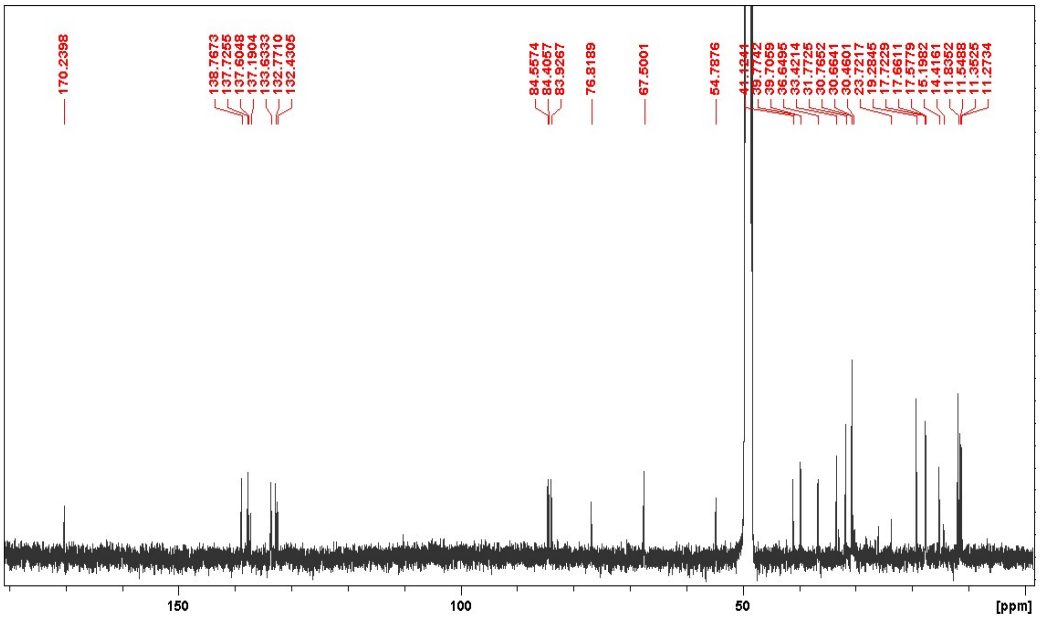
HMBC spectrum (CD_3OD) of muyocopronol B (**4**).



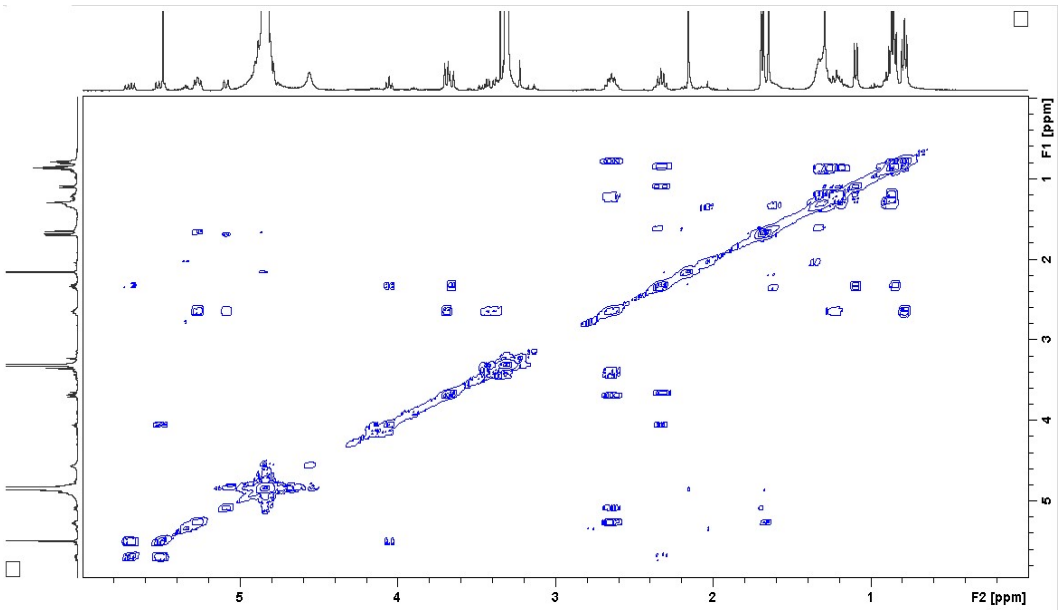
NOESY spectrum (CD_3OD) of muyocopronol B (**4**).



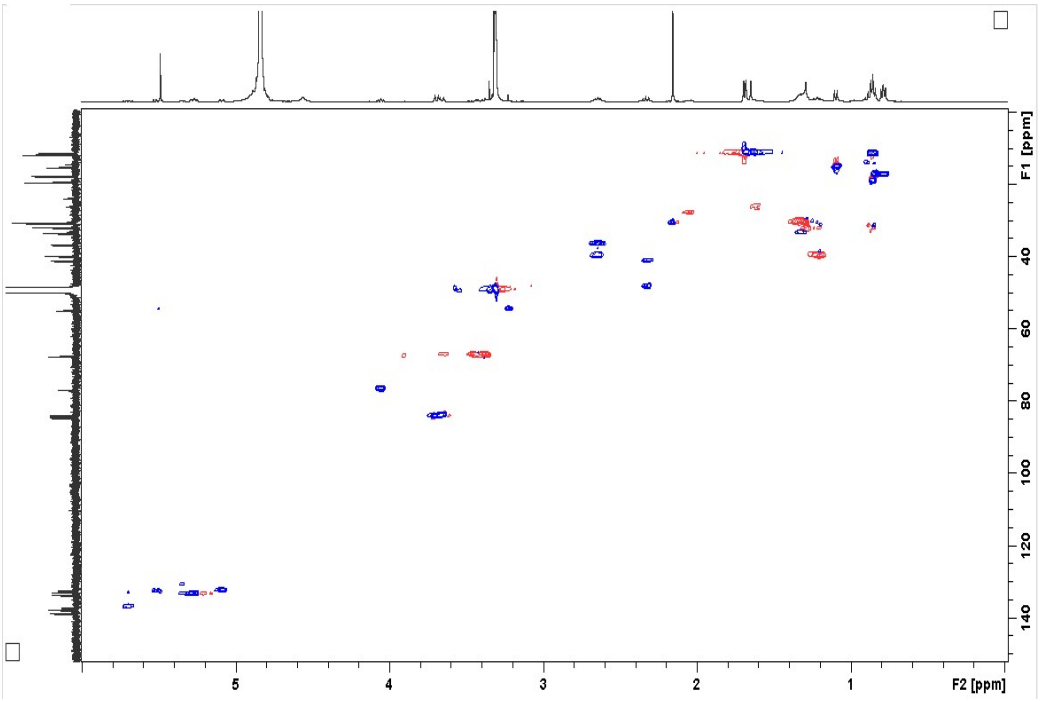
^{1}H NMR spectrum (CD_3OD, 400 MHz) of muyocopronol C (**5**).



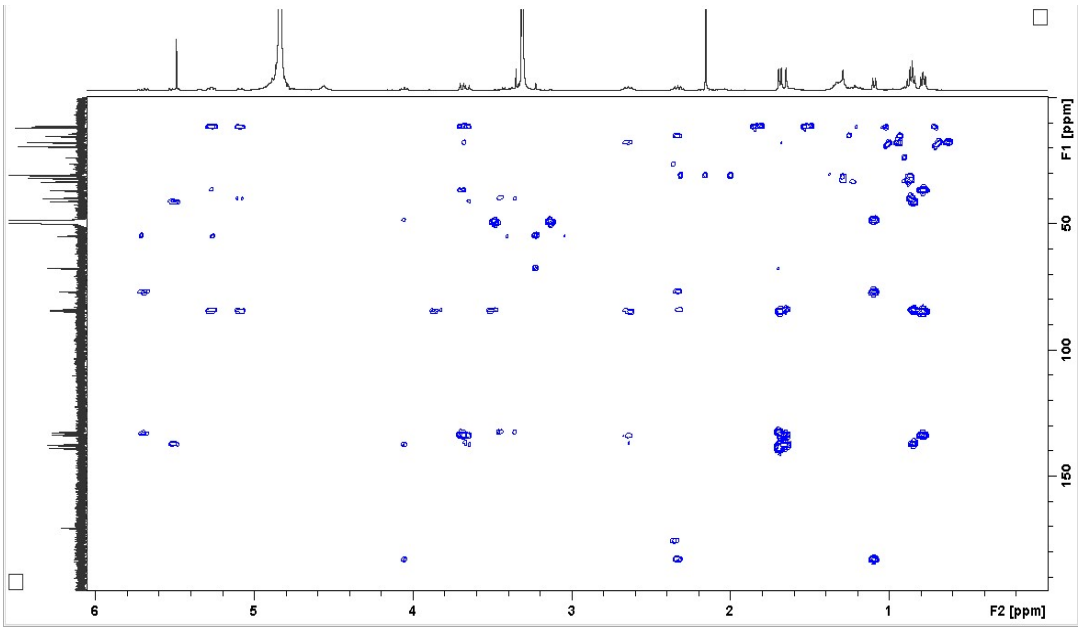
^{13}C NMR spectrum (CD_3OD, 100 MHz) of muyocopronol C (**5**).



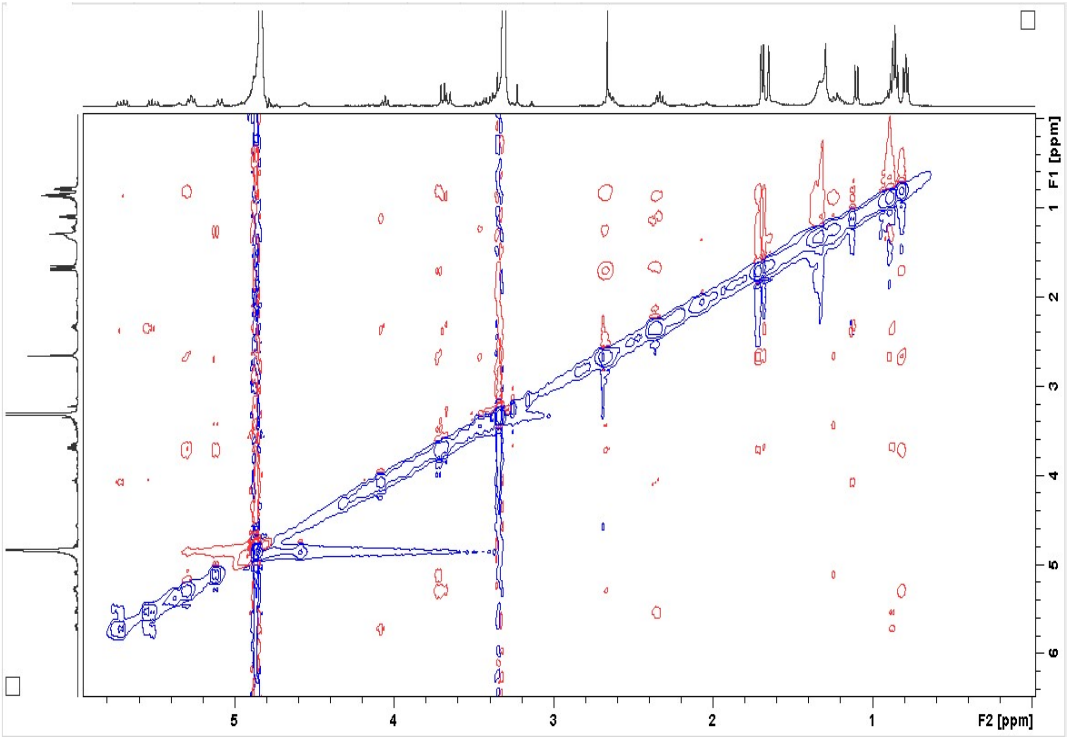
COSY spectrum (CD_3OD) of muyocopronol C (**5**).



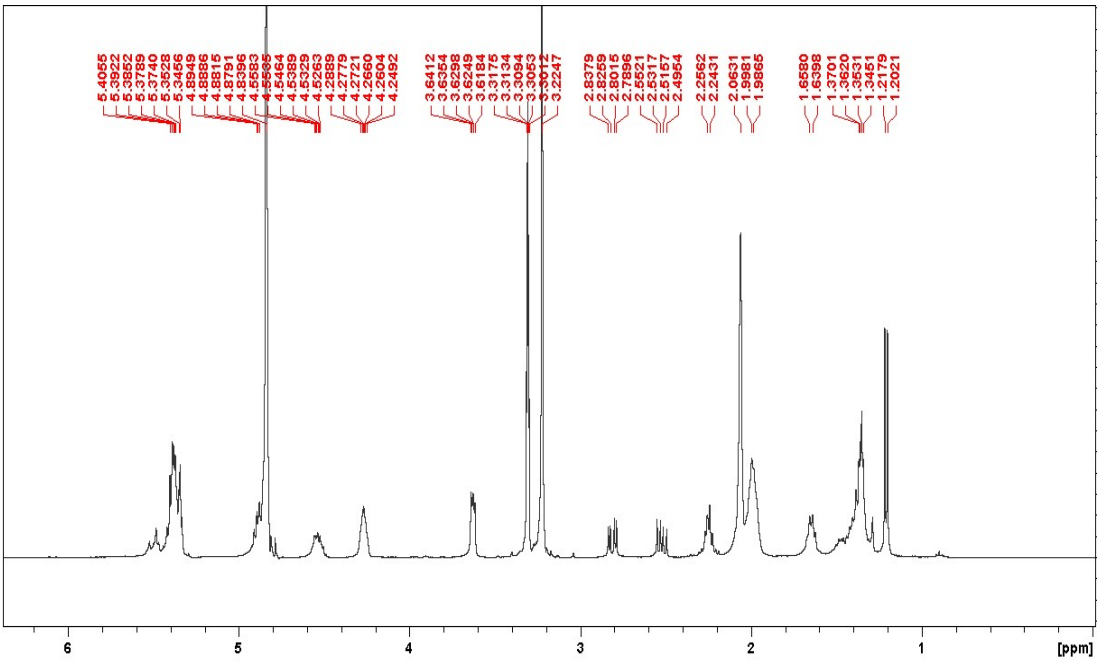
HSQC spectrum (CD_3OD) of muyocopronol C (**5**).



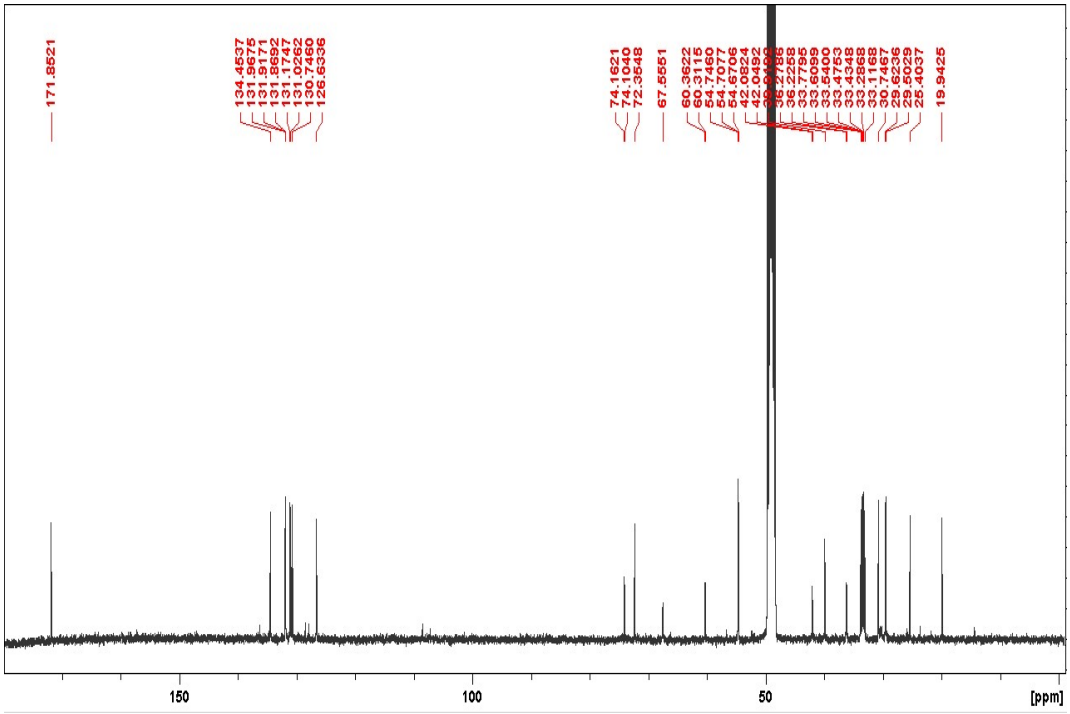
HMBC spectrum (CD_3OD) of muyocopronol C (**5**).



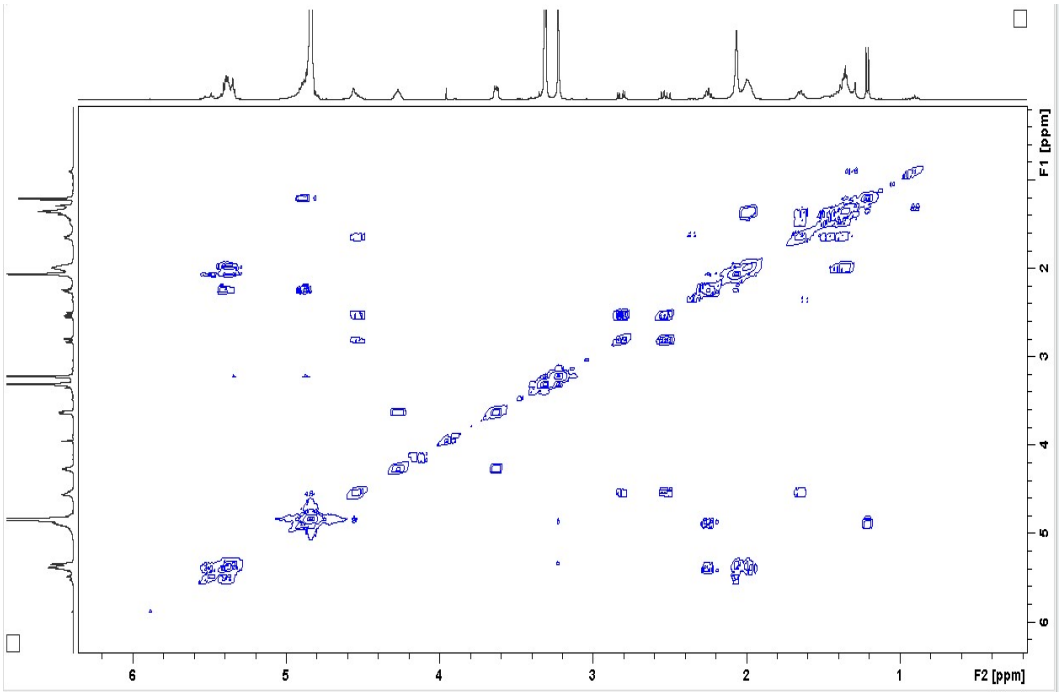
NOESY spectrum (CD_3OD) of muyocopronol C (**5**).



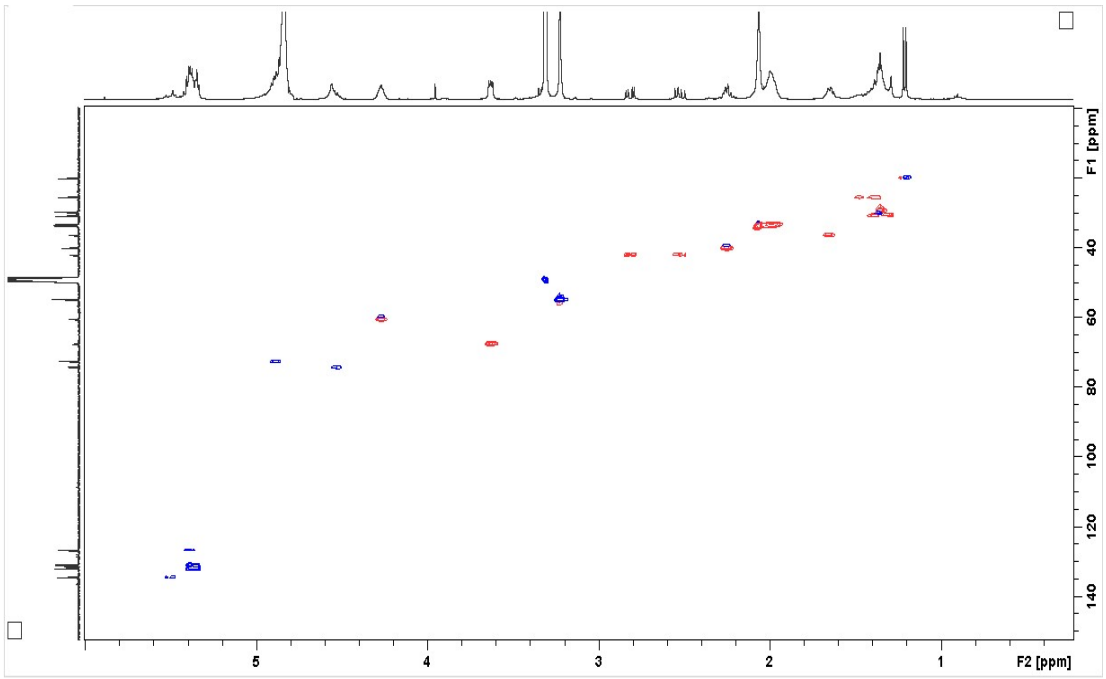
^{1}H NMR spectrum (CD_3OD, 400 MHz) of tropicicolide (**6**).



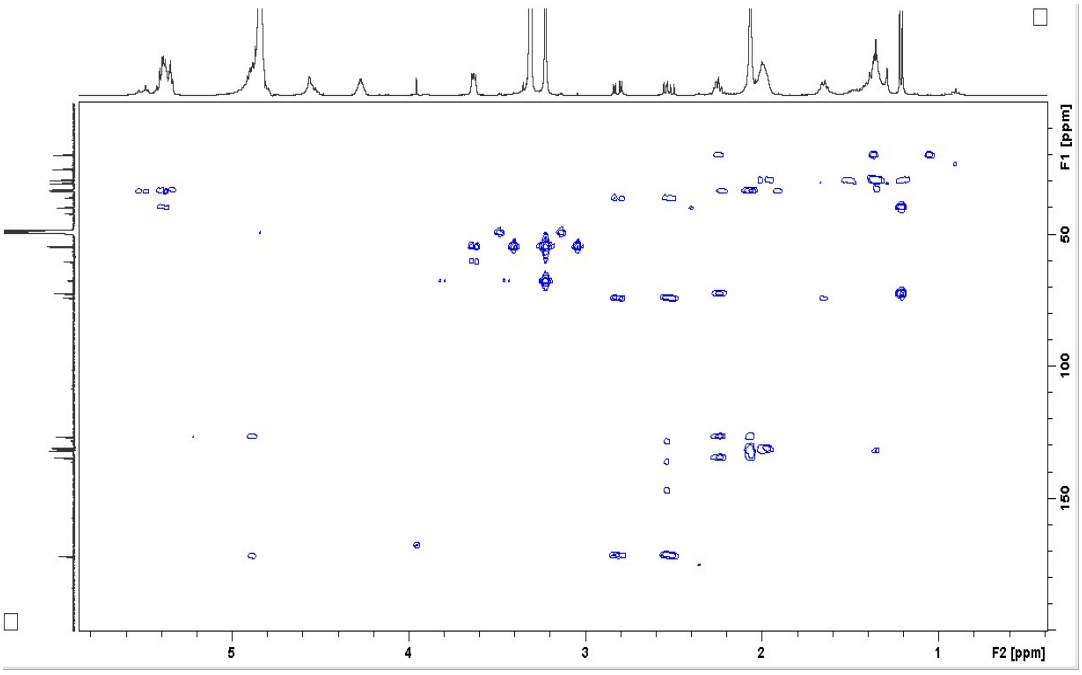
^{13}C NMR spectrum (CD_3OD, 100 MHz) of tropicicolide (**6**).



COSY spectrum (CD_3OD) of tropicicolide (**6**).



HSQC spectrum (CD_3OD) of tropicicolide (**6**).



HMBC spectrum (CD_3OD) of tropicicolide (**6**).

Appendix IX. *Lophiotremataceae* family members used in the phylogenetic analyses

Species	Strain	18S	ITS	28S	RBP2	TEF
***Lophiotrema* sp. F6932**	**F6932**	**ON723936**	**OM791904**	**ON723938**	**ON736762**	**ON736763**
Atrocalyx acutisporus	HHUF 30504	NG_061269.1	NR_153586.1	NG_057141.1	LC194423.1	LC194386.1
Atrocalyx bambusae	MFLU 11-0150	NG_063622.1	NR_153559.1	NG_057116.1	KX672161.1	KX672162.1
Atrocalyx lignicola	CBS 122364	NG_062149.1	NR_153587.1	MH874736.1	LC194424.1	LC194387.1
Crassimassarina macrospora	HHUF 29084	NG_061270.1	NR_153942.1	NG_059030.1	LC194426.1	LC194389.1
Crassiparies quadrisporus	HHUF 30409	NG_061267.1	NR_148185.1	NG_059028.1	-	-
Cryptoclypeus oxysporus	HHUF 30507	NG_062427.1	NR_153943.1	NG_059031.1	LC194427.1	LC194390.1
Cryptoclypeus ryukyuensis	HHUF 30509	NG_061271.1	NR_153944.1	NG_059032.1	LC194429.1	LC194392.1
Cryptocoryneum akitaense	HHUF 30477	NG_065115.1	NR_153935.1	NG_059033.1	LC194430.1	LC096136.1
Cryptocoryneum brevicondensatum	HHUF 30478	NG_065116.1	NR_153936.1	NG_059034.1	LC194431.1	LC096137.1
Cryptocoryneum congregatum	HHUF 30479	NG_065117.1	NR_153937.1	NG_069470.1	LC194435.1	LC096141.1
Cryptocoryneum japonicum	HHUF 30482	NG_065118.1	NR_153938.1	NG_059035.1	LC194438.1	LC096144.1
Cryptocoryneum longicondensatum	HHUF 30486	NG_065119.1	NR_153939.1	NG_069471.1	LC194442.1	LC096148.1
Cryptocoryneum paracondensatum	HHUF 30489	NG_065120.1	NR_153940.1	NG_069472.1	LC194445.1	LC096151.1
Cryptocoryneum pseudorilstonei	CBS 113641	NG_063082.1	NR_153941.1	NG_059036.1	LC194446.1	LC096152.1

Ernakulamia krabiensis	MFLUCC 18-0237	NG_065780.1	NR_163342.1	NG_066314.1	MK434872.1	MK360053.1
Galeaticarpa aomoriensis	HHUF 30505	NG_061272.1	NR_154104.1	NG_059809.1	LC194448.1	LC194393.1
Lophiotrema eburnoides	HHUF 30079	NG_065109.1	NR_138014.1	NG_059801.1	LC194458.1	LC194403.1
Lophiotrema fallopiae	HHUF 30506	NG_065113.1	NR_155339.1	NG_059808.1	LC194459.1	LC194404.1
Lophiotrema mucilaginosis	HMAS 255437	NG_067675.1	NR_164039.1	NG_066426.1	MH822892.1	MH822893.1
Pseudocryptoclypeus yakushimensis	HHUF 30503	NG_061273.1	NR_154379.1	NG_059810.1	LC194472.1	LC194417.1
Pseudolophiotrema elymicola	HHUF 28984	NG_061274.1	NR_154380.1	NG_059811.1	LC194473.1	LC194418.1

Appendix X. Summary of antiSMASH biosynthetic gene cluster predictions for *Lophiotrema* sp. F6932 genome.

Region	Type	From	To	Most similar known cluster	Similarity (%)
Region 1.1	Unknown	116,571	240,321		
Region 1.2	Unknown	308,238	349,085		
Region 1.3	Unknown	432,693	634,304		
Region 1.4	Unknown	1,065,116	1,143,482		
Region 1.5	NRPS	1,171,515	1,281,703	Aspercryptins	13
Region 1.6	T1PKS	1,381,986	1,428,072	(-)-Mellein	100
Region 1.7	T1PKS	1,870,935	1,938,884	4-epi-15-epi-brefeldin A	20
Region 1.8	NRPS	2,447,033	2,661,264	Aflatoxin G1	8
Region 1.9	Unknown	2,671,639	2,772,419		
Region 1.10	T1PKS	2,777,472	2,944,112		
Region 1.11	T3PKS	2,962,163	3,016,966		
Region 1.12	Unknown	3,515,400	3,587,309		
Region 1.13	Unknown	3,618,514	3,705,836		
Region 1.14	Unknown	4,160,513	4,246,489		
Region 1.15	Unknown	4,446,173	4,491,368		
Region 1.16	Unknown	4,577,627	4,632,326		
Region 1.17	Unknown	4,789,180	4,857,556		
Region 1.18	NRPS	5,222,761	5,281,732		
Region 2.1	T1PKS	255,128	450,350		
Region 2.2	Unknown	880,154	1,127,586	Iso-A82775C	16

Region	Type	From	To	Most similar known cluster	Similarity (%)
Region 2.3	Unknown	1,372,944	1,409,598		
Region 2.4	Unknown	1,645,613	1,705,296		
Region 2.5	Unknown	1,714,243	1,775,793		
Region 2.6	Unknown	1,793,048	1,889,962		
Region 2.7	Unknown	1,932,646	1,995,669		
Region 2.8	T1PKS	2,061,057	2,134,556		
Region 2.9	T1PKS	2,281,707	2,394,046		
Region 2.10	Unknown	2,395,946	2,487,387		
Region 2.11	Terpene	2,934,952	3,071,936		
Region 2.12	Unknown	3,347,115	3,422,145		
Region 3.1	Unknown	30,836	143,205		
Region 3.2	Terpene	418,470	515,527		
Region 3.3	Unknown	1,705,730	1,762,477		
Region 3.4	Terpene	1,822,521	1,844,145	Squalestatin S1	40
Region 3.5	Unknown	2,139,179	2,281,614	Communesin A-H	25
Region 3.6	Unknown	2,283,887	2,395,321		
Region 3.7	Unknown	2,549,618	2,613,325		
Region 3.8	Unknown	2,793,702	2,881,126		
Region 4.1	Unknown	457,215	474,184		
Region 4.2	Unknown	483,298	495,204		
Region 4.3	T1PKS	566,832	680,094		
Region 4.4	Unknown	751,992	810,512		
Region 4.5	Unknown	860,351	901,146		

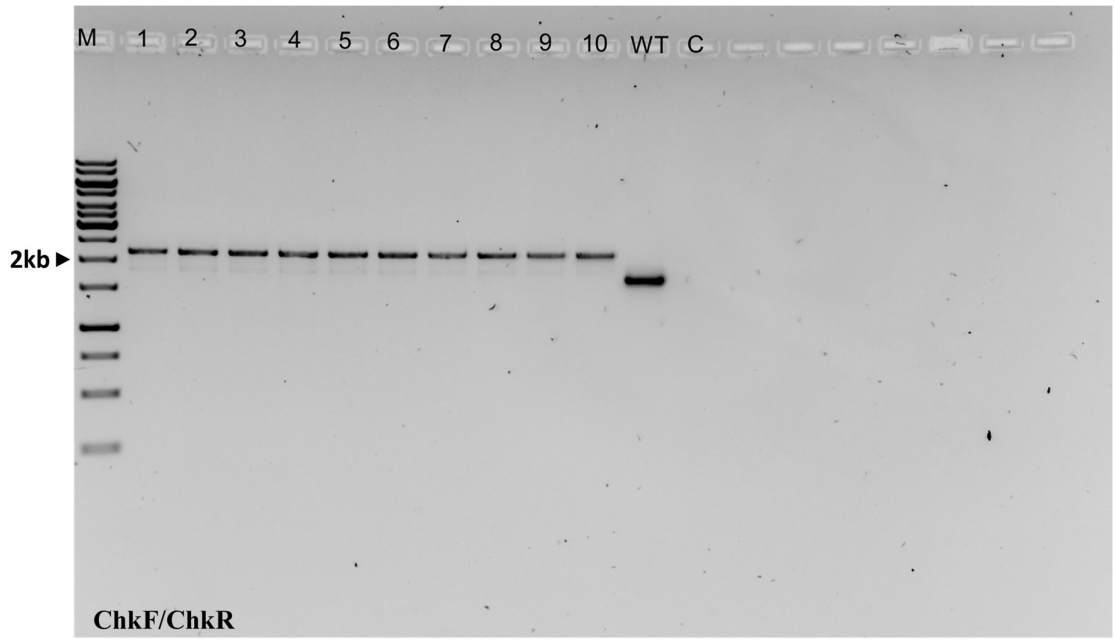
Region	Type	From	To	Most similar known cluster	Similarity (%)
Region 4.6	Unknown	1,045,540	1,176,938		
Region 4.7	Unknown	1,641,587	1,681,905		
Region 4.8	Unknown	2,248,215	2,303,379		
Region 4.9	Unknown	2,692,134	2,731,868		
Region 5.1	Unknown	840,861	866,812		
Region 5.2	NRPS-like	967,342	1,009,332		
Region 5.3	Terpene	1,360,887	1,380,249		
Region 5.4	Unknown	1,781,411	1,822,589		
Region 5.5	Unknown	1,873,117	2,011,129		
Region 5.6	NRPS-like	2,183,662	2,287,559	Chrysoxanthone A-C	13
Region 5.7	Unknown	2,305,795	2,330,488		
Region 5.8	Terpene	2,466,396	2,487,725		
Region 6.1	Unknown	6,374	86,363		
Region 6.2	Unknown	231,336	294,816		
Region 6.3	Unknown	331,211	432,282		
Region 6.4	Unknown	858,769	890,900		
Region 6.5	Unknown	1,502,836	1,549,569		
Region 6.6	Unknown	1,884,513	1,948,799		
Region 7.1	NRPS	2,768	32,737		
Region 7.2	Unknown	35,688	128,694		
Region 7.3	NRPS	226,783	282,973		
Region 7.4	Unknown	714,690	881,396		
Region 7.5	Unknown	1,566,916	1,662,432		

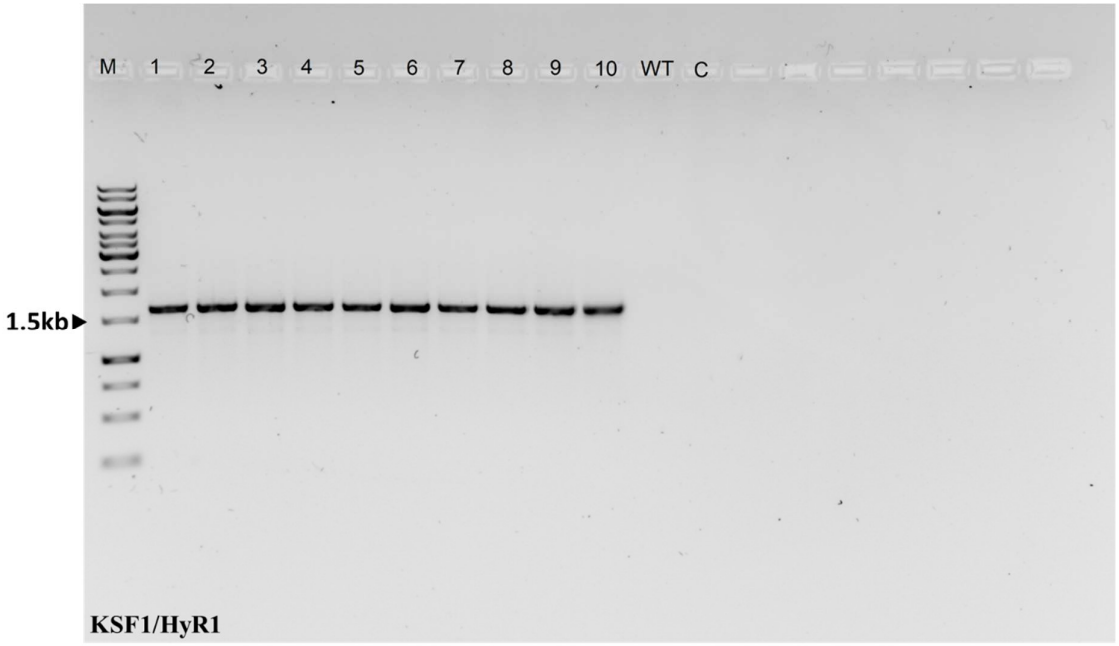
Region	Type	From	To	Most similar known cluster	Similarity (%)
Region 8.1	NRPS-like	14,443	100,700		
Region 8.2	Unknown	784,127	829,974		
Region 8.3	Unknown	1,077,203	1,211,982		
Region 8.4	Unknown	1,377,391	1,417,287		
Region 8.5	NRPS, terpene	1,638,249	1,748,058	Dimethylcoprogen	100
Region 9.1	Unknown	124,754	134,104		
Region 9.2	Unknown	540,894	675,828		
Region 9.3	NRPS-like	700,151	760,776		
Region 9.4	Unknown	1,303,045	1,403,301		
Region 10.1	Unknown	6,883	81,986		
Region 10.2	Unknown	653,390	683,976	Huperzine A	53
Region 10.3	Unknown	1,335,018	1,506,891	Communesin A-H	25
Region 10.4	Unknown	1,571,370	1,634,597		
Region 10.5	Unknown	1,672,504	1,727,169		
Region 11.1	NRPS-like	250,006	293,587		
Region 11.2	T1PKS	545,899	695,482	Melanin	100
Region 11.3	Unknown	868,843	882,655		
Region 11.4	Unknown	1,105,572	1,182,959		
Region 11.5	T1PKS	1,497,182	1,551,787		
Region 12.1	NRPS-like	99,563	291,320		
Region 12.2	Unknown	1,243,504	1,290,587		
Region 12.3	Unknown	1,404,586	1,418,801		
Region 12.4	Unknown	1,422,933	1,485,361		

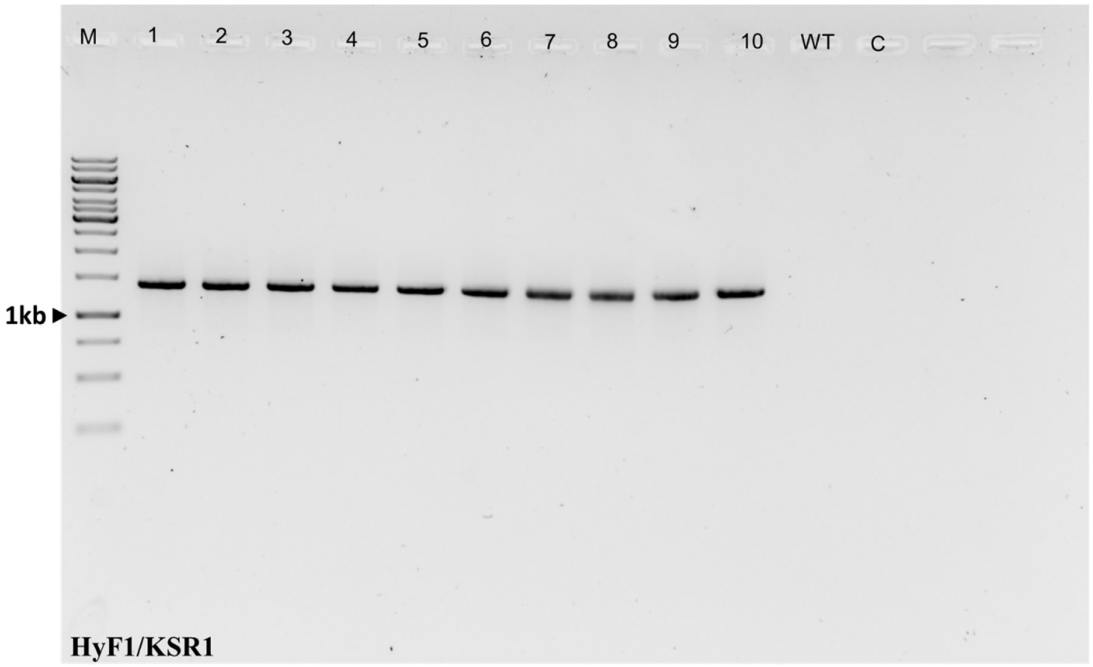
Region	Type	From	To	Most similar known cluster	Similarity (%)
Region 13.1	Terpene	113,501	159,214	Clavaric acid	100
Region 13.2	Unknown	235,017	289,444		
Region 13.3	Unknown	370,195	418,346		
Region 13.4	NRPS-like	420,025	467,503		
Region 13.5	Unknown	528,284	613,438		
Region 13.6	NRPS	1,039,677	1,184,387	KK-1	20
Region 14.1	Unknown	10,225	82,288		
Region 14.2	Unknown	457,583	566,897		
Region 14.3	Unknown	854,261	982,390		
Region 14.4	Unknown	991,694	1,113,490		
Region 14.5	T1PKS	1,206,100	1,269,420		
Region 14.6	Unknown	1,290,264	1,329,360		
Region 15.1	T1PKS	186,603	370,274		
Region 15.2	T1PKS	824,900	958,669		
Region 15.3	Unknown	1,139,387	1,175,056		
Region 15.4	Unknown	1,280,373	1,318,193		
Region 16.1	T1PKS	238,970	393,091		
Region 16.2	Unknown	471,470	607,368		
Region 16.3	Unknown	921,512	989,845		
Region 16.4	Unknown	1,127,976	1,185,333		
Region 16.5	Unknown	1,194,854	1,263,311		
Region 17.1	Unknown	21,987	144,376		
Region 17.2	Unknown	145,953	270,216		

Region	Type	From	To	Most similar known cluster	Similarity (%)
Region 17.3	Unknown	341,735	380,902		
Region 17.4	Unknown	471,775	596,600		
Region 17.5	NRPS-like	747,673	830,967		
Region 17.6	Unknown	1,053,176	1,115,067		
Region 17.7	T1PKS	1,117,243	1,191,476		

Appendix XI. Full-length gel pictures from PCR analysis of *Lophiotrema sp. F6932* CRISPR/Cas9-induced mutants.






M
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WT
C
1kb
HyF1/KSR1