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Characterization of Endophytic Microbiomes of Medicinal Plants for their Antimicrobial Activities

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CHARACTERIZATION OF ENDOPHYTIC MICROBIOMES OF MEDICINAL PLANTS FOR THEIR ANTIMICROBIAL ACTIVITIES

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Ganesh Khadka

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Ganesh Khadka, and entitled Characterization of Endophytic Microbiomes of Medicinal Plants for their Antimicrobial Activities, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2022

DEDICATION

I dedicate this dissertation to my parents, brothers, sisters, wife, and kids.

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ABSTRACT OF THE DISSERTATION

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Emerging new infectious diseases and developing antimicrobial resistance are serious global health threats. New approaches are needed for the discovery and development of new antimicrobial compounds with novel modes of action to overcome the problem of resistant microbial pathogens. There has been increasing interest in the discovery of endophytes, an important component of plant microbiome that live within plant tissues as potential key sources of novel antimicrobial compounds. Medicinal plants are generally considered excellent sources of bioactive compounds produced by endophytes. South Florida with its unique sub-tropical humid climate has botanical gardens, private orchards, and nurseries with vast collections of sub-tropical and tropical medicinal plants species. These plant species can be potential sources of endophytes capable of producing novel antimicrobial compounds. Moreover, increasing loss of biodiversity is a threat to the survival of these native medicinal plant species in South Florida. Therefore, discovering endophytic fungi and bacteria and their biodiversity in native medicinal plants such as *Petiveria alliacea* (Guinea hen weed), *Annona glabra* (Pond apple), *Agave americana* (Century plant), and *Conocarpus erectus* (Buttonwood

mangrove) is crucial for understanding their roles in ecosystems and for the discovery of novel antimicrobials against human, plant, and animal pathogens. Characterization of microbiomes of these four medicinal plant species were conducted using cultural, biochemical, and molecular approaches. Pure cultures of bacterial and fungal endophytes were isolated from different parts of native medicinal plants and was screened for antagonistic activity against bacteria and fungi using dual culture technique. Culture extracts from endophytes showing antagonistic activity were collected and screened for antimicrobial activity against common human and plant pathogens such as *Escherichia coli* ATTC 25902, *Staphylococcus aureus* ATTC 14775, *Bacillus subtilis* NRRL 5109*,* *Candida albicans* ATTC 10231 and *Aspergillus fumigatus* NRRL 5109. Phylogenetic identification of endophytes producing antimicrobial compounds were carried out using 16S rRNA and ITS gene sequence analysis. Characterization of antimicrobial compound/s present in the culture extracts were analyzed using chemical analysis method. Microbial biodiversity of endophytes was assessed using both cultural and molecular methods. Results indicated that the fungal endophytes isolated from all four native medicinal plant South Florida has potential to produce novel bioactive potential to kill or inhibit the growth of disease-causing strain of bacteria and fungi. Moreover, endophytic bacteria produced from medicinal plant of South Florida also contain bacterial endophytes that provide antibacterial properties. It was also found that there is diversity among the culturable and non-culturable fungal endophytes from medical plants of South Florida. Hence, Native medicinal plants of South Florida harbor the endophytic microbiomes capable of producing novel bioactive metabolites.

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ABBREVIATIONS AND ACRONYMS

IR	Isolation Rate
CR	Colonization Rate
HPLC	High Performance Liquid Chromatography
TSA	Tryptic Soy Agar
NA	Nutrient Agar
NB	Nutrient Broth
PDA	Potato Dextrose Agar
MHA	Mueller Hinton agar
PDB	Potato Dextrose Broth
PCR	Polymerase Chain Reaction
CDC	Centers for Disease Control and Prevention
RPM	Revolutions Per Minute
MIC	Minimum Inhibitory Concentration
ITS	Internal Transcribed Spacer

CHAPTER 1 : INTRODUCTION

1.1 Background

Recently, there has been a growing interest in the field of endophytic plant microbiome research because of their potential to contribute to the discovery of new bioactive compounds. They are ubiquitous and colonized in all types of plants and are versatile in all ecosystems globally. The endophytic microbiomes that live inside the host plants have an interesting relationship and contribution to the host plant. The term "Endophytes" was first introduced by De Bary (1886) and are the microorganisms (bacteria or fungi or actinomycetes) that dwell intercellularly and intracellularly in the host plants for at least part of their life cycles without causing harmful effects (Bary, 1886; Doley P & Kha DK, 2015). Endophytic association with host plants dates back to the first terrestrial plants and they have been contributing to host plants for their growth, survival, and evolution. Most of the plants and their endophytes relationships are not known well yet but their relationship is often symbiotic to the broadening on pathogenic (Dudeja et al., 2012). Sometimes, their association can be obligate or facultative with the host plants (Petrini, 1991). An endophyte can infect and dwell in many host genera but still exhibits genotypic specificity within a species. They can get to host plants via many different routes, but most endophytes originate from environmental infection. Similarly, some of them can be transmitted via vegetative means or by the means of seed.

After penetrating to the host plant, endophyte's growth normally controls by plants components, and later endophytes also start adapting gradually according to their living environments. As the relationship is often mutualistic, plants provide different types of

nutrients source vital for the growth and survival of the endophytes and in the meantime, endophytes also produce different types of chemicals and improve plant's physical structures for better growth and development to defense against the external threats (Dudeja et al., 2012). This is done by different mechanisms including the formation of extra hyphae on the root for better nutrient absorption, stimulating root growth, alternating plant metabolism for promoting nutrients uptake, nitrogen fixation and altering root exudates, and modifying soil physical and chemical properties directly (Johnston-Monje, 2011).

As the endophytes are beneficial for the host plant, their population density is highly variable to many reasons. Many different factors contribute to the diversity and density of the endophytes in the host plants. These factors include the genetic make-up of the host plants, developmental stages of the host plants, environmental conditions, and inoculum density of the endophytes.

On the other hand, people from different parts of the world have been facing different types of health problems due to changes in the genetic makeup of the disease-causing agents and there are increased reports of superbug or antibiotic resistance pathogens in different parts of the world (Chokshi et al., 2019). Antibiotic resistance happens when pathogens like fungi and bacteria develop the ability to defeat the drugs which are designed to kill them. That means these germs are not killed and continue to grow and multiply (Centers for Disease Control and Prevention [CDC], 2019). Due to the increased risk of the resistance form of disease-causing microorganisms or superbugs and reoccurrence of the same disease from time to time has pushed advancements in the field of new drug discovery (Demain & Fang, 2000; Strobel & Daisy, 2003).

In order to address that situation, scientists have been researching to find out the curative agents for treating resistance forms of microbes and they have been trying from different sectors of the science to address those problems. The current ongoing field of research includes in situ cultivation, co-culture, genome mining, metabolomics, combinatorial biosynthesis, exploring new reservoirs such as marine and endophytes (Kealey et al., 2017). Among them, one of the main sectors that have a huge probability for finding such chemical agents is the endophytic microbes. Scientists have been able to find some useful antimicrobial and anticancer drugs from plant endophytes. Examples of the drugs discovered from plant endophytes such as Penicillenols, Taxol, Clavatol, Sordarcin, Jesteron, Javanicin, etc. (Gouda et al., 2016). As the recent research demonstrated interesting results in the field of plant antimicrobial, scientists have focused more on plant endophytes as a good field to discover new drugs. Thus, Phyto-endophytes are now being considered as the potential source of novel antimicrobial agents.

In addition to that, people have been using medicinal plants/herbs in traditional medical practice since the prehistoric era (Yousuf et al., 2012). So, it can provide the clue that medicinal plants harbor good chemicals that help humankind to tackle health problem. Furthermore, Medicinal plants synthesize and metabolize many kinds of chemicals that help them for fighting against disease-causing agents such as fungi, bacteria, insects, herbivorous animals (Saxena et al., 2013). Many different phytochemicals with strong bioactive potentials have been discovered and used for treatment purposes. The categorization of the chemicals produced by medicinal plants includes alkaloids, glycosides, phenols, organic acids, resins, gums, saponins, and essential oils. Hence, medicinal plants always have been the resources for new drug discovery. The mutualistic

relationship of medicinal endophytes with the host plants produces various compounds such as antibiotics, antioxidants, anticancer agents, immunosuppressive agents, volatile organic compounds, plant growth-promoting factors, herbicides, insecticides, etc. (Strobel & Daisy, 2003). Endophytes are capable of producing the same or similar chemicals as the host plant produces (Ebada et al., 2016; Uzor et al., 2015). Hence, medicinal endophytes are the reservoir of the novel antimicrobial compounds.

South Florida also plays an important role in the ethnobotanical sector of US history. Tropical and sub-tropical medicinal Plants that are present in South Florida provided an important source of remedy for native Americans and early settlers in Florida (Beare, 2019).

Native people who live in South Florida such as Seminole and Mikasuki tribe still use traditional herbal passed down by their ancestors. Scientists have started researching medicinal plants for therapeutic purposes and they have been successful in finding some important chemicals from South Florida medicinal plants. These include chemicals from saw palmetto fruits to reduce swelling associated with prostate cancer, compounds from mayapple (*Podophyllum peltatum*) have been used in cancer and chemotherapy (Allen et al., 2002).

The rapid loss of natural habitats and biodiversity of medicinal plants have threatened the future finding of novel antimicrobial products from these valuable medicinal plants. So, immediate screening of valuable medicinal plants for their valuable compounds from endophytes is the immediate need for fighting against different types of pathogens and would vitally reason to conserve these valuable plant resources in the region.

1.2 Research Objectives

The purpose of this research is to isolate and characterize the endophytic microbiomes focusing on fungi and bacteria and to investigate the antimicrobial properties and diversity of endophytic fungi on a medicinal plant found in South Florida. Following are the objectives to prove the hypothesis of this project.

- Isolation of endophytic fungi and bacteria from native medicinal plants of South Florida.
- Characterization and purification of the endophytic isolates into pure culture.
- Testing of endophytic isolates with human, animal, and plant pathogens.
- Identification of the isolates and their antimicrobial compound using molecular techniques.
- Identification of the fungal biodiversity from the native medicinal plant of South Florida.

1.3 Significance of the study

People have been misusing antibiotics nowadays without being aware of the negative effects. Consequently, there is an increasing case of untreatable novel cases of pathogens emerging every day. Scientists have been trying from a different aspect of science to address that global problem of antibiotic resistance. One of the potential aspects of science to study novel drugs to discover new medicine is the exploration of endophytic microbiomes. As the endophytes are ubiquitous, they can be isolated from all the host plants. This study is mainly focusing on the medicinal plant as people have been using those plants as a curative agent for different types of diseases. Previous studies have focused on plant parts extracted from medicinal plants to test their antimicrobial potential.

But recent studies have proven that endophytic microbiomes are the producer of medicinal properties of medicinal plants.

The main purpose of this study was to investigate the characteristics of the endophytic microbiome from medicinal plants to see if the endophytic microbiomes from the medicinal plants from South Florida have the bioactive properties. Results from the current study will be beneficial to discover novel antibiotics to address the current problem of antibiotic resistance.

CHAPTER 2 : CHARACTERIZATION OF FUNGAL ENDOPHYTES ISOLATED FROM NATIVE MEDICINAL PLANTS OF SOUTH FLORIDA

Abstract

Medicinal plants have been used since the ancient era to treat some type of disorders but still there is the lack of scientific evidence for their effectiveness. It is not clear yet that medicinal properties of medicinal plant are from plant itself or from the endophytic fungi that harbor inside the tissue of the host plant. In this study, we have investigated the endophytic fungi from four native medicinal plants from South Florida for their potential bioactive properties. These native medicinal plants include *Petiveria alliacea* (Guinea hen weed), *Annona glabra* (Pond apple), *Agave americana* (Century plant), and *Conocarpus erectus* (Buttonwood mangrove). Characterization of endophytic fungi of these four medicinal plant species were conducted using cultural, biochemical, and molecular approaches. Pure cultures of fungal endophytes were isolated into pure culture from different parts of native medicinal plants and was screened for antagonistic activity. Culture extracts from endophytes were screened for antimicrobial activity against common human and plant pathogens such as *Escherichia coli* ATTC 25902, *Staphylococcus aureus* ATTC 14775, *Bacillus subtilis* NRRL 5109*, Candida albicans* ATTC 10231 and *Aspergillus fumigatus* NRRL 5109. Phylogenetic identification of endophytic fungi producing antimicrobial compounds were carried out using ITS gene sequence analysis. Results indicated that some fungal endophytes isolated from all four native medicinal plants of South Florida have potential to produce novel bioactive potential to kill or inhibit the growth of disease-causing strain of bacteria and fungi.

Although, Native medicinal plants of South Florida harbor the endophytic fungi capable of producing novel bioactive metabolites, further studies needed to characterized individual fungal strain with its detail molecular study in larger geographical area in US.

2.1 Introduction

Since the ancient times, medicinal plants have been used as primary source of medicine (Hussain et al., 2022; Sen & Chakraborty, 2017). Even in these modern times, plant remedies are still the most important therapeutics to treat diseases (Bussmann, 2013). According to the survey report of the World health organization, "Almost 80% of the world's population from developing countries still rely on traditional medicine that involves the use of plant extract for their primary health care" (Patwardhan et al., 2004; Singh et al., 2022). Endophytes are an important component of the plant microbiome and are known to be highly abundant and diverse.

Endophytic fungi live asymptomatically within the host plant tissues (Carroll, 1988; Faeth & Hammon, 1997). They can be found in a wide range of host plants and there is at least one endophyte in all the plants studied so far and hundreds or thousands of endophytic fungus species may be present (Faeth & Hammon, 1997; Saikkonen et al., 1998).

Endophytes are known to produce a multitude of secondary metabolites and are considered as natural reservoir of novel bioactive compounds of medical importance (Newman & Cragg, 2020; Pasrija et al., 2022). Currently there is a growing interest in

bioprospecting of endophytes for bioactive compounds and their potential application in biotechnology and biomedicine.

Endophytic fungi produce some of the widely used antibiotic and cancer treatment drugs. Some examples of these antibiotics and anti-cancer drugs include Penicillenols extracted from *Penicillium species*, Taxol extracted from *Taxomyces andreanae*, which is the most effective and popular cancer drugs extracted from endophytic fungi. Similarly, Clavatol from *Torreya mairei*, sordaricin from *Fusarium spp.* Jeserone from *Pestalotipsis jester* and Javanicin from *Chloridium spp*. are well known for possessing strong antifungal and antibacterial properties against few infectious disease-causing agents. Pestacin was isolated from *Pestalotiopsis microspora*, also possesses strong antioxidant properties (Gouda et al., 2016). This evidence suggests that endophytic fungi provide a promising source of novel drugs.

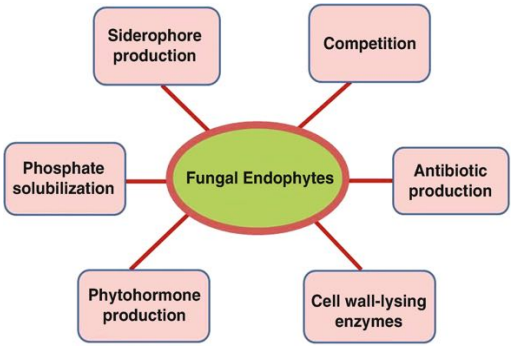

Figure 2.1: Beneficial role of endophytic fungi on medicinal plants (online image) (Sharma et al., 2019).

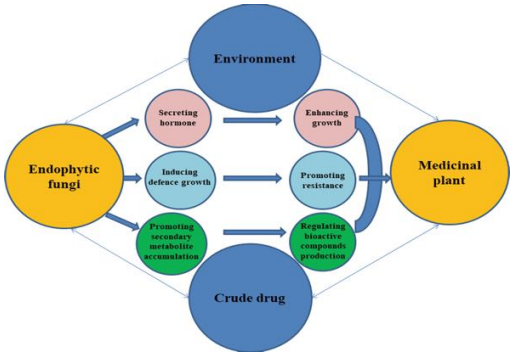

Figure 2.2: Fungal endophytes as a potential source of bioactive compound for plant growth promotion (Online image) (Vyas & Bansal, 2018).

As they are an important source of novel antibiotics, only about a tenth of the estimated one million terrestrial fungal endophytes have been studied yet and a lot more remains unknown (Ganley et al., 2004). The spread of antibiotic-resistant bacteria is a growing problem worldwide, it is critical to find new sources of antimicrobials to address this problem. Recently there is a significant interest in endophytes research due to their immense potential as source of novel bioactive compounds. The choice of the plant to be used for exploring endophytes for alternative sources of bioactive compounds is important (Alvin et al., 2014). Therefore, medicinal plants are a valuable source for bioprospecting endophytes. Traditional ethnobotanical knowledge may assist in narrowing down the plants as targets for investigating the production of novel antimicrobial compounds. However, in certain cases plant availability for commercial production can be a limiting factor. Many bioactive compounds in medicinal plants are produced by their endophytes, hence it is more appropriate to explore the endophytes associated with those medicinal plants (Alvin et al., 2014).

South Florida also plays a crucial part in the ethnobotanical sector of US history. Tropical and sub-tropical medicinal plants that are present in South Florida provided an important source of remedy for native Americans and early settlers in Florida (Beare, 2019). Different varieties of medicinal plants have adapted to grow in this climatic condition. In addition to that, native people who live in South Florida such as Seminole and Mikasuki tribe still use traditional herbal passed down by their ancestors. Scientists have started researching medicinal plants for therapeutic purposes and they have been successful in finding some important chemicals from South Florida native medicinal plants. These include chemicals from saw palmetto fruits to reduce swelling associated with prostate cancer (Sarma et al., 2022).

Although the potential to discover new antimicrobial compounds still exists in South Florida, the rapid loss of natural habitats and biodiversity of medicinal plants threatening the chances of discovering novel antimicrobial products from these plants. So, immediate screening of valuable medicinal plants for their valuable compounds from endophytes is the immediate need for fighting against different types of pathogens and would be the important reason to conserve these valuable plant resources in the region.

Petiveria alliacea which is also known by its common name Guinea hen weed belongs to the family Phytolaccaceace which is the most primitive family in the order of Caryophyllales (Cronquist, 1988). This family has 17 genera and 120 tropical and sub-tropical species that are found across South and north America. Herbs, shrubs, and, on rare occasions, trees with little flowers and alternating leaves are included (Duarte & Fitoterapia, 2005; Joly, 1979).

Figure 2.3: Pictures of four medicinal plants used for sampling. Adult plant of Petiveria alliacea herb with visible seed (a.), Adult plant of Agave americana (b.), Adult plant of Annona glabra with visible fruit (c.) and adult plant of Conocarpus erectus with visible flower(d.)

Research conducted on plant parts extract has demonstrated important information about its bioactive potential. The *Petiveria alliacea* leave contains important chemicals such as antifungal, antiviral, anti-inflammatory compounds (Arogbodo et al., 2021). It is used as an antirheumatic, soothing agent, for restorative purposes (Marini et al., 1993; Williams et al., 1997). The endophytic fungal communities of *Petiveria alliacea* and their bioactive potential have not yet been examined.

Similarly, *Agave americana* is a flowering plant commonly known as the century plant, Maguey, American aloe. It is native to Mexico, the southern part of the US, and has

been growing globally as an ornamental plant (Irish & Irish, 2000). A previous study conducted on the leaf extract of the plant showed antioxidant properties which hints at the possibility of its anticancer property (Ahumada-Santos et al., 2013). Moreover, similar research conducted using an extract made from leaf stem, seed, root has shown the promising effect on drug-resistant clinical isolates, antibacterial effects, antifungal activities, sources of phytonutrients, anticancer ingredients, bioremediation activities (Alipanah et al., 2021; Enweani et al., 2021; Gallegos & Katerine, 2021; Ieven et al., 1979; Johanna et al., 2021; Maurya et al., 2021). All of this research has focused on the extract made from plant parts. None of them have focused on the endophytic microbiome's contribution to these effects. Similarly, *Conocarpus erectus* is a shrub commonly known as Buttonwood or Button mangrove. It is commonly found in the shoreline of the tropical and subtropical region of the world (Beare, 2019). Recent studies have demonstrated that mangroves possess a different type of antibacterial, antifungal, antimalarial activities (That et al., 2019).

The fourth medicinal plant Pond apple (*Annona glabra*) is a tropical fruit plant commonly known as Alligator apple, Swamp apple, Corkwood, Bob wood, and Monkey apple. It is native to Florida, the Caribbean, Central America, South America, and West Africa (USDA, 2009). Most of the research conducted on *Annona glabra* has focused on plant part extract. They have demonstrated good antimicrobial, anticancer, antioxidant, pesticidal, antimalarial, anthelminthic activities from leaf, stem, root, and fruit extract tested with different pathogens (Abdulsalami et al., 2013; Biba et al., 2014; Liu et al., 1999; Sivagnanam et al., 2016; Stanley et al., 2016). Recent research has demonstrated that

endophytic fungi isolated from this plant possess antioxidant agents, antibacterial agents, anti-inflammatory agents, antiseptic properties (Dissanayake et al., 2022; Eze et al., 2019).

The objective of the current study was to investigate endophytic fungal microbiomes from native medicinal plants of South Florida for a potential source of novel bioactive compounds to address the current global problem of antibiotic resistance.

2.2 Methodology

2.2.1 Collection of host medicinal plant

The healthy plant samples of four medicinal plants were collected from Possum trot Tropical Fruit Nursery (25°32′07" N; 80°28′ 37" W) located in Miami's Redland Agricultural district and Fruit and Spice Park (25°32′6" N; 80°29′ 31" W) located at Homestead, Florida. Guinea hen weed (*Petiveria alliacea*) plants were collected from Possum trot farm whereas Century plant (*Agave americana*), Pond apple (*Annona glabra*), Buttonwood mangrove (*Conocarpus erectus*) plants were collected from Fruit and Spice Park. Plant species were identified by Jim Stribling, director and plant specialist from Fruit and Spice Park and the specimen *Petiveria alliacea* were identified by Robert L. Burnum from Possum trot farm who is an expert in native medicinal plants in South Florida. Samples of fresh healthy leaves, stems, and roots of four medicinal plants were collected in separate zipper lock bags, labelled, stored in a cooler on ice, transported to the laboratory and stored at 4ºC until processing as described previously by Tan et al., (2018). Samples were processed within 24 hours of collection.

2.2.2 Isolation of Endophytic Fungi

Isolation and cultivation of endophytic fungi were carried out from the leaf, stem, and root of the sampled plants. Surface sterilization of the plant's parts was performed by modified protocol from Tan et al., (2018). For surface sterilization, the segments were soaked in 70% ethanol for 30 sec, then sodium hypochlorite (2.5% available chlorine) for 5 min, followed by three successive rinses with sterile distilled water and blotted dry using sterile filter paper. Sterile segments were immediately aseptically cut into smaller fragments (0.5cm X 0.5cm) and individual sample fragments were placed horizontally on potato dextrose agar (PDA) supplemented with chloramphenicol (200 µg/ml) and streptomycin sulfate (100 µg/ml). Nine stem/leaf/root fragments per plant sample were seeded onto agar plates. The effectiveness of the surface sterilization was confirmed by the absence of microbial growth on the triplicate PDA plates inoculated with 100 µl aliquots of the final rinse water and on the triplicate, PDA plates with surface imprint of surface sterilized sample segment. Plates were incubated at room temperature (28^{o}C) for more than a week. Inoculated plates were observed every day for fungal growth from plant tissue. Fungal hyphal tips from the growth were transferred onto the fresh PDA and sub-culturing of fungal isolate was continued until a pure culture was obtained. Observations on the morphological characteristics (colony color, surface, margin, pattern etc.,) of the fungal isolates were recorded. Cultures of individual endophytic fungal isolates were stored in 2.0 ml cryovials containing 15% glycerol in PDB medium at −80°C.

2.2.3 Crude extract preparation for secondary metabolites

Individual endophytic fungal isolates extracted from four medicinal plants were grown on PDA medium for 7 days at 28°C. After that, three discs (5 mm) of each isolate were picked and inoculated individually into a 250 mL Erlenmeyer flask containing 80 ml of potato dextrose broth (PDB) and incubated at 28°C for 60 days in an orbital shaker set at 125 rpm. Endophyte extract preparation was carried out following modified protocol from Pansanit & Pripdeevech, (2018) and Sharma et al., (2016). The fungal broth was centrifuged for 5 minutes at 4000g to obtain cell-free supernatant (CFS). The supernatant was decanted into a separate vial, the CFS and the pellet were extracted using 50 ml and 30 ml of ethyl acetate respectively at room temperature for 3 days. The resulting decanted organic phase from CFS and pellet were combined and filtered and concentrated by using a vacuum rotary evaporator (Buchi R-300) at reduced pressure at 45°C, resulting in the 10ml of fungal crude extract of endophyte. Later the solvent from crude extract was further evaporated using a vacuumed rotary evaporator (Buchi R-300) at 45 °C to yield the crude metabolite. The dry solid was dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 10 mg/ml and stored at 4°C. The crude extract and DMSO extract were used for initial and final screening of antimicrobial activity respectively.

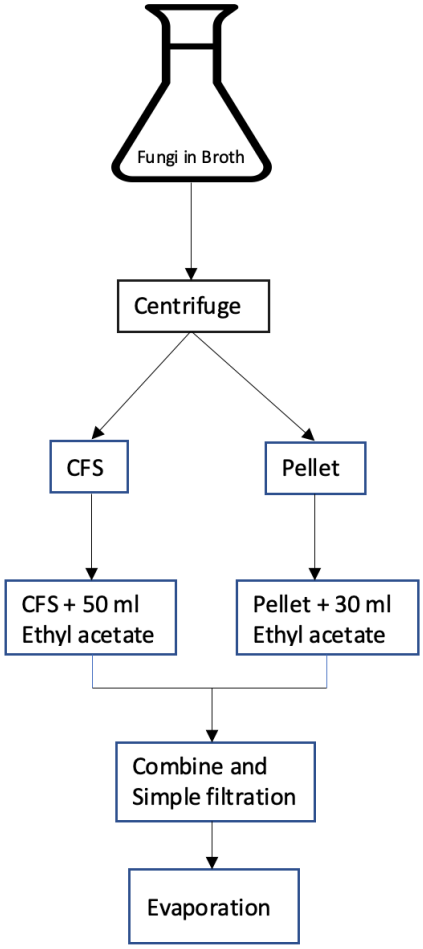

Figure 2.4: Flowchart of the process of fungal crude extract preparation.

2.2.4 Testing of antimicrobial and antifungal activity

The endophytic fungal crude extracts were screened for their antimicrobial activity using the agar diffusion method against Gram-negative (*Escherichia coli* ATTC 25902), Gram-positive (*Staphylococcus aureus* ATTC 14775, *Bacillus subtilis* NRRL 5109), unicellular fungus (*Candida albicans* ATTC 10231), and multicellular filamentous fungus (*Aspergillus fumigatus* NRRL 5109). Tested bacteria and fungi were seeded on Muller-

Hinton agar (MHA) and Potato Dextrose Agar (PDA) plates respectively. On each plate, three equally spaced wells were cut into the surface of agar using a sterile cork-borer (6mm) and 100, 150, and 200 µl of endophytic fungal crude extract or 10mg/ml DMSO were then dispensed into three separate wells then incubated at 28°C (Sharma et al., 2016). Antimicrobial activity was assessed by measuring the inhibition diameter (mm) zones at 24 and 48 hours. Ampicillin sodium 100µg/ml (positive control 1) and Fluconazole 30µg/ml (positive control 2) were employed as positive controls, while 10% DMSO was used as a negative control. The experiment was performed in triplicates.

2.2.5 Sequencing of ITS region of endophytic fungal isolates

The sequence of the ITS region of isolates that showed positive antimicrobial activity were identified by sending the isolates to Azenta life science for their custom service. Briefly, the colony samples were lysed using crude NaOH lysis technique to be directly used in PCR. To amplify ribosomal internal transcribed spacers (ITS), the primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) were employed in a Polymerase chain reaction (PCR) assay (White et al., 1990). Amplified samples were spot checked using gel electrophoresis to check for robust amplification along with a negative control to check for contamination. Following amplification, enzymatic cleanup was performed according to Azenta Life Sciences SOP using Exonuclease I – Shrimp Alkaline Phosphatase (ExoSAP), and dye-terminator sequencing was performed by Azenta Life Sciences Inc. (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. Sequencing-specific primers were used to generate bidirectional reads. The reactions were then run on Applied Biosystem's 3730xl

DNA Analyzer. Thus, obtained results of isolates from Azenta were used to compare the sequences of ITS region of fungal species already in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and accession numbers of the best matched database sequences were noted for each isolate.

Phylogenetic analysis was performed for species in the data base that have the closest identities to the isolates showing strong inhibition (>20mm) based on sequence data from ITS gene using Mega software version 11 and Neighbor-joining phylogenetic tree. The bootstrap value was chosen to 100 for percentage of bootstrap replications supporting the branch.

2.2.6 Statistical analyses

The Shannon-Weiner Index (H') was used to calculate the fungal diversity of endophytic fungi using the formula below:

$H' = -\Sigma(Pi \times \ln Pi)$

Where Pi was calculated as $Pi = \frac{ni}{N}$ and n*i* represents numbers of the individual segment from which fungal endophytic isolates were isolated and N is the total number of segments incubated (Husna et al., 2015; Y. Sun et al., 2011).

Similarly, the Colonization rates (CR%) of the fungal isolates from *Petiveria alliacea* were calculated as follows CR %= Nsc/Nss ×100 where Nsc represents the number of segments infected by fungal isolates and Nss represents the total numbers of plant segment investigated. The isolation ratio (IR%) of the fungal strains were calculated as follows: IR% =Ni/Nt ×100 where Ni is the number of segments from which fungal isolates were isolated and Nt is the total number of plant segment incubated (Hata and Futai, 1995).

$$\text{CR \%} = \frac{No.\ of\ colonized\ segment}{No.\ of\ segment\ studied} \text{ X100}$$

$$\text{IR \%} = \frac{No.\ of\ segment\ from\ which\ fungi\ isolated}{No.\ of\ segment\ incubated} \text{X100}$$

The Diversity index were analyzed by One-Way ANOVA. SPSS version 20.0 was used to perform all statistical analyses (SPSS Inc., Chicago, IL, USA)

2.3 Results and Discussion

2.3.1 Collection of sample and pure culture isolation

The endophytic fungi pose unique and diverse antimicrobial activity (Pelaez, 2004). The biodiversity of the fungi remains unclear, but it is estimated that the total species number can be up to 5 million (Ventola, 2015). Hence, it can be stated that fungi are one of the major resources of novel antimicrobial discovery.

In this study, endophytic fungi associated with four native medicinal plants Guinea hen weed (*Petiveria alliacea*), Century plant (*Agave americana*), Pond apple (*Annona glabra*), Buttonwood mangrove (*Conocarpus erectus*) were evaluated to investigate the production of bioactive compounds. The identification of *Petiveria alliacea* medicinal plant was performed by Robert L. Burnum from Possum trot farm who is an expert in native medicinal plants in South Florida whereas James Stribling of Fruit and Spice Park, a specialist in natural medicinal plants in South Florida, identified the *Agave americana, Annona glabra* and *Conocarpus erectus.*

Table 2.1: Endophytic fungal isolates from *Petiveria alliacea*.

Tissue	Segment studied	Colonized segments	Total Isolates	Endophytic isolates	CR†%	IR‡%	Shanon_H'¥
Root	99	83	86	11	83	86.5	2.366
Stem	99	95	97	9	96	98	2.189
Leaf	99	94	96	10	95	97	2.282

†CR= Colonization rate of fungal isolates

‡IR= Isolation rate of fungal isolates

¥Shanon_*H'*= Shannon- Weiner Index (H')

Table 2.2: Endophytic fungal isolates from *Agave americana*.

Tissue	Segment studied	Colonized segments	Total Isolates	Endophytic isolates	CR%	IR%	Shanon_H
Root	99	82	97	15	0.828	0.979	2.677
Leaf	99	90	104	17	0.909	1.05	2.792

Abbreviations are same as Table 2.1

Table 2.3: Endophytic fungal isolates from *Conocarpus erectus*.

Tissue	Segment studied	Colonized segments	Total Isolates	Endophytic isolates	CR%	IR%	Shanon_H
Root	90	41	45	17	45.55	50	2.79
Stem	99	57	62	17	57.57	62.62	2.78
Leaf	90	39	46	22	43.33	51.11	3.02

Table 2.4: Endophytic fungal isolates from *Annona glabra*.

Tissue	Segment studied	Colonized segments	Total Isolates	Endophytic isolates	CR%	IR%	Shanon_H
Root	99	89	110	20	89.89	95.95	2.962
Stem	99	99	106	20	100	107	2.90
Leaf	99	99	103	17	100	104	2.81

From the 289 sampled plant parts (representing a total of 99 roots, 99 stem, and 99 leaves) from *Petiveria alliacea,* a total of 279 isolates counted and are categorized as 30 morphotypes based on their morphological appearances as listed in the Figure 2.5. The 11 endophytic fungi were morphologically different form each other isolated from root

sample, 9 were from the stem and 10 isolates from leaf sample as described by (Y. Sun et al., 2011). Some culturable morphotypes that showed a good zone of inhibition were identified using ITS rDNA sequence analysis.

The colonization ratios were 89.89,100,100 respectively from the root, stem, and leaf whereas is isolation ratios were 95.95, 197, and 104 respectively from root, stem, and leaf. Shannon-Weiner diversity index was found higher in root followed by stem and leaf samples. Similar findings were reported from the research conducted by Tan et al., (2018).

Figure 2.5: Pure culture of endophytic fungi from four medicinal plants isolated on PDA medium. Sample abbreviations are same as in Table 2.5

Similarly, 17 pure cultures from leaf and 15 morphologically different fungal isolates from root were isolated from 198 total samples processed from the *Agave*

americana plant (Table 2.2). Shannon-Weiner diversity index was found higher in leaf compared to root for this plant as leaf contains more nutrition to support endophytic growth.

In addition to that, 297 sample plant parts were analyzed for *Conocarpus erectus*, and 17, 17, 22 morphologically different isolates were found from root stem and leaf respectively. Shannon-Weiner diversity index was found higher in leaf followed by root and stem for this medicinal plant (Table 2.3). Furthermore, the Shannon-Weiner diversity index was higher in the root sample followed by stem and root for *Annona glabra.* Similar outcomes were reported from the research conducted by Sadeghi et al., (2019) and Tan et al., (2018).

2.3.2 Production and extraction of secondary metabolites

The pure cultures of fungal isolates were used for producing crude extracts. The crude extracts were further concentrated using Buchi Rotavapor (R300) and were extracted with two solvents: ethyl acetate and DMSO.

Figure 2.6: Fermentation of fungal endophytes in broth medium during extract preparation process.

2.3.3 Antimicrobial activity of crude fungal extract using Agar well diffusion method

Most fungal crude extracts have shown promising results with human bacterial strains by showing maximum bioactivity. The antimicrobial screening of the fungal extracts was performed using agar well diffusion methods against gram-positive and gram-negative bacteria and fungal strains.

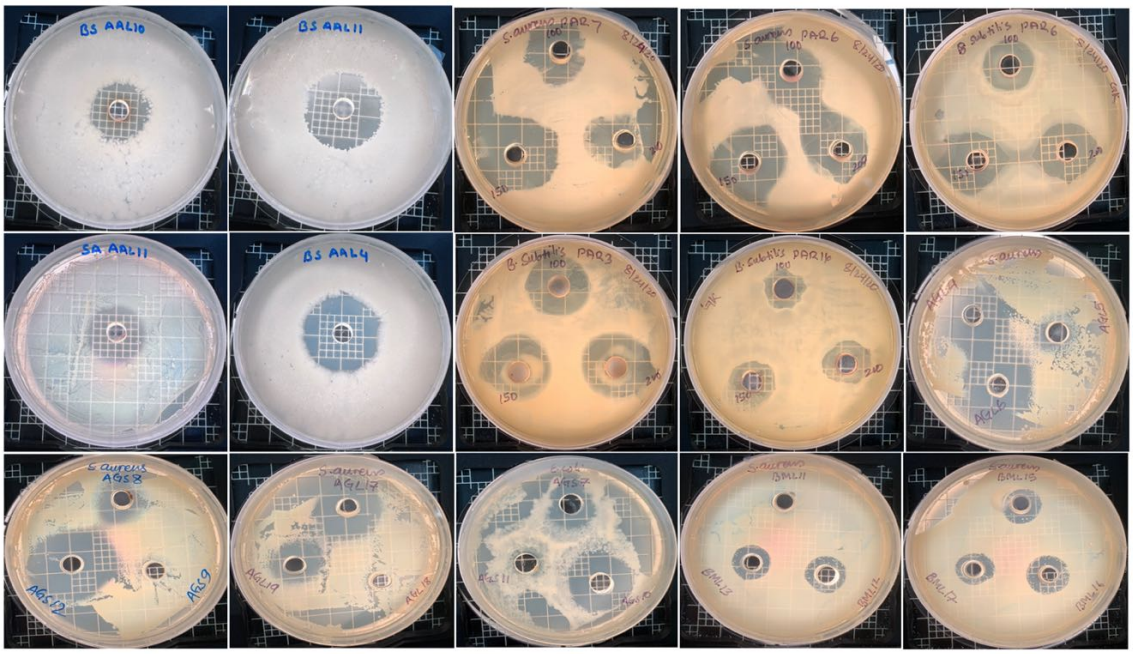

Figure 2.7: Antimicrobial activity of fungal crude extract of endophytic fungi with some bacterial strains.

Table 2.5: Antibacterial activities of the crude extracts of endophytic fungi isolated from four medicinal plants against selected bacterial pathogens (the numbers are the diameters of the zones of inhibition in mm; mean ± SD; n= 3).

Endophytes	*E. coli*			*S. aureus*			*B. subtilis*		
µl	100	150	200	100	150	200	100	150	200
PAL 04	-	-	-	-	-	-	18.3 ± 0.5	22.2 ± 0.3	24.0 ± 0.1
PAL 05	-	-	-	-	-	-	22.2 ± 0.3	23.1 ± 0.4	25.8 ± 0.1
PAL 23	22.3 ± 05	24.5 ± 0.2	27.0 ± 0.1	20.1 ± 0.1	23.3 ± 0.2	25.7 ± 0.1	-	-	-
PAR 3	12.1 ± 0.3	14.2 ± 0.1	18.1 ± .0.3	20.2 ± 0.2	22.0 ± 0.3	23.9 ± 0.2	18.2 ± 0.3	20.1 ± 0.1	22.1 ± 0.2
PAR 6	-	-	-	16.2 ± 0.2	18.1 ± 0.3	20.0 ± 0.2	16.1 ± 0.2	19.3 ± 0.2	22.2 ± 0.1
PAL 13	-	-	-	6.1 ± 0.2	9.0 ± 0.2	10.0 ± 0.2	9.4 ± 0.1	10.2 ± 0.1	11.3 ± 0.2
PAR 16	12.0 ± 0.3	14.0 ± 0.1	16.2 ± 0.3	20.2 ± 0.1	22.2 ± 0.3	24.1 ± 0.2	13.3 ± 0.3	16.1 ± 0.1	18.0 ± 0.2
PAS 04	16.3 ± 0.1	18.2 ± 0.3	20.1 ± 0.2	14.1 ± 0.3	16.1 ± 0.1	18.2 ± 0.1	17.8 ± 0.1	20.1 ± 0.2	22.2 ± 0.3
PAS 13	18.1 ± 0.3	20.2 ± 0.3	22.0 ± 0.3	16.1 ± 0.3	17.2 ± 0.3	17.5 ± 0.5	16.2 ± 0.3	18.2 ± 0.2	20.0 ± 0.1
PAS 14	-	-	-	24.1 ± 0.3	25.9 ± 0.4	27.8 ± 0.5	2.0 ± 0.1	3.9 ± 0.1	5.8 ± 0.3
PAS 26	-	-	-	23.7 ± 0.5	26.0 ± 0.3	27.5 ± 0.4	14.2 ± 0.3	16.1 ± 0.3	17.5 ± 0.3
AAS1	26 ± 0.2	28 ± 0.4	30 ± 0.1	20 ± 0.2	22 ± 0.3	24 ± 0.1	12 ± 0.4	14 ± 0.3	16 ± 0.1
AAL4	26 ± 0.1	28 ± 0.2	30 ± 0.2	17 ± 0.3	20 ± 0.0	23 ± 0.3	18 ± 0.1	20 ± 0.1	22 ± 0.2
AAL10	26 ± 0.0	28 ± 0.2	30 ± 0.1	28 ± 0.1	30 ± 0.1	32 ± 0.2	12 ± 0.2	14 ± 0.4	16 ± 0.3
AAL11	12 ± 0.2	14 ± 0.3	16 ± 0.2	18 ± 0.1	20 ± 0.1	22 ± 0.0	18 ± 0.1	22 ± 0.1	26 ± 0.1
AAL14	32 ± 0.1	34 ± 0.3	36 ± 0.0	21 ± 0.3	23 ± 0.1	25 ± 0.2	16 ± 0.2	18 ± 0.2	20 ± 0.3
AGS 8	20 ± 0.1	22 ± 0.3	23 ± 0.5	11 ± 0.3	12 ± 0.2	13 ± 0.2	11 ± 0.3	12 ± 0.0	13 ± 0.3
AGS12	24 ± 0.3	26 ± 0.4	27 ± 0.4	21 ± 0.6	22 ± 0.3	24 ± 0.1	17 ± 0.3	18 ± 0.4	20 ± 0.4
AGL4	23 ± 0.4	25 ± 0.1	26 ± 0.3	19 ± 0.2	20 ± 0.3	22 ± 0.1	17 ± 0.3	18 ± 0.2	20 ± 0.1
AGL10	5 ± 0.1	6 ± 0.3	7 ± 0.3	5 ± 0.1	6 ± 0.2	7 ± 0.1	-	-	-
AGL11	18 ± 0.3	20 ± 0.1	21 ± 0.4	19 ± 0.1	20 ± 0.1	22 ± 0.3	10 ± 0.4	12 ± 0.1	14 ± 0.0
AGL20	25 ± 0.4	27 ± 0.0	28 ± 0.0	17 ± 0.3	18 ± 0.0	19 ± 0.1	18 ± 0.4	20 ± 0.3	21 ± 0.1
AGR1	6 ± 0.1	7 ± 0.1	8 ± 0.2	10 ± 0.3	12 ± 0.1	13 ± 0.4	6 ± 0.1	7 ± 0.1	8 ± 0.0
AGR5	8 ± 0.3	10 ± 0.1	12 ± 0.0	9 ± 0.3	10 ± 0.0	12 ± 0.3	-	-	-
BML2	9 ± 0.3	10 ± 0.4	12 ± 0.5	8 ± 0.3	10 ± 0.3	11 ± 0.3	-	-	-
BML12	10 ± 0.0	12 ± 0.1	14 ± 0.1	9 ± 0.3	10 ± 0.0	12 ± 0.0	-	-	-
BML15	9 ± 0.3	10 ± 0.3	11± 0.0	9 ± 0.3	10 ± 0.0	10 ± 0.3	-	-	-
BMR5	11 ± 0.3	12 ± 0.0	14 ± 0.4	-	-	-	-	-	-
BMR11	12 ± 0.0	14 ± 0.3	16 ± 0.3	10 ± 0.3	11 ± 0.0	12 ± 0.1	-	-	-
BMS11	14 ± 0.3	16 ± 0.3	17 ± 0.5	11 ± 0.3	12 ± 0.2	14 ± 0.1	4 ± 0.3	6 ± 0.4	7 ± 0.5
Positive control 1	15.0 ± 0.1	16 ± 0.2	16.9 ± 0.2	16.0 ± 00	16.3 ± 0.2	16.9 ± 0.2	20.0 ± 0.1	20.1 ± 0.2	20.5 ± 0.2
Positive control 2	-	-	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-	-	-

Some abbreviations from the table are as follow:

PAL= *Pateveria alliacea* leaf sample

PAS= *Pativeria alliacea* stem sample

PAR= *Pateveria alliacea* root sample

AAL=*Agave americana* leaf sample

AAS= *Agave americana* stem sample

AGS= *Annona glabra* stem sample

AGL= *Annona glabra* leaf sample

AGR= *Annona glabra* root sample

BML=Buttonwood mangrove leaf sample

BMS= Buttonwood mangrove stem sample

BMR= Buttonwood mangrove root sample

¶µl= Microliter of the sample used

Numbers are mean ±standard error.

Table 2.6: Antifungal activities of the crude extracts of endophytic fungi isolated from four medicinal plants against selected fungal pathogens (the numbers are the diameters of the zones of inhibition in mm; mean ± SD; n= 3) Positive control 1: Ampicillin sodium (100 µg/ml), Positive control 2: fluconazole (30 µg/ml); Negative control: 10% DMSO.

Endophytes	*C. albicans*			*A. fumigatus*		
µl¶	100	150	200	100	150	200
PAL 04	-	-	-	-	-	-
PAL 05	-	-	-	1.0 ± 0.2	2.0 ± 0.2	4.1 ± 0.2
PAL 23	-	-	-	-	-	-
PAR 3	-	-	-	-	-	-
PAR 6	6.1 ± 0.2	8.1 ± 0.1	9.8 ± 0.3	-	-	-
PAL 13	-	-	-	-	-	-
PAR 16	-	-	-	-	-	-
PAS 04	-	-	-	-	-	-
PAS 13	-	-	-	-	-	-
PAS 14	-	-	-	-	-	-
PAS 26	-	-	-	-	-	-
AAS1	-	-	-	-	-	-
AAL4	3 ± 0.3	5 ± 0.1	8 ± 0.2	-	-	-
AAL10	2 ± 0.1	5 ± 0.2	8 ± 0.3	-	-	-
AAL11	-	-	-	-	-	-
AAL14	-	-	-	-	-	-
AGS 8	-	-	-	-	-	-
AGS12	-	-	-	-	-	-
AGL4	-	-	-	-	-	-
AGL10	-	-	-	-	-	-
AGL11	-	-	-	-	-	-
AGL20	-	-	-	-	-	-
AGR1	-	-	-	-	-	-
AGR5	-	-	-	-	-	-
BML2	-	-	-	-	-	-
BML12	-	-	-	-	-	-
BML15	-	-	-	-	-	-
BMR5	-	-	-	-	-	-
BMR11	-	-	-	-	-	-
BMS11	-	-	-	-	-	-
Positive control 1	-	-	-	-	-	-
Positive control 2	24.1 ± 0.3	24.1 ± 0.3	24.6 ± 0.3	17.1 ± 0.3	17.4 ± 0.1	17.8 ± 0.3
Negative control	-	-	-	-	-	-

Abbreviations are same as Table 2.5

The antimicrobial activities of crude extracts from 30 endophytic fungal isolates from *Petiveria alliacea* were evaluated against test organisms. Crude extracts from majority (83%) of the isolates showed positive antimicrobial activity in the initial screening Table 2.5. Out of the fungal endophyte crude extracts that showed antimicrobial activity, 65% of the leaf endophyte extracts, 88% of the stem endophyte extracts and 71% of the root endophyte extracts showed activity against at least one of the bacterial strains tested. Only 10% of the fungal crude extract prepared from leaf endophytes demonstrated activity against at least one of the fungal strains tested. None of the crude fungal extract prepared from stem and root sample showed activity against fungal strains tested. The largest zone of inhibition was observed against *S. aureus* (27.8 ± 0.5 mm) inhibited by PAS-14 isolate, *E. coli* (27 ± 0.1 mm) inhibited by PAL-23 isolate and *B. subtilis* (25.8 ± 0.1 mm) inhibited by PAL-05 isolate crude extracts. Crude extracts from two endophytes PAL-05 from leaf and PAR-06 from root showed broad spectrum activity against both bacteria and fungi. Isolate PAL-05 showed maximum inhibition zone of 25.8 ± 0.1 mm and 4.1 ± 0.2 mm against *B. subtilis* and *A. fumigatus* respectively. Similarly, isolate PAR-06 (pure colony picture in Figure 2.5) showed a maximum inhibition zone of 20.0 ± 0.2 mm, 22.2 ± 0.1 mm, and 9.8 ± 0.3 mm against *S. aureus*, *B. subtilis* and *C. albicans* respectively. Three of the endophytes isolates PAR-16, PAS-04, PAS-13 (pure colony picture in Figure 2.5) demonstrated antimicrobial activity against both gram-negative and gram-positive bacteria but did not show any activity against the fungal strains tested. None of the isolates examined inhibited the growth of all test organisms.

Moreover, 80% of fungal isolates from the stem and 82% of fungal isolates from the leaf of *Agave americana* showed some inhibition activity against at least one of the organisms tested. The ethyl acetate extract of 12 fungal isolate demonstrated bioactivity against the tested all three bacterial strains. Endophytic fungal strains AAS1, AAS2, AAS5, AAL4, AAL10, AAL11, AAL14, AAL15, AAL17 are the example of some fungal endophytes that have shown broad spectrum antimicrobial activity against gram positive and gram-negative bacterial strains tested (Figure 2.5). Similarly, endophytic fungal isolates such as AAL10, AAL11, AAL15, AAL17 have demonstrated activity against bacterial strain as well as fungal strain tested. Similarly, 42% of the total endophytic fungal isolates demonstrated some antimicrobial activity against bacterial strain from *Annona glabra* and 43% of the fungal isolates from *Conocarpus erectus* showed bioactivity against bacterial strain tested (Table 2.5).

Table 2.7: Antimicrobial activity of the DMSO extracts of endophytic fungi against selected microbial pathogens (the numbers are the diameters of the zones of inhibition in mm; mean ± SD; n= 3) Positive control 1: Ampicillin sodium (100 µg/ml); Positive control 2: fluconazole (30 µg/ml); Negative control: 10% DMSO.

Isolates	*E. coli*	*S. aureus*	*B. subtilis*	*C. albicans*	*A. fumigatous*
PAL4	-	-	5.1 ± 0.2	-	-
PAL5	-	-	6.0 ± 0.1	-	-
PAL23	4.2 ± 0.1	2.2 ± 0.2	-	-	-
PAR8	8.1 ± 0.2	11 ± 0.2	8.1 ± 0.1	-	-
PAR14	8.2 ± 0.2	14.2 ± 0.2	-	-	-
PAR16	7.2 ± 0.2	13.2 ± 0.1	-	-	-
PAS4	-	10.1 ± 0.2	-	-	-
PAS7	8 ± 0.2	11.3 ± 0.3	13.0 ± 0.1	-	-
PAS21	-	11.1 ± 0.1	-	-	-
AAS1	3.02 ± 0.1	-	-	-	-
AAS5	4.01 ± 0.2	3.4 ± 0.2	2.8 ± 0.1	-	-
AAL4	12.04 ± 0.3	11 ± 0.2	11 ± 0.3	-	-
AAL10	11.2 ± 0.2	6.3 ± 0.1	4.2 ± 0.2	-	-
AAL11	5.2 ± 0.2	6.0 ± 0.3	14.1 ± 0.3	-	-
AAL14	13.2 ± 0.1	13.3 ± 0.2	7.2 ± 0.3	-	-
AAL16	8 ± 0.2	-	-	-	-

Following initial antimicrobial activity screening, the metabolites of endophytes in DMSO extract (10 mg/mL) of selected fungal crude extracts showing promising bioactive activities against test organisms were further evaluated (Table 2.7). The endophytic fungal isolates PAL-4 and PAL-5 showed inhibition zone of 5.1 ± 0.2 mm and 6.0 ± 0.1 mm against *B. subtilis* only. The extracts from isolates PAR-8 and PAS-7 in contrast demonstrated inhibitory activity against all three bacterial strains tested. The fungal endophytic isolate PAR-14 demonstrated largest zone of inhibition of 8.2 ± 0.2 mm against *E. coli* and 14.2 ± 0.2 mm against *S. aureus* compared to ampicillin control with an inhibition zone of 16 ± 0.2 mm and 16.3 ± 0.2 mm. Among the endophyte isolates tested against *B. subtilis* only DMSO extracts from isolate PAS-07 showed largest zone of inhibition of 13.0 ± 0.1 mm with ampicillin control showing 21.1 ± 0.2 mm zone of

inhibition. None of the isolates showed significant antimicrobial activity against fungal stains tested at the concentration of 10 mg/ml in DMSO.

Similarly, the quantification of activity of crude extract of *Agave americana* was also evaluated using 10 mg/ml of extract prepared in DMSO solvent. As the data shown in the Table 2.7, the 10 mg/ml DMSO fungal extract showed activity with bacterial pathogenic strain tested. The endophytic fungal isolates AAS1 and AAS5 isolated from root of Agave plant showed activity of 3.02 ± 0.1mm and 4.01 ± 0.2mm with *E. coli*. The fungal isolates AAS5 showed activity 3.4 ± 0.2mm and 2.8 ± 0.1mm with *S. aureus* and *B. subtilis.* Similarly, isolates AAL10, isolated from leaf demonstrated highest antimicrobial activity of 11.2 ± 0.2mm, 11 ± 0.2mm and 11 ± 0.3mm against *E. coli, S. aureus* and *B. subtilis* respectively. The isolates AAL11 and AAL14 showed broad spectrum activity against all three bacterium pathogens tested. None of these isolates showed significant antimicrobial activity with fungal strain tested at the concentration of 10 mg/ml in DMSO.

Bioactivity is defined as high inhibition of growth of one type of target microbial strain with little or no activity against others, is of particular interest in novel drug discovery. It indicates the presence of metabolites that have specific modes of action as opposed to highly toxic compounds that are often of little use as medication (Kaczorowski et al., 2011; Maharachchikumbura et al., 2011).

The medicinal herb *Petiveria alliacea* has received considerable attention in recent years and its extracts possess many metabolites which have the potential to kill or inhibit many types of disease-causing pathogen (Lateef et al., 2018; Musah et al., 2005). Endophytic isolates of *Petiveria alliacea* from South Florida is particularly interesting as

there is no such research conducted previously (Lateef et al., 2018). This is the reason; this medicinal plant was chosen for study.

Earlier studies of antimicrobial activity on fungal crude extract have been conducted and proven by many studies (Garcia et al., 2012; Idris et al., 2013). Some of the extracts were effective against all the bacterial strains and some fungal strains tested in this study. These results might be attributed that the extract may contain a high concentration of bioactive metabolites. The fungal extract that showed low antimicrobial activity in the bioassay might have active bioactive compounds but in low or very concentrations or might yield more active compounds it can be further purified (Idris et al., 2013). But the low level of bioactivity can be concluded as fungal isolate does not have any activity.

The fungal endophytes isolated from four medicinal plants were tested against human and animal pathogens such as *E. coli, S. aureus, B. subtilis, C. albicans* and *A. fumigatous* to test their production of antimicrobial compounds by using dual culture essay and isolates have shown positive interaction against the tested organism. That had hinted at the fact that those isolates might pose usual bioactive compounds. They were tested with agar well diffusion assay techniques. Zone of inhibition calculation was based on agar well diffusion techniques as it was more reliable than the dual culture bioassay technique.

Some extracts demonstrated different activities on tested organisms. The performance of the extract on strain can be affected by the structural differentiation of the cellular structure of the strain selected for testing as it might be effective in other microorganisms (Tortora et al., 2005). More trials with more isolates might be needed to conclude the poor performance of extract on some bacterial strains and fungal strain tested.

The extract of endophytic fungi also showed some bioactivity with fungal strain tested. The isolate PAL05 showed some inhibitory activity of 22.2 ± 0.3 mm with *B. subtilis* and 1.0 ± 0.2mm with *A. fumigatus* at 100µl of extract. It demonstrates that Isolate PAL05 possesses broad spectrum with bacterial and fungal strain tested. Similarly, the fungal isolates PAR06 showed activity against *S. aureus* (16.2 ± 0.2mm), B. subtilis (16.1 ± 0.2) mm and *C. albicans* (6.1±0.2mm). That has clearly illustrated that isolates PAR06 has broad-spectrum capabilities against pathogens. Similarly, three isolates PAR16, PAS04, PAS13 showed activities against gram-negative bacteria and gram-positive bacteria but no activity with fungal strain tested. None of these isolates showed activities against all gram-positive, gram-negative, and fungal strains tested. Similarly, isolates AAL4 was effective against all bacterial strains tested and with *Candida albicans* fungal strain as well. So, that has shown broad-spectrum activity against tested microbial strains.

Table 2.8: Sequencing results of *Petiveria alliacea* endophytic fungal isolates selected based on their zone of inhibition activity. Accession number of best matched database sequence is shown for each isolate.

Isolates	Closest relative NCBI data base	Tissue	ITS identity	Phylum, Class, Order	Fungal species with best matched ITS sequence	Accession number of best matched ITS sequence
PAL05	*Fusarium solani* isolate 18 FS	Leaf	99.62%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium solani*	KX929305.1
PAL23	*Fusarium oxysporum* isolate K_MNSO2_3_2	Leaf	92.68%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium oxysporum*	MN452004.1
PAS04	*Fusarium oxysporum* isolate N648	Stem	99.62%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium oxysporum*	MT032687.1
PAS10	*Fusarium oxysporum* strain WM 08.30	Stem	88.34%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium oxysporum*	KP068931.1
PAS13	*Fusarium solani* strain GFR21	Stem	100%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium solani*	MT447526.1
PAS26	*Fusarium proliferatum* isolate 286	Stem	78.85%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium proliferatum*	KC577193.1
PAR03	*Fusarium solani* isolate 182WS	Root	99.82%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium solani*	KJ207394.1
PAR06	*Fusarium oxysporum* clone SF_789	Root	100%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium oxysporum*	MT530065.1
PAR13	*Fusarium solani* isolate 182WS	Root	99.64%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium solani*	KJ207394.1
PAR16	*Fusarium oxysporum* clone SF_993	Root	100%	Ascomycota, Sordariomycetes, Hypocreales,	*Fusarium oxysporum*	MT530269.1

Table 2.9: Sequencing results of *Agave americana* endophytic fungal isolates selected based on their zone of inhibition activity. Accession number of best matched database sequence is shown for each isolate.

Isolates	Closest relative NCBI data base	Tissue	ITS identity	Phylum, Class, Order	Fungal species with best matched ITS sequence	Accession number of best matched ITS sequence
AAL4	*Colletotrichum siamense* isolate UPBT-BCS06	Leaf	100%	Ascomycota, Sordariomycetes, Glomerellales	*Colletotrichum siamense*	MK880376.1
AAL11	*Penicillium citrinum* isolate F23-02	Leaf	99.82%	Ascomucota, Eurotiomycetes, Eurotiales,	*Penicillium citrinum*	KX664347.1
AAL10	*Neofusicoccum batangarum* strain COUFAL0163	Leaf	100%	Ascomycota, Dothideomycetes, Botryosphaeriales	*Neofusicoccum batangarum*	MH251952
AAL14	*Colletotrichum siamense* isolate AXCD1	Leaf	99.82%	Ascomycota, Sordariomycetes, Glomerellales	*Colletotrichum siamense*	MK014832.1
AAL16	*Curvularia senegalensis* isolate 15-833	leaf	99.82%	Ascomycota, Dothideomycetes, Pleosporales	*Curvularia senegalensis*	MT476857.1
AAS1	*Pleosporales sp.* Strain TUCIM 6194	Stem	97.64%	Ascomycota, Dothideomycetes, Pleosporales	*Pleosporales sp.*	MT663379.1
AAS5	*Dothideomycetes sp.* Genotype 752 JMUR-2016	Stem	100%	Ascomycota, Dothideomycetes, Pleosporales	*Dothideomycetes sp.*	KX908956.1
AAS11	*Colletotrichum sp.* isolate RS_75	Stem	78%	Ascomycota, Sordariomycetes, Glomerellales	*Colletotrichum sp.*	MK332489.1

Table 2.10: Sequencing results of *Annona glabra* endophytic fungal isolates selected based on their zone of inhibition activity. Accession number of best matched database sequence is shown for each isolate.

Isolates	Closest relative NCBI data base	Tissue	ITS identity	Phylum, Class, Order	Fungal species with best matched ITS sequence	Accession number of best matched ITS sequence
AGR1	*Diaporthe tulliensis* strain Y. H. Yeh I1001	Root	100%	Ascomycota; Sordariomycetes; Diaporthales	*Diaporthe tulliensis*	MK336513.1
AGR5	*Diaporthaceae sp.* isolate DMW1048	Root	99.64%	Ascomycota; Sordariomycetes; Diaporthales	*Diaporthaceae sp*.	KU593525.1
AGS8	*Neofusicoccum batangarum* culture CPC:29624	Stem	100%	Ascomycota; Dothideomycetes; Botryosphaeriales	*Neofusicoccum batangarum*	MT587474.1
AGS9	*Diaporthe pseudomangifera* isolate 133	Stem	99.82%	Ascomycota; Sordariomycetes; Diaporthales	*Diaporthe pseudomangiferae*	MG576129.1
AGS12	*Fusarium solani* strain GFR43	Stem	99.64%	Ascomycota; Sordariomycetes; Hypocreales	*Fusarium solani*	MT447548.1
AGL4	*Colletotrichum sp.* isolate CMM2936	Leaf	100%	Ascomycota; Sordariomycetes; Glomerellales	*Colletotrichum sp.*	MF383467.1
AGL6	*Colletotrichum siam*ense isolate AXCD1	Leaf	100%	Ascomycota; Sordariomycetes; Glomerellales	*Colletotrichum siamense*	MK014832.1
AGL9	*Colletotrichum fructicola* strain HEV132A	Leaf	99.64%	Ascomycota; Sordariomycetes; Glomerellales	*Colletotrichum fructicola*	MT470570.1
AGL11	*Colletotrichum siamense* isolate UPBT_BCS05	Leaf	100%	Ascomycota; Sordariomycetes; Glomerellales	*Colletotrichum siamense*	MK880375.1

Table 2.11: Sequencing results of *Conocarpus erectus* endophytic fungal isolates selected based on their zone of inhibition activity. Accession number of best matched database sequence is shown for each isolate.

Isolates	Closest relative NCBI data base	Tissue	ITS identity	Phylum, Class, Order	Fungal species with best matched ITS sequence	Accession number of best matched ITS sequence
BML2	*Fusarium oxysporum* isolate N648_ITS1_F04_011.ab1	Leaf	99.89%	Ascomycota; Sordariomycetes; Hypocreales	*Fusarium oxysporum*	MT032687.1
BML12	*Fusarium circinatum* strain A47	Leaf	96.10%	Ascomycota; Sordariomycetes; Hypocreales	*Fusarium circinatum*	OK510252.1
BML13	*Fusarium oxysporum* isolate G220_ITS1_H02_001.ab1	Leaf	100%	Ascomycota; Sordariomycetes; Hypocreales	*Fusarium oxysporum*	MT032647.1
BML15	*Fusarium oxysporum* clone SF_967	Leaf	100%	Ascomycota; Sordariomycetes; Hypocreales	*Fusarium oxysporum*	MT530243.1
BML17	*Fusarium incarnatum* isolate SRRB-54	Leaf	100%	Ascomycota; Sordariomycetes; Hypocreales	*Fusarium incarnatum*	MT003065.1
BMR5	*Aspergillus n*iger isolate agarose gel electrophoresis	Root	100%	Ascomycota; Eurotiomycetes; Eurotiales;	*Aspergillus niger*	MK307680.1
BMR11	*Aspergillus tubingensis* isolate CNUML 003	Root	81.45%	Ascomycota; Eurotiomycetes; Eurotiales;	*Aspergillus tubingensis*	MN818622.1
BMS7	Unidentified	Root		Unknown	Unknown spp.	-

[§]ITS= Internal transcribe spacer
Sample abbreviations are same as Table 2.5

The endophytic fungus can possess unique and diverse antimicrobial activity (Pelaez, 2004). The diversity of fungus is unknown; the number of endophytic fungi is estimated to be approximately one million based on the 6:1 ratio of fungal–plant species (X. Sun & Guo, 2012). However, in tropical and subtropical regions the ratio could be up to five times higher (Gamboa et al., 2003). In this study, endophytic fungi isolated from medicinal plant *Petiveria alliacea* were evaluated to investigate the potential for producing novel bioactive compounds.

The medicinal herb *Petiveria alliacea* has gotten a lot of attention in recent years and its extracts possess many metabolites which have the potential to kill or inhibit many types of disease-causing pathogens (Garcia et al., 2012; Idris et al., 2013). Previous studies have shown that the benzyl-containing thiosulfinates from *P. alliacea* exhibited the broadest spectrum of antimicrobial activity against bacteria and fungi (Musah et al., 2005). The present study was initiated to understand the association of fungal endophytes with *Petiveria alliacea* in South Florida as there was no such research conducted previously.

Several studied have been conducted and established that endophytic fungal crude extracts have antibacterial property (Garcia et al., 2012; Idris et al., 2013). These results might be attributed that the extract may contain a high concentration of bioactive metabolites. The fungal extract that showed very low antimicrobial activity in the bioassay might have contained active bioactive metabolites in low or very low concentrations or might have yielded more active compounds if it was further purified (Garcia et al., 2012). Various factors might influence the qualitative and quantitative aspects of bioactive metabolite production by endophytic fungi under laboratory conditions. The spectrum and degree of inhibitory activity of endophytic fungal metabolites can also be affected by the type of solvent used (Elghaffar et al., 2022).

The fungal endophytes isolated from *Petiveria alliacea* were tested against *E. coli, S. aureus, B. subtilis, C. albicans and A. fumigatous* to test their production of antimicrobial compounds by using dual culture essay initially and isolates have shown positive interaction against the tested organism. That had suggested that those isolates might produce bioactive compounds. They were then tested with agar well diffusion assay

techniques. Zone of inhibition calculations was based on agar well diffusion techniques as it was more reliable than the dual culture bioassay technique.

Sequencing data from the endophytic fungi that has demonstrated promising results with the strain tested showed that they were the species of *Fusarium*. Endophytic strains of Fusarium are known for producing many different types of useful bioactive compounds. The antimicrobial drug PTOX (podophyllotoxin) was produced by the endophytes *F. oxysporum* isolated from *Juniperus recurve* (Vasundhara et al., 2016). Another natural product produced by endophytic fungi *Fusarium* is Taxol which is the first billion-dollar drug produced by *F. proliferatum* from Taxol plant (X. M. Tan et al., 2014; Xiong et al., 2013). Crude extracts of endophyte *F. equiseti* isolated from Mikania cordata showed significant broad spectrum of antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* (Jayatilake & Munasinghe, 2020). A polyketide fusaequisin A extracted from *F. equiseti*, endophyte of Ageratum conyzoides was found to be inhibitory to *S. aureus* and *P. aeruginosa* (Shiono et al., 2013). *Fusarium spp*. are also known to produce antimicrobial compounds such as PTOX and beauvericin and subglutinol A and B (Tan et al., 2018).

Additional anti-bacterial substance produced by endophytes *Fusarium spp*. identified from medicinal plants are 2-methyl butyraldehyde-substituted α-pyrone, beauvericin, and sub-glutinol A and B (Brady & Clardy, 2000; Lee et al., 1995; Y. Sun et al., 2011). Endophytic fungi such as *F. solani*, *F. oxysporium* and *F. proliferatum* have potential to produces different types of bioactive metabolites and is supported by studied

of other medicinal plants all around the world (Farhat et al., 2019; Gintig et al., 2013; Sogra Fathima & Balakrishnan, 2014; Tayung K et al., 2011; Wei et al., 2019).

Similar studies have also demonstrated that endophytic fungi isolated from medicinal plants have antibacterial, antifungal, anticancer, antioxidant properties and it might provide the opportunities to explore more endophytic fungi for discoveries in the field of antimicrobials (Cui et al., 2015; Strobel et al., 2004). However, more studies are needed to address the dynamical changes of endophytic communities (Cui et al., 2015) and unculturable fungal communities (Tejesvi et al., 2011).

Similarly, the extract of endophytic fungi from *Agave americana* also showed some bioactivity with fungal strain tested (Table 2.5). The isolate *Colletotrichum siamense* (AAL04) shows the inhibitory activity of 26 ± 0.1 mm with *E. coli*, 17 ± 0.3mm against *S. aureus*, 18 ± 0.3 mm against *B. subtilis*, and 3 ± 0.3 mm with *C. albicans* at 100µl of fungal extract tested. Similarly, the isolate *Neofusicoccum batangarum* (AAL10) shows the inhibitory activity of 26 ± 0.2 mm with *E. coli*, 28 ± 0.5mm against *S. aureus*, 12 ± 0.2mm against *B. subtilis*, and 2 ± 0.1 mm with *C. albicans* at 100µl of fungal extract tested. Similar result demonstrated by isolate AAL 15 and AAL17 inhibiting both bacterial strains and fungal strains. It demonstrates that isolate AAL04, AAL10, AAL15 and AAL17 possesses a broad spectrum with bacterial and fungal strain tested. Similarly, three isolates AAS1, AAS2, AAS5, AAL11, AAL14 showed activities against gram-negative bacteria and gram-positive bacteria but no activity with fungal strain tested. None of these isolates showed antimicrobial activities against all of the gram positive, gram negative and fungal strain tested.

The present data showed that *A. americana* leaf contains the highest diversity of fungal endophytes. It is found that Ascomycota being a ubiquitous phylum of fungi also found in the plant globally (Egbuta et al., 2016; Tan et al., 2018). Moreover, Sequenced date from this study revealed that Sordariomycetes, Eurotiomycetes and Dothideomycetes are the classes of endophytic fungi associated with *Agave americana*. Similar research conducted on medicinal plant *Chloranthus japonicus* has revealed that Sordariomycetes was the most prevalent class of endophytic fungi followed by Eurotiomycetes and Dothideomycetes (An et al., 2020). Hence, it can be concluded that medicinal plant *Agave americana* harbor high diversity of endophytic fungi.

This study also found that 47% of morphologically different fungal isolates were isolated from root whereas 53% from leaf sample studied. Isolates also have different genetic makeup in all tissues (form those we sequenced), It can be suggested that these endophytes have adapted the distinct tissue environment overtime resulting in specific tissue specificity among the fungal endophytes as indicated by previous studies from Indian medicinal plants (Kaul et al., 2013; Mishra et al., 2012).

The fungal crude extracts demonstrated antimicrobial activities against tested organisms. The performance of the extract on strain can be affected by the structural differentiation of the cellular structure of the strain selected for testing (Tortora GJ et al., 2005). This fact might explain the poor performance of extract on some bacterial strains and fungal strains tested.

As the sequencing data revealed different endophytic fungi contributed bioactive potential of *Agave americana.* Endophytic fungi *Colletotrichum siamense* isolated from *Piper nigrum* is known for many different types of useful bioactive compounds such as

antioxidant agent, antibacterial and antifungal agents (Munasinghe et al., 2017). Moreover, *Colletotrichum sp.* isolated from medicinal plant *Buxus sinica* has demonstrated antibacterial activities (Wang et al., 2016). Similarly, the endophytic fungi *Penicillium citrinum* isolated from *Azadirachta indica* is known to produce various bioactive compounds such as antioxidant, antibacterial compound against different pathogens (Kumari et al., 2021). Similarly, the endophytic fungi *Neofusicoccum batangarum* was reported from medicinal plants such as *Myrciaria floribunda, Alchornea castaneifolia,* and *Eugenia aff. Bimarginata* (Vaz et al., 2012). Recently published study has demonstrated that endophytic fungi *Neofusicoccum batangarum* produces various metabolic compounds such as botryoisocoumarin A, botryosphaerones, cyclobotryoxide and isosclerone (Salvatore et al., 2021). Endophytic fungi *Curvularia senegalensis* are known to produce various metabolites such a sclerotiorin, isochromophilone VI, pencolide and penicillic acid with good antimicrobial properties isolated from medicinal plants such as *Calotropis procera, Catharanthus roseus and Brazilian cerrado* (Chowdhury et al., 2018; Takahashi et al., 2008). Moreover, endophytic fungi *Pleosporales* sp. are reported to produced various antibacterial, antioxidant and cytotoxic compounds such as an abscisic acid, asterric acid from mangrove *Kandelia candel* (Wen et al., 2019). Various bioactive compounds such as cytotoxic, antibacterial, and anti-Aβ42 aggregation, polyketides, Azaphilones were reported to produce by endophytic fungi *Dothideomycetes sp*. isolated from medicinal plants *Tiliacora triandra, Trichilla elegans* (Hewage et al., 2014; Rhoden et al., 2012). Hence, it is clear that medicinal plant *Agave americana* is a reservoir of endophytic fungi with potential of novel bioactive compounds. Similar studies have also demonstrated that endophytic fungi isolated from medicinal plants have antibacterial, antifungal, anticancer,

antioxidant properties and it might provide the opportunities to explore more endophytic fungi for discoveries in the field of antimicrobials (Cui et al., 2015; Strobel et al., 2004).

Additionally, 57 endophytic fungal isolates from *Annona glabra* were chosen as morphologically different isolates. It is also found from Shannon-Weiner diversity index that root harbor high diversity of endophytic fungi followed by stem and leaf. Morphologically diverse isolates were chosen for further antimicrobial activity with the five bacterial and fungal strains. 42% of the fungal isolates demonstrated antimicrobial activity against bacterial strain from *Annona glabra* and no isolates showed activity against fungal strains tested. The fungal isolate AGL20 showed highest activity of 25 ± 0.4 mm against *E. coli*, 17 ± 0.3 mm against *S. aureus* and 18 ± 0.4 mm against *B. subtilis* at 100µl of aqueous fungal extract used.

Furthermore, the medicinal plant *Conocarpus erectus* also demonstrated the interesting result from this study. From the 279 sample parts studied, 56 morphologically distinct endophytic fungi were isolated and higher endophytic diversity was found in leaf followed by root and stem. 43% of the fungal isolates from *Conocarpus erectus* showed bioactivity against bacterial strain tested and no antimicrobial activity were detected with fungal strains tested. The endophytic fungi BMR11 identified as *Aspergillus tubingensis* demonstrated antimicrobial activity against *E. coli* and *S. aureus* bacterial strains tested. Moreover, endophytic fungi BMS11 which is not identified yet and showed activity against all three bacterial strains tested.

2.3.4 Fungal diversity and phylogenetic analysis

The present data showed that *P. alliacea* root contains the highest diversity of fungal endophytes. We found that 37% of morphologically different fungal isolates were isolated from root whereas 33% from leaf and 30% from stem sample studied. Isolates that have the strongest antimicrobial activities in three tissues also have similar genetic makeup (form those we sequenced), Previous research from Indian medicinal plants imply that these endophytes have evolved to the different tissue environment throughout time, resulting in particular tissue specificity among the fungal endophytes (Kaul et al., 2013; Mishra et al., 2012)

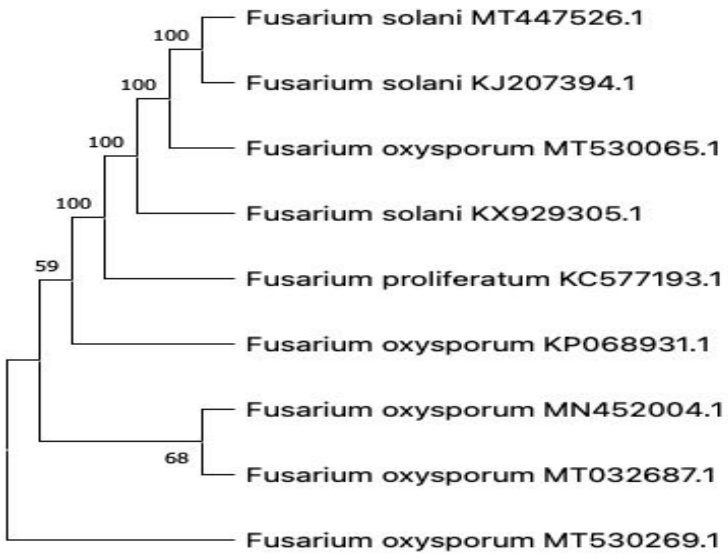

Figure 2.8: Phylogenetic (Neighbor-joining) tree based on sequence data from ITS gene of Petiveria alliacea. The value on each branch is the percentage of bootstrap replications supporting the branch.

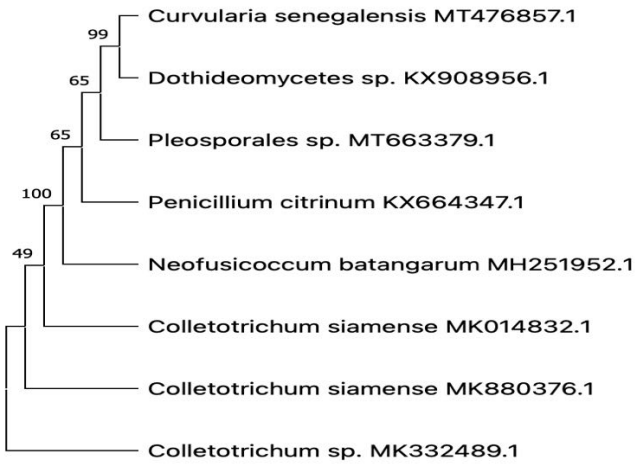

Figure 2.9: Phylogenetic (Neighbor-joining) tree based on sequence data from ITS gene of Agave americana.

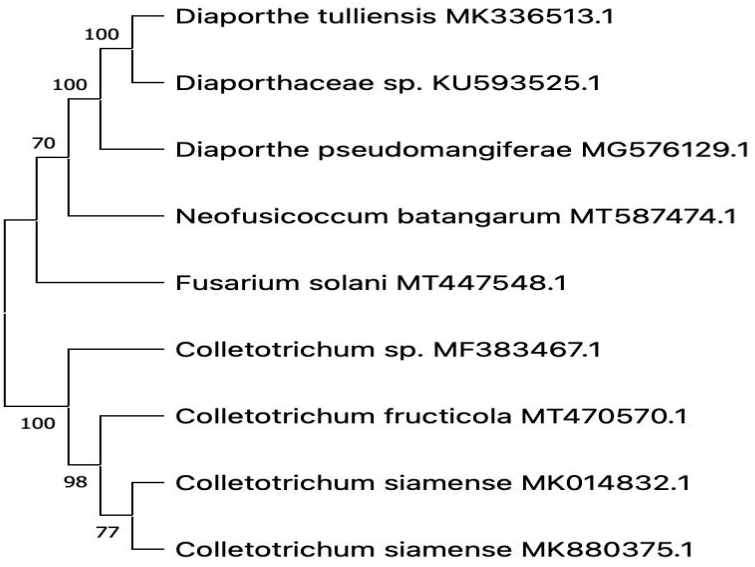

Figure 2.10: Phylogenetic (Neighbor-joining) tree based on sequence data from ITS gene of Annona glabra.

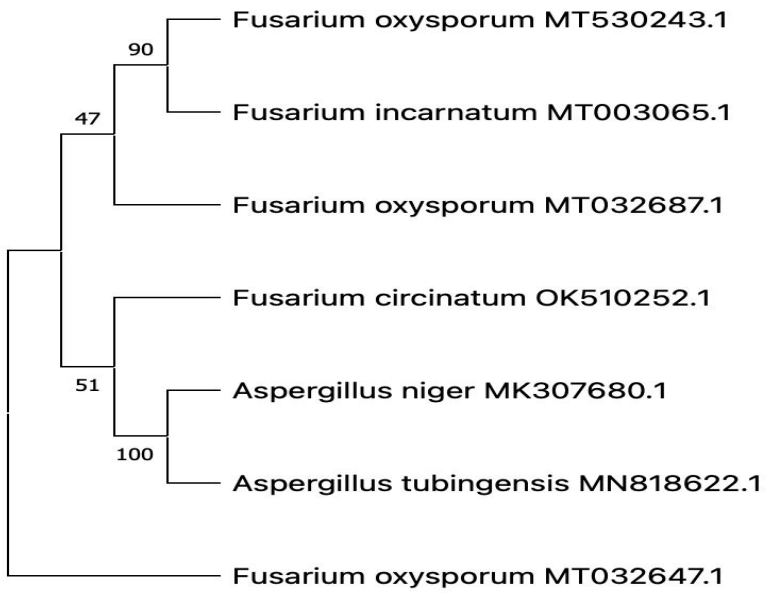

Figure 2.11: Phylogenetic (Neighbor-joining) tree based on sequence data from ITS gene of Conocarpus erectus.

The eight isolates of *Petiveria alliacea* showing strong antimicrobial activity (>20mm) from crude extract were chosen to sequence the internal transcribed spacer region (ITS) of ribosomal DNA. Sequencing data revealed that all the isolates were from the phylum Ascomycota. Sequenced data reveled that *Fusarium solani, F. oxysporium* and *F. proliferatum* are the main endophytic fungi that are responsible for antimicrobial activity. Phylogenetic tree analysis demonstrated that *Fusarium solani* MT447526.1 formed the group with *F. solani* KJ207394.1 with the strong bootstrap support of 100%. Similarly, *F. oxysporium* MN452004.1 and *F. oxysporum* MT032687.1 form a group with the bootstrap value of 68%. Results indicate that all the sequenced isolates belong to the same genus *Fusarium*.

The present data showed that *A. americana* leaf contains the highest diversity of fungal endophytes. We also found that Ascomycota being a ubiquitous phylum of fungi

also found in the plant globally. Moreover, Sordariomycetes was the most prevalent class of endophytic fungi in *A. americana* (Bhunjun et al., 2021). Similarly, we found other classes such as Eurotiomycetes, Dothideomycetes from the root and leaf sample. Hence, it can be concluded that Endophytic fungi of Agave contain high diversity of endophytic fungi.

Similarly, eight isolates showing strong antimicrobial activity (>20mm) from crude extract were selected for sequencing and the data has revealed that seven of the isolates were from the phylum Ascomycota. According to the sequenced data *Colletotrichum, Penicillium, Neofusicoccum, Curvularia, Pleosporales, Dothideomycetes* are the main genus of endophytic fungi responsible for antimicrobial activity. Phylogenetic tree analysis demonstrated that *Colletotrichum siamense* MK014832.1 formed the group with *Colletotrichum siamense* MK880376.1 with the strong bootstrap support of 97%. Similarly, *Curvularia senegalensis* MT476857.1 and *Dothideomycetes sp.* KX908956.1form a group with the bootstrap value of 94%.

We also found that 47% of morphologically different fungal isolates were isolated from root whereas 53% from leaf sample studied. Isolates also have different genetic makeup in all tissues (form those we sequenced), It can be suggested that these endophytes have adapted the distinct tissue environment overtime resulting in specific tissue specificity among the fungal endophytes as indicated by previous studies from Indian medicinal plants (Kaul et al., 2013; Mishra et al., 2012).

Hence, All the four medicinal plants studied in this project demonstrated antimicrobial activity against tested microorganisms. Further studied needed to identify the

bioactive metabolites from the endophytic fungi. Isolates with inhibition activity should be tested with ESCAPE group of drug resistance pathogens and other pathogens of interest.

2.4 Conclusion

This study investigated the antimicrobial activity of extract from fungal endophytic microbiomes from four native medicinal plants in South Florida. It has also studied the overall endophytic fungal diversity of the culturable fungal in an artificial medium. The root of *Petiveria alliacea* and *Annona glabra* have higher species diversity whereas leaf samples contain higher species diversity in *Conocarpus erectus* and *Agave americana*. The isolates that showed a good zone of inhibition were sequenced for their identification. We were unable to investigate all the isolates for sequencing and GCMS analysis for their entire metabolic characterization. Future study needs to investigate all the culturable isolates as well as unculturable isolates using molecular methods for their identification (Sánchez Márquez et al., 2007; Y. Wang et al., 2011). Expanding the testing of identified isolates for more pathogens and drug resistance strains would help to solve the global problem of antibiotic resistance.

CHAPTER 3 : CHARACTERIZATION OF ENDOPHYTIC BACTERIA FROM NATIVE MEDICINAL PLANTS

Abstract

Antimicrobial-resistant bacteria are currently considered as an emergent global disease and a major global health challenge. Exploration of endophytic microbiomes from host plant is one of the promising methods of discovering novel antimicrobials. In the current study, different plant parts such as root, stem, and leaf of two medicinal plant *Petiveria alliacea* and *Agave americana* were used for isolation, identification, and characterization of endophytic bacterial isolates using morphological, biochemical, and molecular sequencing technique. Endophytic bacterial extract was used to test the antimicrobial activity of bacterial and fungal pathogens such as such as *Escherichia coli* ATTC 25902, *Staphylococcus aureus* ATTC 14775, *Bacillus subtilis* NRRL 5109*, Candida albicans* ATTC 10231 and *Aspergillus fumigatus* NRRL 5109. Phylogenetic identification of endophytic bacteria producing antimicrobial compounds were carried out using 16s rRNA gene sequencing technique. Results indicated that some bacterial endophytes isolated from native medicinal plants of South Florida such as *Agave American* and *Petiveria alliacea*, have potential to produce novel bioactive potential to kill or inhibit the growth of disease-causing strain of bacteria. Hence, native medicinal plants of South Florida harbor the endophytic bacteria capable of producing novel bioactive metabolites, further studies still needed to characterized individual bacterial strain with its detail molecular study in larger geographical area in US.

3.1 Introduction

Bacterial endophytes are the important microbial components of host plants. They coexist with fungal endophytes without harming each other (Christina et al., 2013). Hence, their relationship with the host plants is often mutualistic. Endophytic bacteria generally enter through roots or aerial portions of the host plant. At the point of entry, they colonies locally and enters to the inside cells or intercellular spaces of host plant tissue (Bell et al., 1995). Endophytic bacteria are mainly distributed in almost all plant species and scientists have abled to isolate them from roots, leaves, and stems and only few from other plants parts such as flowers, seeds, and fruits (Lodewyckx et al., 2002).

The diversity of endophytic bacteria that colonizes in the host plant ranges from gram positive to gram negative such as *Achromobacter, Acinetobacter, Agrobacterium, Bacillus, Brevibacterium, Microbacterium, Pseudomonas, Xanthomonas* etc. (Sun et al., 2012). After lodging inside the host plants, they start producing various types of metabolic organic products important for growth and survival for host plants. Hence, natural products produced by endophytic bacteria often known to produce metabolites which are broad spectrum antimicrobial effective for wide varieties of bacterial and fungal pathogens (Roh et al., 2009). They also have potential to promote plant growth, controlling the soil borne pathogens and help plant to overcome the environmental stresses (Mastretta et al., 2006; Ryan et al., 2008; Taghavi et al., 2009). Moreover, the beneficial interactions between host plant and endophytic bacteria help to ecosystem restoration process (Glick et al., 1995). It will help host plants the ability to use nutrients from the soil which can assist host plant to

increase root development, better nutrient uptake capabilities and control the soil-borne pathogens (JM Whipps, 2001).

Many endophytic bacteria isolated from medicinal plants have revealed promising bioactive potentials with different pathogens. These plants include *Tinospora cordifolia* Miers, *Memecylon edule* Roxb., *Dipterocarpus tuberculatus* Roxb. and *Phyllodium pulchellum* Benth (Bhoonobtong et al., 2012). Hence, endophytic bacteria have huge potential to provide antimicrobial chemicals against many different pathogens.

As medicinal plants demonstrate antimicrobial activity, it is very important to find out if the medicinal properties produced is by plant itself or from the endophytic microbiomes that lives inside the host plants. As South Florida harbors many different tropical and subtropical native medicinal plants which will prove to be the important contributor for the global effort to fight against different types of pathogens.

Petiveria alliacea plant commonly known as Guinea henweed, Anamu, Apacin, Guine, Mucura, Tapi and is natively found natively in Florida, Texas, central America, Caribbean, tropical parts of South America and some area of Africa (Gann et al., 2007). It is popular in folk medicine as it is said that it has capacity of healing different types of disorders.

Research conducted on plant parts extract have demonstrated important information about its bioactive potential. *Petiveria alliacea* leave contains important chemicals such as antifungal, antiviral, anti-inflammatory compounds. It is used as antirheumatic, soothing agents, restorative purposes. Many results from pharmacological study have demonstrated that it contains immunomodulatory that provides bone and joint comfort, promotes joint mobility, antioxidant, fight against free radical damage, effective

against immune system health and muscle spasm and as insect repellent etc. (Marini et al., 1993; Rossi et al., 1993; Williams et al., 1997). So, those evidence have suggested that research conducted on these medicinal plants have mainly focused on plant extracts, not in the endophytic bacteria and its extract for testing their bioactivities.

Similarly, *Agave americana* which is commonly known as century plant, Maguey or American aloe is the species of flowering plant native to Mexico and some parts of US and is being grown as ornamental plants throughout the world (Bailey, 1976). It has also demonstrated bioactive potential from its extract prepared from plant parts. Previous study conducted on the leaf extract of plant showed antioxidant properties which hints the possibility of its anticancer property (Ahumada-Santos et al., 2013). Moreover, similar research conducted using extract made from leaf stem, seed, root has shown promising effect on drug resistant clinical isolates, antibacterial effects, antifungal activities, sources of phytonutrients, anticancer ingredients, bioremediation activities (Alipanah et al., 2021; Enweani et al., 2021; Gallegos & Katerine, 2021; Ieven et al., 1979b; Johanna et al., 2021; Maurya et al., 2021; Rosas-Taraco et al., 2010; Shegute & Wasihun, 2020). All of these research have focused on plant extract properties not the endophytic microbiomes contributions on these properties.

3.2 Materials and Methods

3.2.1 Collection, isolation, and morphological illustration of endophytic bacteria

Different researchers have followed different protocol for isolation and identification of pure colony of endophytic bacteria. The best and modified methodology was utilized for isolation steps. Medicinal plants for this study were collected randomly

from various places of South Florida. The collection of *Petiveria alliacea* plant was focusing mainly from Possum trot farm (25°32′7" N; 80°28′ 37" W) and *Agave americana* plant was collected from Fruit and Spice Park (25°32′6" N; 80°29′ 31" W) located at Homestead, Florida. Plant species *Agave americana* was identified by Jim Stribling, director and plant specialist from Fruit and Spice Park whereas *Petiveria alliacea* was identified by Robert L. Burnum from who is expert in native medicinal plant in South Florida. The isolation of endophytic bacteria was conducted following modified protocols from Das et al., 2018. Two grams of different sampled tissues (leaf, stem, root) of medicinal plants was washed thoroughly in running tap water to get rid of dirt and soil particles. Surface sterilization was performed by dipping in to 70% ethanol for 60 seconds, followed by submerging in 2.5% sodium hypochlorite for 90 seconds and 30 seconds in 100% ethanol. After that, tissues were washed with sterile distilled water for 5 times. Tissues sample was dried out in sterile condition inside the safety hood. Grounding of tissues was performed with the help of sterile mortar and pestle and paste solution was incubated in 6 ml of 0.9% NaCl for 3 hours at 24°C to allow proper discharge of endophytic bacteria from the tissues.

The supernatant was diluted to 10 and 100-fold with 0.9 % NaCl solution and spread over the different solidified agar media (NA, TSA, R3A) and incubate it for 1-2 weeks for 28°C to isolate endophytic bacteria. The colony characteristics (size, color, form, margin, elevation) of the isolated bacteria was recorded with standard methods (Ngoma et al., 2014). Pure culture was isolated based on the morphological difference among the colony and continuously subculture until pure colony was achieved.

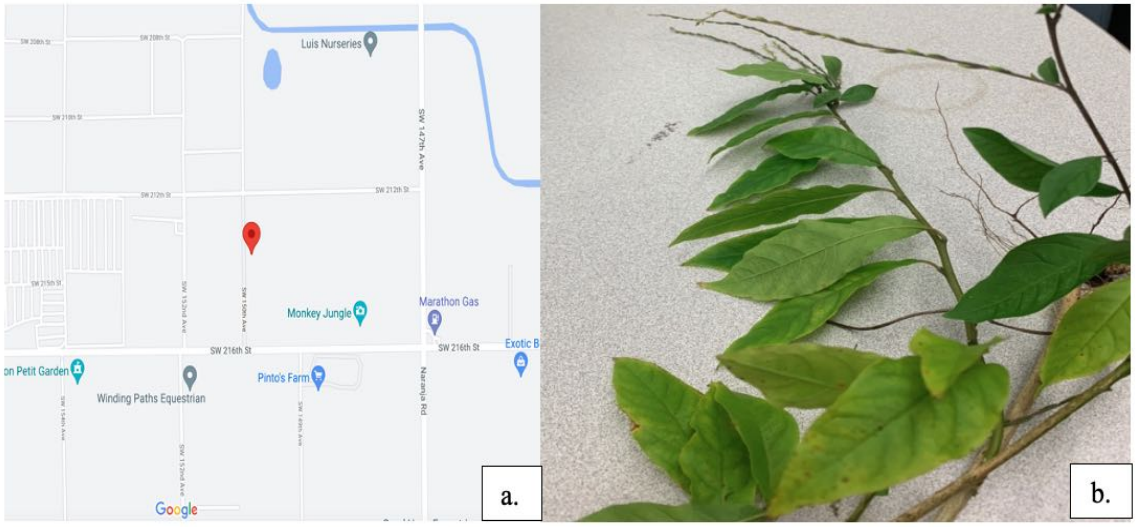

Figure 3.1: Google map view of Possum trot farm from where the medicinal plant *Petiveria alliacea* was collected (a). *Petiveria alliacea* plant at the time of sample collection (b).

3.2.2 Preparation of extract from endophytic bacteria

The extraction of endophytic bacterial extract was performed following modified protocol from El-Deeb et al., 2013. The selected bacterial isolates were grown in Nutrient Broth (100 ml) in a 250 ml Erlenmeyer flask and incubated in an orbital shaker (125 rpm) at 28°C for 48 hours. Bacterial cells were removed from culture media by centrifugation at 10,000 rpm for 10 min. The cell free supernatant was obtained by filtering through 0.45 µm Millipore filter. Thus, formed extracts were utilized for antimicrobial activity against bacterial and fungal strains and store at 4°C for further antimicrobial and chemical analysis.

3.2.3 Evaluation of antimicrobial activity of endophytic bacteria

The antimicrobial activity of the bacterial isolates was tested against pathogenic bacterial strains such as (*Escherichia coli* ATTC 25902, *Staphylococcus aureus* ATTC 14775, *Bacillus subtilis* NRRL 5109) and two fungal strains (*Candida albicans* ATTC 10231 and *Aspergillus fumigatus* NRRL 5109). These are the important pathogenic

microorganisms that are causing serious health problems. The initial antimicrobial activity was assessed with the dual culture technique (Daungfu et al., 2019). Cell suspension of pathogenic microorganisms was swabbed to the surface of different agar medium. Then, each of the endophytic bacterial specimen was streaked with four lines (2 cm long) on four side of the petridish (90mm) and was incubated at room temperature for 48 hours to check zone of inhibition around the four streaks of endophytic bacteria. The antagonistic effects were confirmed using agar well diffusion methods. In this technique, pathogen suspension was swabbed on the Nutrient Agar medium and after that, three wells (6mm diameter and 8 mm high) were made on each plate of media and 100, 150 and 200 µl (10^8 cfu/ml) of endophytic bacterial extracts were added in each well. Nutrient Broth medium was used as negative control without endophytic bacteria whereas ampicillin sodium (100 µg/ml) was taken as positive control 1 and fluconazole (30 µg/ml) as positive control 2. All plates were incubated at 28°C (room temperatures) for 48 hours to measure diameter of zone of inhibition. The experiment was performed in triplicates. The isolates with effective zone of inhibition were chosen for the molecular identification.

3.2.4 Identification of isolates

For the identification of isolates of interest, molecular study was performed by Azenta life science for each isolate to identify their genus and species. The brief protocol as follow. Firstly, extraction of DNA was performed from their broth using Quick-DNA Fungal/Bacterial Kits (Zymo Research, USA) following manufactured protocols. DNA specimen will be amplified using 16S rRNA genes using universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3')

(Daungfu et al., 2019). PCR products were analyzed electrophoretically in 1% w/v agarose gel staining with ethidium bromide. DNA sequence obtained from molecular sequencing were compared with the NCBI database (http://blast.ncbi.nlm.nih.gov) and accession numbers were assigned to each sequence.

3.3 Results and discussion

3.3.1 Pure culture of bacterial endophytes

In this study, we examined antimicrobial activities focusing on antibacterial and antifungal activity of the bacterial endophytes isolated from *Petiveria alliacea* and *Agave americana* medicinal plants sampled from South Florida. Fresh plant materials (leaf, stem, and root) were used in this project. Surface sterilization was very critical in this project to remove all epiphytic microorganisms from the sampled plant part. The control was used as the third distilled water used in surface sterilization process and it proved to be sterile as there was no growth after few days of incubation with 100 µl in the Nutrient agar medium. The number of colonies observed was also adequate from the sample plant parts on Tryptic Soy Agar medium after 24 to 48 hours of incubation at 37°C. Bacterial isolates were isolated into pure culture based on their morphological differentiation. The colony with distinct growth pattern and morphology were chosen for the new isolate.

For preliminary identification of the isolates from the sampled parts, wide range of morphological, physiological, and physiological analysis were carried out. The result indicates that bacterial endophytes identified were different. For morphological differentiation, colony characteristics were performed, and isolates exhibited diverse colony in shape, color, margin, texture i.e., white, yellow, brownish colony, circular and

irregular shapes, regular and wavy margins etc. Three isolates were gram negative and remaining were gram positive in gram staining procedure.

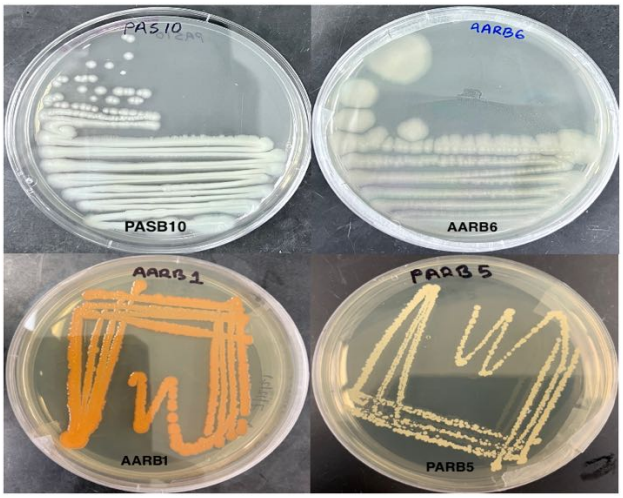

Figure 3.2: Pure colony of some endophytic bacteria isolated from medicinal plants.

Table 3.1: Endophytic bacteria isolated from Agave americana

Tissue	Segment studied	Colonized segment	Total Isolates	Endophytic species	CR†%	IR‡%	Shannon-index¥
Leaf	90	61	66	8	0.67	0.73	2.065
Root	90	90	98	10	1.00	1.08	2.277

†CR= Colonization rate of bacterial isolates
‡IR= Isolation rate of bacterial isolates
¥Shanon_*H'*= Shannon- Weiner Index (H')

Table 3.2: Bacterial endophytes isolated from Petiveria alliacea.

Tissue	Segment studied	Colonized segment	Total isolates	Endophytic species	CR†%	IR‡%	Shannon-index¥
Root	99	84	89	8	85	89.8	2.04
Stem	99	49	56	6	49	56.5	1.78
Leaf	99	98	63	4	98.9	46	1.35

Abbreviations are same as in the Table 3.4

3.3.2 Antimicrobial activity of isolate with bacterial and fungal strain

The endophytic bacteria were isolated from medicinal plant explants such as root, stem, and leaf. Endophytic bacteria isolation from medicinal plant have been reported

previously by many scientific reports such as Thymes vulgaris (Abdelshafy Mohamad et al., 2020), Glycyrriza uralensis (Li et al., 2018), Alkanna tinctoria (Rat et al., 2021), Carica papaya (Sarjono et al., 2021), Dicoma anomala (Makuwa & MH Serepa-Dlamini, 2021). The endophytic bacteria exhibited antibacterial and antifungal properties that inhibits the growth of bacterial and fungal pathogens. It is reported that they are the major source of novel bioactive metabolites for therapeutic purposes (R. X. Tan & Zou, 2001). Furthermore, Strobel & Daisy, (2003) emphasized for further endophytic study on medicinal plant and its endophytic bacteria as they are expected to harbor rare and potential endophytes with novel bioactive metabolites. This study also showed that medicinal plant *Agave americana* and *Petiveria alliacea* harbor bacterial endophytic isolates that have potential to kill or inhibit the growth of bacterial strain tested.

Inhibition activity is expressed in the form of clear zones and is measured by the term of the digital numbering to know the size of the inhibition. The positive test results were compared to 4 groups of inhibition of antimicrobial compounds. The diameter of > 20 mm was categorized as very strong inhibition, 10-20 mm including strong category, 5-10 mm including medium category, and the inhibition of > 5 mm including weak category (Simarmata & Lekatompessy, 2007).

Table 3.3: Antagonistic activity of the bacterial endophytes isolated from Century plant (Agave americana) and Guinea hen weed (Petiveria alliacea) against bacterial strains (the numbers are the diameters of the zones of inhibition in mm; mean ± SD; n= 3) Positive control 1: Ampicillin sodium (100 µg/ml), Positive control 2: fluconazole (30 µg/ml); Negative control: Nutrient broth.

Endophytes	*E. coli*			*S. aureus*			*B. subtilis*		
µl	100	150	200	100	150	200	100	150	200
AARB6	13.5 ± 0.2	14.0 ± 0.1	14.6 ± 0.3	-	-	-	-	-	-
PARB 12	16.0 ± 0.3	17.9 ± 0.1	19.2 ± 0.3	24.2 ± 0.1	26.2 ± 0.3	28.1 ± 0.2	-	-	-
PASB 10	-	-	-	28.2 ± 0.4	30 ± 0.4	30.9 ± 0.5	2.0 ± 0.1	4.1 ± 0.1	5 ± 0.2
Positive control 1	15.0 ± 0.1	16.0 ± 0.2	16.9 ± 0.2	16.0 ± 0.0	16.3 ± 0.2	16.9 ± 0.2	20 ± 0.1	20.1 ± 0.2	20.5 ± 0.2
Positive control 2	-	-	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-	-	-

Table 3.4: Antagonistic activity of the bacterial endophytes isolated from Century plant (Agave americana) and Guinea hen weed (Petiveria alliacea) against fungal strains (the numbers are the diameters of the zones of inhibition in mm; mean ± SD; n= 3) Positive control 1: Ampicillin sodium (100 µg/ml), Positive control 2: fluconazole (30 µg/ml); Negative control: Nutrient broth.

Endophytes	*C. albicans*			*A. fumigatus*		
µl	100	150	200	100	150	200
AARB6	-	-	-	-	-	-
PARB 12	-	-	-	-	-	-
PASB 10	-	-	-	-	-	-
Positive control 1	-	-	-	-	-	
Positive control 2	24.1 ± 0.3	24.1 ± 0.3	24.6 ± 0.3	17.1 ± 0.3	17.4 ± 0.1	17.8 ± 0.3
Negative control	-	-	-	-	-	-

Some abbreviations from the table are as follow:
AARB= Agave americana root bacteria
PARB= *Petiveria alliacea* leaf bacteria
PASB= *Petiveria alliacea* stem bacteria
"-" = no inhibition activity
¶µl= Microliter of the sample used
Numbers are mean ± standard error.

After morphological characterization, antimicrobial activity of the bacterial isolate extracts was performed. To achieve that objective, three bacterial strains were selected that have potential for disease causing capabilities. Of the bacterial strain selected, one was gram negative and other two were gram positive. Similarly, two fungal strains were chosen

for testing antifungal activity by endophytic bacteria. The isolated bacterial endophytes obtained from the medicinal plant showed bioactivity against some bacterial strains (Table 3.3). Regarding the antimicrobial testing of *Agave americana,* seventeen isolates did not show any types of antagonistic activity against bacterial and fungal strain tested. One isolates AARB6 which was isolated from root explant of *Agave* showed promising result with *E. coli.* It showed 14.6 ± 0.3 mm of zone of inhibition from its extract prepared from bacterial isolate grown in nutrient broth medium for 48 hours.

Similarly, sixteen endophytic bacterial isolates from *Petiveria alliaceae* were selected for testing with all five microbial strains tested. That includes six endophytes from stem sample, eight endophytes from root sample and four endophytes from leaf sample. Only two of extract from endophytic bacteria showed antimicrobial activity against the bacterial strain tested. The endophytic bacterial strain PARB12 which is sequenced as *Arthrobacter woluwensis* showed zone of inhibition of 17.9 ± 0.1mm against *E. coli* using 150 µl of sample. Similarly other sample PASB10 showed antimicrobial activity of 28.2 ± 0.4 mm and 4.1 ± 0.1mm against *S. aureus* and *B. subtilis* respectively. None of the endophytic bacterial isolates showed antagonistic activities against fungal strain tested Table 3.4).

3.3.3 Sequencing of endophytic bacteria

Three endophytic bacterial isolates showing strong zone of inhibition were chosen for sequencing. For accurate identification of isolates of interest, 16sRNA sequencing analysis were performed at GENEWIZ from Azanta life sciences LLC. Multiple sequenced alignment methods were conducted using a freely available alignment program.

Endophytic Isolates identification were based on 16s rRNA gene sequencing similarity (Halda-Alija, 2004). Sequences are than compare with National Center for Biotechnology Information (NCBI) database. The bacterial isolates reported in this article have been reported in the GenBank database under accession numbers MT071300.1 *(Arthrobacter woluwensis*); MN309878.1 (*Arthrobacter woluwensis*) and the bacterial isolate AARB6 identified as *Bacillus velezensis*.

Table 3.5: Sequencing results of selected endophytic bacterial isolates. Accession number of best matched database sequence is shown for each isolate.

Isolate	Closest relative NCBI data base	Tissue	ITS identity	Phylum, Class, Order	Bacterial species with best matched sequence	Accession number of best matched database sequence
PASB10	*Arthrobacter woluwensis* strain TDS9	Stem	99.87%	Terrabacteria, Actinobacteria, Micrococcales	*Arthrobacter woluwensis*	MT071300.1
PARB12	*Arthrobacter woluwensis* strain MCM353	Root	100%	Terrabacteria, Actinobacteria, Micrococcales	*Arthrobacter woluwensis*	MN309878.1
AARB6	*Bacillus velezensis* strain BFAM3	Root	99.88%	Firmicutes, Bacilli, Bacillales	*Bacillus velezensis*	MT882024.1

Abbreviations are same as in the Table 3.3.

Strains of *Arthrobacter woluwensis* were previously reported as rhizospheric bacteria form other plant like soyabean plant (Khan et al., 2019). Other species of *Arthrobacter* was isolated as endophytic bacteria from Korean turf grass (Chung et al., 2010). Similar study conducted on strawberry discovered *Arthrobacter woluwensis* as an endophytic bacterium (de Pereira et al., 2012). As there are limited studies conducted on endophytic bacteria, it is very clear that bacteria like *Arthrobacter woluwensis* is present as endophytic bacteria in wide variety of plants. Similarly, the endophytic bacteria AARB6 was identified as *Bacillus velezensis* from sequencing. Recent research conducted on

endophytic bacteria *Bacillus velezensis* isolated from different plants species from different geographical distribution has demonstrated antibacterial, antifungal and plant growth promoting factors (Cai et al., 2016; Chen et al., 2019; L. Cui et al., 2020; Gao et al., 2017; Harun-Or-Rashid et al., 2017; Rabbee et al., 2019). Hence it can be considered that the endophytic bacteria present in *Petiveria alliacea* and *Agave americana* are wide range of distribution. The phylogenetic study of 16s rRNA sequence of the isolates along with the sequences retrieved from the NCBI was carried out with MEGA 11 using the neighbor-joining method with 1,000 bootstrap replicates. The phylogenetic results indicated that there is the distinct clustering of isolates as shown in Figure 3.3.

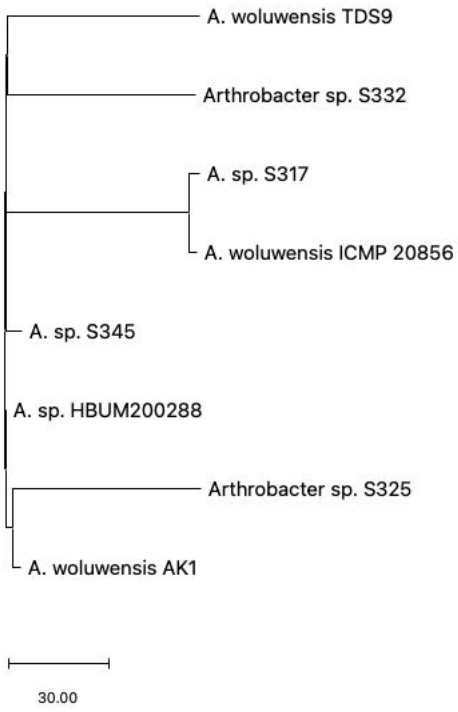

Figure 3.3: Phylogenic analysis of the endophytic bacteria from Petiveria plant along with its closely related sequences of NCBI. The analysis was conducted on MEGA 11 using neighbor-joining method.

3.4. Conclusion

Endophytic bacteria isolated from medicinal plants have potential to kill or inhibit the growth of potential human, animal, and plant pathogens. Hence, they are the good candidates for exploring the novel antimicrobials required to fight against novel drug resistance pathogens. It can be studied for its novel antimicrobial agents, medicines, biofertilizers, biopesticides and many other aspects of human welfare. One endophytic bacterium (AARB6) from *Agave americana* identified as *Bacillus velezensis* had good antagonistic activity against *E. coli*. Similarly, two endophytic strains of *Petiveria alliacea* sequenced as different strains of *Arthrobacter woluwensis* had antibacterial property against tested bacterial strains. Other endophytic bacterial strain that we isolated did not showed antimicrobial activity against the bacterial and fungal strain that we tested. This study provides the future research encouragement on native medicinal plants to explore possible endophytic microbiomes that have potential to produce bioactive metabolites to fight against global microbial resistant problem.

CHAPTER 4 : ENDOPHYTIC FUNGAL BIODIVERSITY IN MEDICINAL PLANTS

Abstract

Endophytic fungi are the major components of plant microbiomes as they harbor host plant without causing any harmful effect. Majority of the studied have not characterized the medicinal endophytic fungi systematically. In this study, 30 fungal endophytes were isolated from native medicinal plant *Petiveria alliacea* of South Florida using traditional morphological differentiation method. The colonization rate, isolation rate and Shannon-wiener species diversity index were studied from culturable fungi. Exploration of diversity of unculturable endophytes were achieved by fungal ITS targeted sequencing techniques. The results showed that endophytic fungi isolated from *Petiveria* plant exhibits high diversity, host specificity and spatial heterogenicity. Direct PCR and ITS sequencing of plant parts revealed substantially greater diversity richness compared to culture-based method. Future study should focus on exploration of undiscovered endophytic microbiomes using advanced molecular techniques from more medicinal plant from South Florida.

4.1 Introduction

Endophytes are ubiquitous and have been reported in most plant that have been studied and are also considered niche specific. Endophytic fungi are an important component of host plants as they contribute to different aspects of plant growth and survival. They are the group of fungi that infect and colonize the internal tissue of the host plant without causing any harmful effects (Hirsch & Braun, 1992). Recent research studies

have shown that at least 1 million endophytic fungi live in a plant (Dreyfuss & IH Chapela, 1994). The fungal component of the endophyte is a very important and quantifiable element of plant-fungal biodiversity. Fungal endophytes are also known to affect host plant community diversity and structures as well (Gonthier et al., 2006; IR Sanders, 2004; Krings et al., 2007). To this date, only 80,000 to 100,000 plant-fungal species have been described so far (Hawksworth et al., 1995; Hawksworth & AY Rossman, 1997). So, there is still more research is needed to explore their characteristics.

Endophytic fungi form a large and phylogenetically diverse group of tissue colonizing fungi. They are different from the mycorrhizal fungi in that they live entirely within the plant tissues (Stone & Polishook, 2004). Four classes of fungal endophytes have been distinguished so far and these include: 1) The Clavicipitaceous endophytes that colonize grasses, 2) Non-Clavicipitaceous endophytes that colonize whole plants, 3) Non-Clavicipitaceous endophytes that colonize shoots and 4) Non-Clavicipitaceous endophytes that colonizes roots (Rodriguez et al., 2009). Recent studies have found that all the terrestrial plants have been infected with class three endophytes which are horizontally transmitted (Davis et al., 2003; Saikkonen et al., 1998). The representative isolates from four of these classes colonize above ground tissues through localized infection of host plants and are generally in high diversity even in the same host plants (Arnold & Lutzoni, 2007; Higgins et al., 2007). Previous studies have attempted to show that endophytes are host and tissue-specific, but results are inconsistent indicating both host-specific and host generalism (Arnold & Lutzoni, 2007; Cao et al., 2002; Higgins et al., 2007; Sun et al., 2012).

The endophytic relationship with the host plant can be described using different terminologies such as Host specificity, host recurrence, host selectivity, or host preference (Cao et al., 2002; Cohen, 2006). Host specificity is defined as "the relationship in which endophytic fungus is only restricted to only one host or group of related species" (Holliday, 2001). Similarly, Host-recurrence is the predominant occurrence of the endophytic fungi on the host or host ranges (Cao et al., 2002). Host selectivity is defined as "the relationship of single fungal endophytes with two different plant species but always demonstrates the preference for one host" (Cohen, 2004, 2006). The difference in preference of endophytes to different host plants might be due to the chemical differences of host plants (Bettucci et al., 2004; Paulus et al., 2006).

Many evolutionary processes and earlier fossils records have demonstrated an association between endophytic fungi and the various groups of host plants. The relationship between endophytes and the host plant often results in adaptation, plant growth promotion, uptake of nutrients, and production of various types of metabolites and bioactive products needed for plant growth and development because they protect the plant against biotic and abiotic stress (Rai et al., 2014).

Fungal endophytes that we are able to isolate in our laboratory are categorized as culturable fungi. Some culturable fungi are highly selective in certain types of growth medium and cannot be isolated in general-purpose media. There is no single medium that supports the growth of all the endophytic fungi and if we use many different types of media for isolation, there will still be a lot of other fungal endophytes that can't grow in the medium of our interest. So, the molecular techniques are being used by scientists to identify the microbes which we can't culture in vitro.

Hence, utilizing these molecular techniques, fungal endophytes belonging to diverse genera including Acremonium, Alternaria, Aspergillus, Berkleasmium, Chaetomium, Cladosporium, Claviceps, Colletotrichum, Cryptococcus, Curvularia, Fusarium, Geomyces, Glomus, Leptospora, Metarhizium, Microdochium, Neotyphodium, Ophiognomonia, Paecilomyces, Penicillium, Phaeomoniella, Phyllosticta, Piriformospora, Rhizoctonia, Rhizopus, Rhodotorula, Talaromyces, Trichoderma, Wallemia, and Xylaria, have been successfully isolated and identified from various host plant (Cronquist, 1988)

Petiveria alliacea which is also known by its common name Guinea hen weed belongs to the family Phytolaccaceace which is the most primitive family of the Caryophyllales (Cronquist, 2002). It is a perennial herb naively found in North America (Florida and Texas), Mexico, Central America, West Indies, and South America. It is a fast-growing herb with the capacity to tolerate a wide range of environmental stress (Alegre & Clavo, 2007). Only a few studies have been devoted to exploring the endophytic community of *Petiveria alliaceae*. Endophytes are isolated from many traditional medicinal plants such as Turmeric, Malay Apple, Acanthus used in Chinese and Indian herbal medicine are known to harbor several beneficial endophytes such as *Lasiodipodias spp*. which are known to treat different health problems such as hepatitis, lymphadenectasis and bacterial infections (Tanvir et al., 2017).

Research conducted on plant parts extract has demonstrated important information about its bioactive potential. *Petiveria alliacea* leave contains important chemicals such as antifungal, antiviral, anti-inflammatory compounds. It is used for antirheumatic, soothing agents, restorative purposes. Many results from the pharmacological study have demonstrated that it contains immunomodulatory agents that helps for bone and joint

comfort, promotes joint mobility, antioxidant, fight against free radical damage, immune system health, muscle spasm, insect repellent, etc. (Marini et al., 1993; Rossi et al., 1993; Williams et al., 1997). Hence, it is very important to know about its endophytic microbial diversity.

The main goal of this study is to isolate and characterize the endophytic fungal communities from native medicinal plant *Petiveria alliacea* to explore its endophytic fungal diversity. Following questions have been addressed from this study: 1) How diverse the endophytic fungal communities in *Petiveria alliacea*? 2) What is the taxonomic identity of fungal endophytes isolated from the medicinal plant? 3) Is there is any difference in endophytic fungal isolates in culturable and unculturable isolates?

4.2 Materials and Methods

4.2.1 Host plant collection and localization of the sampling sites

Endophytic fungi that colonize the host plant tissues are mostly influenced by the environmental condition surrounding the host plant such as soil type, soil pH, alkalinity of soil, and climate. They may develop in a small amount and are generally in a confined location in the plant. Hence it is very difficult to determine the relationship of fungal endophytes to their host plant. For separation and isolation of fungal endophytes, special care must be taken to prevent unwanted contamination by epiphytic microorganisms (R. N. Singh et al., 2016).

The healthy plant samples of *Pativeria alliacea* were collected from Possum trot Tropical Fruit Nursery (25°32′7" N; 80°28′ 37" W) located in Miami's Redland Agricultural district, Florida. Samples of fresh healthy leaves, stems, and roots of *Pativeria*

alliacea were collected in separate zipper lock bags, labelled, stored in a cooler on ice, transported to the laboratory and stored at 4ºC until processing as described previously by Tan et al., 2018. Samples were processed within 24 hours of collection. Specimens were identified by Robert L. Burnum from Possum trot farm who is expert in native medicinal plant in South Florida.

4.2.2 Endophytic fungal isolation and identification

Healthy shoots, roots, and leaves of medicinal plants were washed in running tap water and surface sterilization was achieved by subsequent immersion into 70% ethanol for 60 seconds, followed by submerging in 2.5% sodium hypochlorite for the 90s and 30s in 100% ethanol. After that, plant tissues were washed with sterile distilled water for 5 times for getting rid of the chemicals used in the sterilization procedure (Davis et al., 2003). Surface sterilization eliminates epiphytic bacteria, yeasts, and rapid-growing Zygomycetes (Arnold et al., 2000). Surface sterilization success was confirmed by imprinting surface-sterilized tissue in sterile Potato Dextrose agar (PDA) plates (If no fungal growth was observed then it is considered surface-sterilized). Tissues were dried out in sterile condition inside the safety hood. Sample tissues were cut into 6mm X 6mm pieces, and 9 segments of plant part were placed in each 90 mm Petri dish containing PDA. PDA was enriched with 0.2 gm/L of Chloramphenicol and 0.1 gm/L of streptomycin sulfate to inhibit the growth of bacteria. All plates including control were incubated at 28°C for 2-6 weeks until the fungal growth was visible. Isolates were categorized as different morphotypes and they were continuously sub-cultured until the pure culture was obtained (Y. Wang et al., 2011). Selected morphologically different isolates were identified using molecular methods from

Azenta life science. The brief the experimental procedure is a follow; the colony samples were lysed using crude NaOH lysis technique to be directly used in PCR. To amplify ribosomal internal transcribed spacers (ITS), the primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) were employed in a Polymerase chain reaction (PCR) assay (White et al., 1990). Amplified samples were spot checked using gel electrophoresis to check for robust amplification along with a negative control to check for contamination. Following amplification, enzymatic cleanup was performed according to Azenta Life Sciences SOP using Exonuclease I – Shrimp Alkaline Phosphatase (ExoSAP), and dye-terminator sequencing was performed by Azenta Life Sciences Inc. (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. Sequencing-specific primers were used to generate bidirectional reads. The reactions were then run on Applied Biosystem's 3730xl DNA Analyzer. Sequences were than compared with National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and accession numbers were assigned to each sequence.

4.2.3 Targeted fungal ITS sequencing of unculturable endophytic fungi

For ITS sequencing of culturable and unculturable fungal endophytic fungi, plant samples were collected from Possum trot farm located at Homestead, Florida. To estimate the fraction of endophytes that are unculturable, the molecular DNA extraction, PCR and sequencing were performed from Zymoresearch molecular facilities. Briefly, DNA was extracted directly from the plant parts (shoot, root, and leave) using the ZymoBIOMICS® DNA Miniprep Kit (Zymo Research, Irvine, CA). The ITS and 5.8S rDNA regions were

amplified using fungal-specific primers ITS2. The PCR products were sequenced from the Zymoresearch® facility for fungal targeted ITS sequencing to get information about all the fungal DNA from the plant parts which are culturable as well as unculturable. Fungal ITS gene-targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit with custom ITS2 primers substituted for 16S primers. Cut off values were chosen as 98.5% for OTUs (Operational taxonomic units) and their gene accession numbers were assigned (Callahan et al., 2016; Caporaso et al., 2010; Segata et al., 2011).

4.2.4 Statistical analysis

The fungal diversity of endophytic fungi was calculated using the Shannon-Weiner Index (H') with the following formula.

H'= -Σ(Pi X In P*i*)

Where Pi was calculated as Pi = and n*i* represents numbers of individual segment from which fungal endophytic isolates were isolated and N is the total number of segments incubated (Y. Sun et al., 2011).

Similarly, the Colonization rates (CR%) of the fungal isolates were calculated as follows CR %= Nsc/Nss ×100 where Nsc represents the number of segments infected by fungal isolates and Nss represents the total number of plant segments investigated. The isolation ratio (IR%) of the fungal strains was calculated as follows:(IR% =Ni/Nt ×100 where Ni is the number of segments from which fungal isolates were isolated and Nt is the total number of plant segments incubated (Hata & Futai, 1995).

$$\text{CR \%} = \frac{No.\ of\ colonized\ segment}{No.of\ segment\ studied} \quad X100$$

$$\text{IR \%} = \frac{No.\ of\ segment\ from\ which\ fungi\ isolated}{No.of\ segment\ incubated}\ X100$$

Similarities of endophytic fungal communities between different sampling sites were calculated using the Jaccard similarities coefficient (Jaccard, 1912). The Diversity index were analyzed by One-Way ANOVA. All statistical analysis were calculated using SPSS 20.0 (SPSS Inc., Chicago, IL, USA)

4.3 Results and Discussion

4.3.1 Collection and pure culture isolation

The fungal endophytes harbor unique and diverse antimicrobial activity (Pelaez, 2004). The biodiversity of the fungi in plants remains unclear, but it is estimated that the total number of fungal species number can be up to 5 million (Ventola, 2015). Hence, it can be indicated that a lot of endophytic fungi are needed to explore their fungal biodiversity.

4.3.2 Biodiversity of culturable fungal endophyte

Table 4.1: Endophytic fungal isolates from Petiveria alliaceae.

Tissue	Segment studied	Colonized segments	Total Isolates	Endophytic isolates	CR%	IR%	Shanon_*H'*
Root	99	83	86	11	83	86.5	2.366
Stem	99	95	97	9	96	98	2.189
Leaf	99	94	96	10	95	97	2.282

As in the Table 4.1, from the 289 sampled plant parts (representing a total of 99 roots, 99 stems, and 99 leaves), a total of 279 isolates were counted and are categorized as 30 morphotypes based on their morphological appearances. 11 endophytic fungi were morphologically different from each other isolated from root sample, 9 were from the stem and 10 isolates from leaf sample as described by Wang et al., 2011. Some culturable

morphotypes that showed a good zone of inhibition were identified using ITS rDNA sequence analysis.

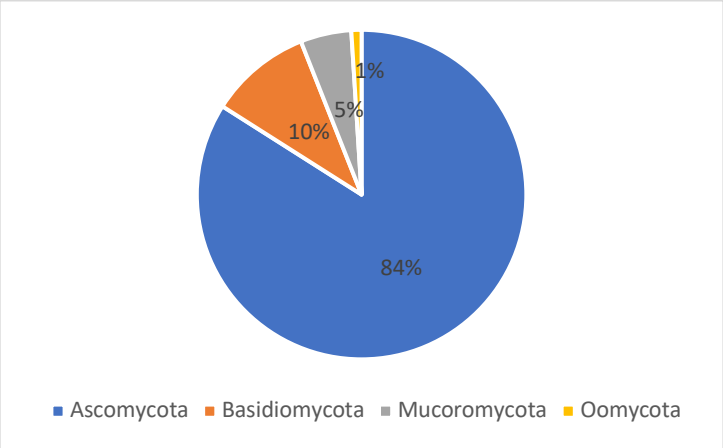

Figure 4.1: Abundance of endophytic fungi belonging to different phyla isolated from various plants (Nisa et al., 2015).

To estimate the total endophytic fungal population from the plant, fungal ITS sequencing was performed to identify all the endophytic fungal DNA found inside the plant parts including culturable as well as unculturable.

Among the endophytic fungi studied so far, Ascomycota, Basidiomycota, Mucoromycota, and Oomycota are the main phyla identified in the plant studied and the overall distribution of endophytic fungi reported mostly form phylum Ascomycota followed by Basidiomycota. The least isolated fungal strains have been reported from the phylum Oomycota (Nisa et al., 2015).

The present data as in the Table 4.1 showed that *P. alliacea* root contains the highest diversity of fungal endophytes. It is also found that Ascomycota was the major phylum on *Petiveria* plant as it is also being a ubiquitous phylum of fungi found in the plant globally.

Moreover, the most common class of endophytic fungus was Sordariomycetes in *P. alliacea.* It is also found from this study that 37% of morphologically different fungal isolates were isolated from root whereas 33% from leaf and 30% from stem sample studied. Isolates also have similar genetic makeup in all three tissues (from those we sequenced). Previous research from Indian medicinal plants imply that these endophytes have evolved to the different tissue environment throughout time, resulting in particular tissue specificity among the fungal endophytes (Kaul et al., 2013; Mishra et al., 2012)

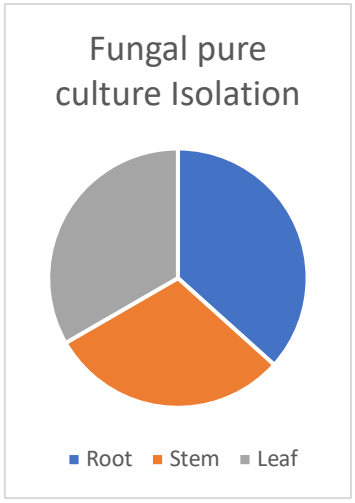

Figure 4.3: Relative frequencies of different fungal endophytes from root, stem, and leaf.

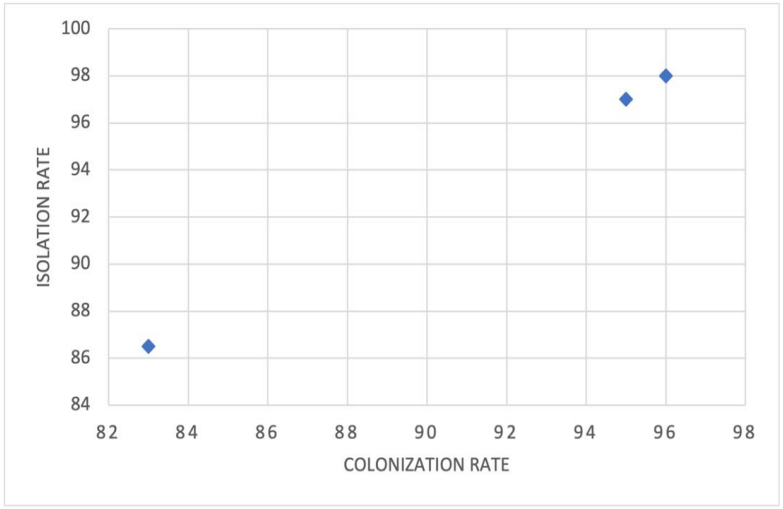

Figure 4.2: The relationship between colonization rate and isolation rate of endophytic fungi.

Colonization factor (CF%); also called isolation frequency or colonization frequency were calculated as the total number of plants segments colonized by fungi divided by the total number of plants segments incubated (expressed in percentage) (Hata & Futai, 1995). The colonization rate for endophytic fungi from *Petiveria alliacea* were found 83, 96 and 95 from root, stem and leaf respectively. Similarly, isolation factor was

calculated as the ratio of number of segments from which fungi isolated to the number of segments incubated. Isolation rate was found as 86.5, 98, and 97 from root, stem and leaf respectively from the plant studied. Species diversities were evaluated using Shannon's diversity index (Shannon CE, 1948). Higher species diversity was found in root followed by leaf and stem from the plant investigated. Similar results were reported from endophytic fungi studied in different medicinal plant from different locations (H. Jin et al., 2013; Z. Jin et al., 2017; Pawłowska et al., 2014). Sequenced data of some of the isolates from *Petiveria alliacea* were *Fusarium solani, F. oxysporium and F. proliferatum* belongs to phylum Ascomycota.

4.3.3 Targeted fungal ITS sequencing for unculturable endophytic fungi

The endophytic fungi which are impossible to cultivate in artificial medium were investigated by molecular method. The ITS and 5.8S rDNA regions were amplified using fungal-specific primers ITS2. The DNA from endophytic fungi from plant parts were sequenced from the Zymoresearch® facility for fungal targeted ITS sequencing to get information about all the endophytic fungal DNA from the plant parts which are culturable as well as unculturable. The final library was sequenced on Illumina® MiSeq™ with a V3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in.

Table 4.2: Absolute Abundance Table, shows the absolute abundance of fungal (ITS) DNA measured in the samples which contains data for gene copies, calculated genome copies, and calculated amount of DNA.

Sample label	Sample id	Ct value	Gene copies per µl	Genome copies per µl	DNA ng per µl
Sample1(LEAF)	ZR5963-1ITS2	17.30	1415500	7078	0.0930883
Sample2 (STEM)	ZR5963-2ITS2	17.44	1288188	6441	0.0847106
Sample3 (ROOT)	ZR5963-3ITS2	16.40	2594385	12972	0.1706048

Table 4.3: Illustration of the fungal composition at different taxonomy levels from phylum to species.

Taxonomic ID	Total	Leaf	Stem	Root
Unassigned; Other; Other; Other; Other; Other; Other	99.3%	98.7%	0.99%	100%
K_Fungi; Other; Other; Other; Other; Other; Other	0.0%	0.1%	0.0%	0.0%
k_Fungi;**p__Ascomycota**; Other; Other; Other; Other; Other	0.1%	0.0%	0.3%	0.0%
k_Fungi;p__Ascomycota;c__**Dothideomycetes;o__Botryosphaeriales**;f__Phyllostictaceae;g__Phyllosticta;Other	0.2%	0.5%	0.2%	0.0%
k_Fungi;p__Ascomycota;c__**Dothideomycetes;o__Pleosporales**;f__unidentified;g__unidentified;s__unidentified	0.0%	0.0%	0.1%	0.0%
k_Fungi;p__Ascomycota;c__**Eurotiomycetes;o__Chaetothyriales**;Other;Other;Other	0.0%	0.0%	0.1%	0.0%
k_Fungi;p__Ascomycota;c__**Sordariomycetes;o__Diaporthales**;f__Diaporthaceae;g__Diaporthe;s__unidentified	0.0%	0.0%	0.0%	0.0%
k_Fungi;p__Ascomycota;c__**Sordariomycetes;o__Glomerellales**;f__Glomerellaceae;g__Colletotrichum;Other	0.0%	00.0%	0.1%	0.0%
k_Fungi;p__Ascomycota;c__Sordariomycetes;o__Glomerellales;f__Glomerellaceae;g__**Colletotrichum;s__Colletotrichum cliviicola**	0.2%	0.5%	0.1%	0.0%
k_Fungi;p__Ascomycota;c__**Sordariomycetes;o__unidentified**;f__unidentified;g__unidentified;s__unidentified	0.0%	0.1%	0.0%	0.0%

From the data from Table 4.3, 99.3% of endophytic fungal DNA were unidentified from the primer ITS2 that was used in this purpose. This data is supported by the fact that only the fraction of endophytic fungi has been investigated yet and still more studies are needed to discover them (Ganley et al., 2004). Four different classes of endophytic fungi were discovered in the medicinal plant *Petiveria alliacea* that include Dothideomycetes, Eurotiomycetes, Sordariomycetes from the phylum Ascomycota from the study conducted

on different locations (Hoffman et al., 2010; Soltani & Hosseyni Moghaddam, 2015). Previous studies on other medicinal plants have also shown that Ascomycota was the dominant phylum (Glynou et al., 2015; Toju et al., 2012). Hence, it can be concluded that medicinal plant that we studied in South Florida contains diverse endophytic fungi.

The identification of the endophytic fungi can be varied by the choice of primer in sequencing. The primers ITS1 and ITS4 were used in culturable endophytic fungi whereas ITS2 were chosen in targeted ITS sequencing. Result have demonstrated that the Ascomycota was the highest dominant phylum among all primers used which was consistent with previous studies (Glynou et al., 2015; Toju et al., 2012).

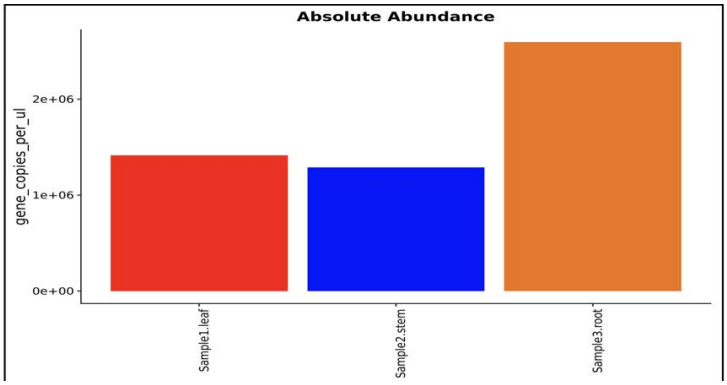

Figure 4.4: Absolute abundance graphs of sample parts from Petiveria alliacea.

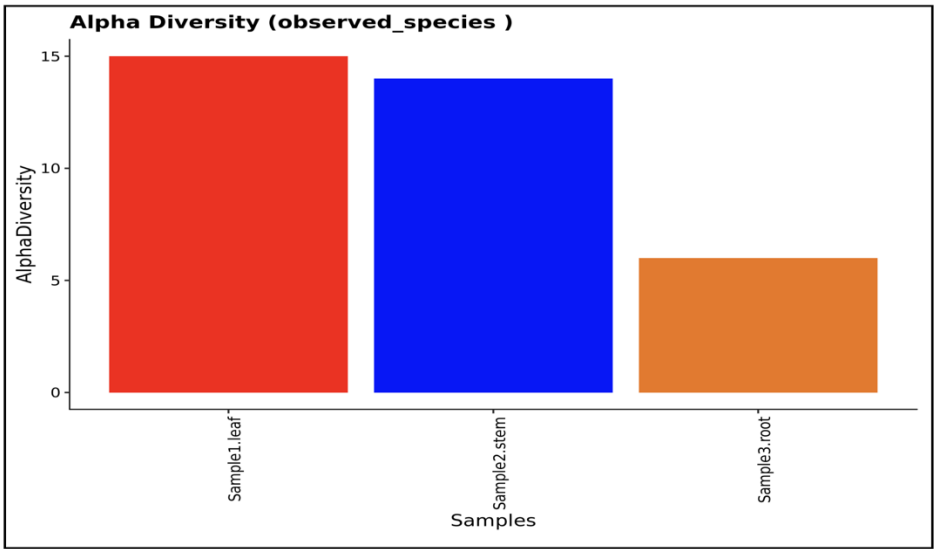

Figure 4.5: Graph of alpha diversity of sample plant parts.

The Alpha diversity refers to the diversity within particular ecosystem and is usually express as species richness in the ecosystem (Whittaker, 1972). The alpha-diversity indices of sample parts of *Petiveria alliacea* indicated that the species diversity of the endophytic fungal community from the leaf sample is highest followed by stem and root (Figure 4.5). Similar results were found the study of endophytic microbiomes from medicinal plants in other studies (Z. Jin et al., 2017; Yao et al., 2017).

Similarly, Absolute abundance data from Table 4.2, which contains the data from gene copies, calculated genome copies, and calculated amount of DNA from the sample parts studied. Absolute abundance analysis is more effective than relative abundance calculation which utilizes more traditional methodologies (Barlow et al., 2020). Data revealed as in the Figure 4.4 that absolute abundance was found higher in root followed by leaf and stem in the given sample. Similar result was observed from sequencing of culturable endophytic fungi.

4.4 Conclusion

This study investigated the endophytic fungal diversity in medicinal plant *Petiveria alliacea* from South Florida. For the culturable endophytic fungal diversity, the 30 morphologically different endophytic fungal isolates were chosen and eight of them were sequenced. Sordariomycetes was the main classes of fungi investigated from the phylum Ascomycota. Similarly, from unculturable endophytic fungal diversity analysis, four different classes Dothideomycetes, Eurotiomycetes, Sordariomycetes from the phylum Ascomycota were discovered. Moreover, root harbors highest diversity of endophytic fungi followed by leaf and stem. Hence, the medicinal plant *Petiveria alliacea* harbor diverse group of endophytic fungi. The method that we implemented was method-dependent process (Guo et al., 2001) and the fungi we isolated were dependent on our methodology. We were unable to identify the all the culturable and unculturable fungal isolates. Future study needs to focus on identifying all the fungal DNA of endophytic fungi. Potentially successful methods that can be implemented in the future study include DNA cloning (Guo et al., 2001), Total genome analysis (Gianoulis et al., 2012).

CHAPTER 5 : SUMMARY AND CONCLUSION

Exploration of new reservoir such as endophytic microbiomes are of great interest to fight against current global problem of antibiotic resistance. Scientists are now being focused on studying endophytic microbiomes as they are being proven as the source of various novel antimicrobial compounds. Therefore, it is very crucial to focus on exploration of native medicinal plants for bioactive compound as it has been used as curative agent since ancient period. Medicinal properties of plant could be sometime contributed by the endophytic microbiomes that lives inside the host plant. Hence more research is needed to explore endophytes from medicinal plants from all around the world for possible discovery of noble and effective drugs against common and resistance microorganisms. In this study, native medicinal plants in South Florida such as *Petiveria alliacea, Agave americana, Annona glabra*, and *Conocarpus erectus* were selected to characterize the endophytic fungi for their possible novel antimicrobial properties. Results from the study demonstrated that medicinal plants that we studied here in South Florida harbors endophytic fungi that have potential to inhibit the growth of different types of microbial strains that cause diseases. Sequence data have revealed that endophytic fungi that was isolated in our project contains similar genetic makeup that have already proven effective against wide variety of pathogens and health conditions. In this study, two medicinal plants were chosen for possible endophytic bacteria that have potential to provide useful antimicrobial compounds. *Petiveria alliacea* and *Agave americana* were selected for exploring endophytic bacteria and the results revealed that some of the

endophytic bacteria isolated from native medicinal plants of South Florida possess potential to kill or inhibit the growth of pathogenic bacteria and fungi.

Sequenced data have proven that endophytic bacteria have similar genetic combination with bacteria which have already proven effective against different varieties of microorganisms. In this study, endophytic fungal biodiversity was also studied in *Petiveria alliacea* from culturable and unculturable endophytic fungi. Results from this study have demonstrated that there is high diversity of endophytic fungi and only the fraction of them were able to grow them in the artificial medium. The result from this study showed that native medicinal plants of South Florida harbor useful endophytic fungi and bacteria that are capable of inhibiting the growth of pathogenic microorganisms which are effective in tackling current global problem of drug resistance pathogens. More research needs to be conducted to characterize all the endophytic microbiomes with more pathogens of interest from the field of health, agriculture, and environment.

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