Isolation, screening and biological activity Of *Ocotea bullata* and *Aloe lettyae*`s endophytes and metabolites

CK Marokane Radebe

orcid.org/ 0000-0003-3596-7142

Thesis accepted in fulfilment of the requirements for the degree *Doctor of Philosophy in Science with Microbiology* at the North-West University

Promoter: Prof RA Adeleke

Co-promoter: Prof SO Amoo

Graduation: October 2025

Acknowledgments

First and foremost, I give all glory and thanks to God Almighty for granting me the strength, perseverance, and wisdom to undertake and complete this research journey. Without His guidance and grace, this work would not have been possible.

I would like to express my deepest gratitude to my promoters, Prof Adeleke and Prof Amoo, for their invaluable mentorship, constructive feedback, and continuous support throughout the course of this study. Your guidance has shaped both the direction of my research and my development as a scholar.

Sincere thanks also go to the academic and technical staff of the Department of Microbiology Faculty of Natural and Agricultural sciences, North West University, for their assistance, encouragement, and provision of the facilities and expertise necessary for this work. I am also grateful to the support staff whose behind-the-scenes contributions made the research process smoother.

I gratefully acknowledge the financial support provided by National Research Foundation (NRF), Agricultural Research Council in collaboration with Department of Agriculture Land Reform and Rural Development ARC/DALRRD and the North West University Unit for Environmental Sciences and Management (UESM), which made this research and related activities possible.

Dedication

This thesis is dedicated to remarkable individuals who have shaped and supported my PhD journey;

Chapter1: To my mommy (Francinah Marokane). Although this chapter is placed at the beginning of my thesis, much of it was written last, inspired by your encouragement for me to pursue my PhD and your constant demand for progress. Just as you would always check my school reports, highlight your concerns and propose methods for improvement, you have consistently pushed me to achieve my goals. I dedicate this chapter to you, as it represents both the beginning and the culmination of my doctoral journey. It confirms that I have met the objectives I set out to achieve. I love you, my Queen.

Chapter2: To my husband and sons (Thabo, Ofentje, Amo, and Kgotso), I dedicate this chapter to you in recognition of your firm support throughout my research journey. Your support, from as early as choosing my research plants to searching for them in the wild, has meant everything to me. Thank you, my dearest, for standing by me every step of the way. The time taken from you will surely be compensated. I deeply appreciate you, my beloveds.

Chapter3: To my former supervisors (Prof Gerhard Prinsloo and the late Prof Jacques Vervoort). You always appreciated my proactive nature, and that forever increased my confidence, curiosity and always reminded me to never think that there is ever a stupid question. I am deeply grateful for all you have taught me in the field of metabolomics. Thanks to your guidance, I now confidently undertake and complete metabolomics tasks with little to no supervision, and I am more eager than ever to explore metabolites, particularly secondary ones, for their potential applications in both human health and agriculture.

Chapter4: To Dr. Raimi and my microbiology lab mates, Class of 2022, thank you for fostering a collaborative and supportive working environment. The mini talk sessions during lab encounters, where methods were questioned and refined, remain both memorable and invaluable in shaping my writing. I also appreciate the seamless sharing of reagents; there was never a moment when progress stalled due to unavailable supplies. Efforts to minimize waste were consistently upheld, and I am especially grateful to “Moreki,” Prof. Rasheed Adeleke, for always ensuring that all necessary resources were available.

Chapter5: To my sisters (Sinah, Melidah and Sandra), you always reminded me that small steps matters, and that planning for something big tomorrow is not as important as the little things you do today. Today, I dedicate this chapter to you because, as I began writing my thesis, this has become my icebreaker. Though brief, it has allowed me to begin, and it

continued to keep me going, one step at a time. Thank you, my boss babes. I adore you dearly.

Chapter6: To my esteemed promoters, Prof Rasheed Adeleke and Prof Stephen Amoo. Your unwavering encouragement and steadfast belief in my abilities have been a guiding light throughout my thesis journey. Your words of wisdom and insistence on perseverance echo in my ears, shaping not just my understanding of my research project but also nurturing my growth as a researcher. Today I dedicate this massive chapter of my thesis to you as a testament to your mentorship and the invaluable lessons you have imparted. Thank you for inspiring me to strive for excellence and to embrace every challenge with determination.

Chapter7: To my brothers (John, David and Khomotjo). You believe in me so deeply, seeing strength in me that I sometimes struggle to see in myself. Today, I dedicate this important and challenging chapter of my thesis to you. Reaching this point proves that I am everything you believe I am and perhaps even more. Thank you my ‘wolf pack’. I love you to the moon and back.

Abstract

This study investigated the potential of endophytes from endangered plants to support sustainable agriculture, species conservation efforts, and human health applications, focusing on *Ocotea bullata* (*O. bullata*) and *Aloe lettyae* (*A. lettyae*). Endophytes, known for synthesizing medicinal, agricultural, and biotechnological metabolites, may provide sustainable solutions for conservation efforts. In addition to the primary focus on the endangered *A. lettyae*, the study included *Aloe longibracteata* (*A. longibracteata*), a closely related non-endangered species, as a comparative model to identify traits beneficial for conservation and stress resilience. Comparative analysis revealed a diverse array of plant growth-promoting metabolites, such as indole-3-acetic acid (IAA) and 5-hydroxy indole-3-acetic acid, with significant implications for species conservation and agricultural applications. Endophytes from both *Aloe* species, predominantly belonging to the *Bacillus* and *Enterobacter* genera, exhibited key plant growth-promoting traits including: 100% of isolates that fixed nitrogen, 35% that solubilised phosphate, 76% that produced siderophores, and IAA production ranging from 10 to 75 µg/mL. These traits collectively contribute to plant health and stress tolerance. This study also emphasized the importance of sustainable resource utilization, particularly by demonstrating the feasibility of harvesting metabolites from the leaves of the endangered *O. bullata*. The results showed that the leaves contain a diverse range of metabolites with potential human health applications, including ascorbate, gentisic acid, gallic acid, and chlorogenic acid, providing a sustainable alternative to harvesting the bark and thereby contributing to the protection of the species. Additionally, the endophytes isolated from *O. bullata* exhibited strong plant growth-promoting properties, including enhancing germination in tomato seeds and exhibiting antimicrobial activity, which could support the plant's health and resilience. The inclusion of *A. longibracteata* also allowed for the assessment of its potential as a donor of beneficial endophytes for transfer to *A. lettyae*, offering a novel conservation strategy for the endangered species. Future research should leverage the optimised metabolite extraction methods to explore additional applications while ensuring that the safety of using these *Aloe* species is thoroughly evaluated. The findings highlight the potential of metabolites derived from these plant species for biofertilizer and biocontrol applications, offering opportunities to develop eco-friendly agricultural solutions while contributing to the preservation of biodiversity.

Keywords: microbial communities, metabolite profiling, sustainability, plant growth promotion, bio stimulants, conservation, bio fertilizers, bio control.

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Preface

The rapid loss of biodiversity, driven by habitat degradation, overharvesting, and climate change, underscores the urgent need for innovative approaches to plant conservation and sustainable resource use. Endophytes, which are microorganisms that live within plant tissues without causing harm are increasingly recognized for their capacity to produce valuable metabolites that support plant growth, enhance stress tolerance, and offer therapeutic potential. This research was conceived at the intersection of conservation biology, microbiology, and sustainable biotechnology, with a focus on two ecologically and medicinally significant South African species: *Ocotea bullata*, a vulnerable medicinal tree, and *Aloe lettyae*, an endangered succulent. To provide a meaningful benchmark for conservation-oriented research, the study also included *Aloe longibracteata*, a closely related but non-endangered species, as a comparative model to *A. lettyae*. Analysing the similarities and differences in endophytic communities and metabolite profiles between *A. lettyae* and *A. longibracteata* allows the identification of traits that may be transferable to support the growth, resilience, and long-term survival of *A. lettyae*. In parallel, sustainable alternatives to harvesting *O. bullata* bark were investigated by evaluating the medicinal potential of its leaves. The work presented here integrates field sampling, microbial isolation, metabolite profiling, and functional assays, offering insights with potential applications in eco-friendly agriculture, medicine, and biodiversity preservation.

Chapter 1

General Introduction

Conservation of endangered plant species faces growing challenges due to habitat loss, climate change, and overharvesting. This research was inspired by a growing interest in the unique potential of plant microbiome, specifically endophytes as allies in promoting plant resilience and adaptability under adverse conditions. While extensive research has demonstrated the benefits of endophytes in agricultural and forestry contexts, their application in supporting endangered plants remains underexplored. This study focused on two threatened South African plant species, *Ocotea bullata* (*O. bullata*) and *Aloe lettyae* (*A. lettyae*), each facing pressures that demand innovative conservation strategies.

The idea for this work grew from observing the resilience that some non-endangered plants exhibit under harsh conditions, leading to the question: could the microbial partners of these hardy plants help enhance the survival and adaptability of endangered species? With advances in metabolomics and genomic sequencing, it has become possible to identify and characterize these endophytes in ways that reveal their potential to support endangered plants in dynamic, natural habitats. This thesis aimed to bridge conservation and microbiology, exploring how interspecies microbial support could assist in re-establishing vulnerable plant populations and informing sustainable practices for preserving plants with medicinal value.

The chapters in this thesis thus present a comprehensive investigation into endophyte driven conservation, extending the relevance of this microbial support to other vulnerable plant species. Chapter 2 begins with a literature review providing a foundational exploration of endophytes in endangered plants, examining their ecological roles and potential applications in conservation. This chapter introduces *O. bullata* and *A. lettyae*, highlighting the pressures these species face and the ecological value of their conservation. Drawing on literature across endophyte biology, plant-microbe interactions, and conservation science, this chapter sets the stage for understanding how endophytes might be leveraged to enhance plant resilience, support population growth, and aid in habitat restoration.

Chapter 3 then focuses on a comparative analysis of endophytic communities between endangered *A. lettyae* and the non-endangered *Aloe longibracteata* (*A. longibracteata*). Using metabolomic and next-generation sequencing (NGS) techniques, this chapter investigates the

diversity and functions of endophytes in these two *Aloe* species. By profiling the metabolites and genomic potential of these microbes, the study aimed to identify specific traits that support plant growth, resilience, and adaptation. This comparison not only sheds light on the microbial partners that might aid in the conservation of *A. lettyae*, but also highlights ways in which similar endophytes could be used to promote health and resilience in other *Aloe* species.

Chapter 4 builds on the findings from Chapter 3, exploring the *in vitro* potential of using endophytes from *A. longibracteata* to support the growth and stress tolerance of *A. lettyae*. Conservation efforts for *A. lettyae* are hindered by its limited adaptability to different environments and its current endangered status. By exploring the potential of endophytes from the closely related but non-endangered *A. longibracteata* to support the growth and stress tolerance of *A. lettyae* under less favourable conditions, this chapter investigated whether these associated microbes could have a potential to help *A. lettyae* survive and thrive under such conditions. This interspecies microbial support strategy is an innovative approach to conservation, offering a potential pathway for re-establishing *A. lettyae* in a broader range of habitats.

Chapter 5 investigated the seasonal dynamics of metabolites in *O. bullata*, a plant valued for its medicinal bark. Overharvesting of *O. bullata* bark has led to its endangered status, underscoring the need for sustainable use and conservation strategies. This chapter begins by comparing the metabolite profiles in the leaves and bark of *O. bullata* to evaluate whether the leaves might serve as an alternative medicinal resource. Following this, seasonal variations in the leaf metabolites are analysed to pinpoint periods of peak concentration, providing guidance for conservative harvesting practices. By identifying optimal time for harvesting, this study promotes strategies that allow O. *bullata* to regenerate, preserving its ecological and medicinal value.

Chapter six examines the seasonal patterns of plant growth-promoting endophytes within the leaves and bark of *O. bullata*. Understanding how endophyte communities fluctuate with the seasons can offer insights into the microbial factors that influence plant health and resilience over time. This chapter characterizes the growth-promoting properties of these endophytes, revealing how these microbial partners might contribute to the survival and regeneration of *O. bullata*. The findings may also provide guidelines for the timing of endophyte-assisted re-establishment efforts, aiming to maximize the benefits of microbial support during critical

growth periods for this endangered species. Additionally, this chapter evaluated the potential of endophytes isolated from *O. bullata* to promote seed germination through tomato seed priming experiments, highlighting their broader applicability in enhancing plant growth.

1.1 Research Aim and Objectives

1.1.1 Aim

The study aimed to investigate the diversity, metabolite production, and potential applications of endophytic microorganisms associated with the endangered *O. bullata* and *A. lettyae*, with a focus on enhancing plant conservation strategies, sustainable medicinal use, and resilience under environmental stress.

1.1.2 Objectives

The objectives of this study are as follows:

- To characterize the diversity and taxonomy of endophytic bacteria in *O. bullata*, *A. longibracteata* and *A. lettyae*.
- To compare the metabolite profiles of endophytic bacteria from endangered *A. lettyae* and non-endangered *A. longibracteata*.
- To evaluate the ability of endophytes from *A. longibracteata* to promote growth and enhance stress tolerance in *A. lettyae* under *in vitro* conditions, assessing compatibility for potential future applications under challenging environmental conditions.
- To explore whether the leaves of *O. bullata* hold medicinal value similar to that of the bark, and to assess the seasonal variation of bioactive compounds in the leaves to support sustainable harvesting practices.
- To study the seasonal dynamics of plant growth-promoting endophytic bacteria from the leaves and bark of *O. bullata*.

1.2 Hypothesis

Endophytes from *O. bullata* and *A. lettyae* are hypothesized to exhibit unique growth-promoting and stress tolerance traits, with potential applications in conservation, sustainable agriculture, and ecosystem resilience. Endophytes isolated from the non-endangered *A. longibracteata* are further hypothesized to have the potential to promote the growth and stress tolerance of *A. lettyae* under less favourable conditions.

1.3 Significance of the study

This study addresses critical conservation concerns by investigating microbial partnerships that may support the resilience and adaptability of endangered plants such as *A. lettyae* and *O. bullata*. Both species face on-going threats due to environmental pressures, overharvesting, and limited natural habitats, leading to their endangered status (Williams et al., 2013, van Wyk et al., 2019, Kremer-Köhne et al., 2020a). By identifying and characterizing beneficial endophytes, this study aimed to provide an innovative approach to conservation that could help maintain and even improve the survivability of these plants in the wild. Endophytic microbes, which live symbiotically within plant tissues, have shown potential in promoting plant growth and increasing tolerance to various stresses, making them promising allies in species preservation efforts (Santoyo et al., 2016, Kumari et al., 2023, Vishwakarma et al., 2021, Sharma et al., 2023, Phurailatpam and Mishra, 2020).

The research into *O. bullata* specifically seeks to balance medicinal demand with ecological preservation by examining the leaves and bark for metabolites composition. Historically, bark harvesting has led to the overexploitation of this species (Vermeulen et al., 2012). By comparing the metabolite compositions of the leaves and the bark (commonly used for medicinal purposes), this study aimed to explore whether the leaves, (a more sustainable plant part), contain similar or even more literature confirmed beneficial metabolites associated with the medicinal properties of *O. bullata*. Furthermore, by analyzing seasonal variations in metabolites composition, it provides a scientific basis for harvesting practices that could minimize ecological damage, allowing *O. bullata* to regenerate naturally and continue its role in traditional medicine and biodiversity.

Beyond individual species, this research underscores the role that native plants and their associated microbes play in ecosystem stability. In addition to studying the endangered *A. lettyae*, inclusion of *A. longibracteata* as a closely related, non-endangered counterpart allowed comparing the two species providing insight into whether microbial partnerships and metabolite profiles differ between an endangered and a non-endangered *Aloe* species, contributing to our understanding of plant resilience and adaptation. Endophytes have been known to improve plant growth and adaptability, traits which are essential for plant species at risk from unfavourable environmental challenges (Kamran et al., 2022, Muhammad et al., 2024, Verma et al., 2021). The findings from this research could have practical applications for restoration and re-wilding projects, contributing not only to conservation but also to local economies that rely on sustainable plant resources. Additionally, insights gained from

endophytes isolated from the *Aloe* species may extend to related plants, suggesting methods to enhance agricultural resilience in environments where stress factors such as drought or salinity are common (Akinsanya et al., 2015, Ameen et al., 2021, Silva et al., 2020)

Finally, this study advances conservation science by integrating microbiology with plant ecology, opening a novel approach to preserving endangered species. Endophytes are an emerging area of interest in conservation biology, but they are rarely applied to endangered species in practical contexts (Smith et al., 2008, Johnston-Monje and Raizada, 2011, Dubey et al., 2020). Therefore, investigating how microbial interactions can support plant health and stress tolerance, this research provides a model that could be applied to other vulnerable species facing similar challenges. The potential for endophytes to contribute to resilience in threatened plant populations represents a promising field of study, highlighting a shift toward ecosystem-based conservation approaches.

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Chapter 2

A literature review on the application of endophytes in biodiversity conservation, plant growth promotion, and medicinal compound discovery: A case study of *Ocotea bullata* and *Aloe lettyae*

Abstract

Endophytic microorganisms, known for their symbiotic relationships with host plants, play critical roles in plant growth, development, and resilience to environmental stresses. While extensive research has been conducted on endophytes in various plant species, the diversity, composition, and functional potential of endophytic communities in endangered plants remain largely unexplored. This review focuses on *Ocotea bullata* and *Aloe lettyae*, two endangered species of significant ecological and economic importance, for which no endophyte studies have yet been reported except for our previous work on Next-Generation Sequencing (NGS) of *Aloe lettyae*. Through a comprehensive literature search, limited studies on endophytes in these species were found, underscoring the urgent need to initiate such research. This gap highlights the potential for discovering novel microbial communities that could contribute to the conservation and biotechnological exploitation of these endangered plants. Few studies on other species within the *Lauraceae* and *Asphodelaceae* families where *Ocotea bullata* and *Aloe lettyae* belong, respectively, have demonstrated the production of bioactive compounds and plant growth promotion by their endophytes, suggesting that *Ocotea bullata* and *Aloe lettyae* may harbour similar, yet unexplored, microbial communities with valuable applications in sustainable agriculture, biotechnology, and medicine. This literature review underscores the importance of exploring these unique endophytic communities to uncover their potential contributions to conservation efforts and the development of novel bioactive compounds.

2.1: Introduction

Endophytic microorganisms have long been recognized as valuable symbiotic partners of plants, residing within plant tissues without causing apparent harm to their hosts (Wani et al., 2015, Hardoim et al., 2015). They play crucial roles in plant growth, development, and adaptation to various environmental stresses (Santoyo et al., 2016). Therefore, endophytes are of particular interest when studying endangered plant species because they can provide crucial support for the survival and adaptation of host plants in challenging environments (Bothe et al., 2010). Endangered plant species often face stresses such as habitat loss, climate change, and limited resources (Leimu et al., 2010). However, endophytes, through their symbiotic relationship can enhance host plant's stress tolerance through various mechanisms enabling the plant to better cope with drought, pests, and diseases (Yuan et al., 2010).

The mechanisms by which endophytes support the host plants include modulation of plant hormone levels, such as increasing the production of stress-related hormones. An example of such hormone is abscisic acid, which helps plants cope with drought and salinity (Salvi et al., 2022). Endophytes can also produce antioxidant enzymes that mitigate oxidative stress caused by environmental pressures (Eid et al., 2019). Additionally, they improve the host plant's nutrient uptake by facilitating access to essential nutrients such as nitrogen, phosphorus, and potassium (Vishwakarma et al., 2021). Some endophytes produce bioactive compounds that protect plants from pathogens and herbivores, further enhancing the plant's ability to thrive in adverse conditions (Ogbe et al., 2020). Therefore, investigating the endophytes of endangered plants allows for the exploration of alternative conservation strategies. Such techniques involve harnessing microbial benefits rather than harvesting plant materials. *Ocotea bullata*, commonly known as the black stinkwood, is a significant and highly valued timber species indigenous to the temperate forests of South Africa (Geldenhuys, 1996). It belongs to the *Lauraceae* family and is recognized for its dark, lustrous wood, which has been extensively used in the furniture industry due to its durability and aesthetic appeal (Gush et al., 2015). Unfortunately, this extensive use, coupled with habitat loss, has contributed to its status as an endangered species, necessitating urgent conservation efforts (Geldenhuys, 2004, Lübbe and Mostert, 1991). Beyond its commercial value, *O. bullata* has a rich history of traditional medicinal use. The stem bark and leaves have been utilized for treating a wide range of human ailments, including diabetes, inflammation, nervous disorders, and headaches (Ogundajo et al., 2018, Hutchings and Scott, 1996, Zschocke et al., 2000). This therapeutic application highlights the potential of *O.*

bullata as a source of valuable compounds that have pharmaceutical relevance. Recent studies have focused on the plant's bioactive compounds, which may hold the key to developing novel therapeutic agents (Ogundajo et al., 2018, Drewes et al., 1997, Horn, 1996).

The medicinal properties of *O. bullata* are believed to be linked to its complex chemical profile, including alkaloids, flavonoids, and other bioactive compounds (Sharma et al., 2022). Given the increasing evidence that endophytic microorganisms can influence or contribute to the production of such bioactive compounds, exploring the endophytic community within *O. bullata* may provide insights into how microorganisms contribute to the therapeutic potential of the host plant. For instance, the efficacy of *O. bullata* in treating urinary bladder infections which are commonly caused by bacterial pathogens such as *Escherichia coli* (Zschocke et al., 2000), highlight the importance of understanding the presence of beneficial microorganisms within plant tissues. While specific microorganisms have not been identified in this context, it is hypothesized that these beneficial microbes may synthesize bioactive compounds that inhibit pathogenic bacteria or enhance the plant's defense mechanisms, supporting its medicinal uses (Strobel et al., 2004, Gouda et al., 2016). Moreover, convincing evidence indicates that adaptation of plants to diverse environmental conditions can enhance their potential to harbour essential microbes with significant benefits for agriculture and medicine (Alsharif et al., 2020, Komaresofla et al., 2019, Soussi et al., 2016, Rho et al., 2018). Similarly, *Aloe lettyae,* a member of the *Aloe* genus and currently classified as endangered, is another plant of significant medicinal interest, though it has not yet been extensively studied. *Aloe* species are renowned for their therapeutic properties, particularly in skin treatment and wound healing (Grace et al., 2008). The classification of *A. lettyae* as endangered is largely due to habitat destruction and alien invasive plants (Reynolds, 1982). *Aloe lettyae* is uniquely adapted to the diverse wood bush granite grassland of Haenertsburg in Limpopo province (Kremer-Köhne et al., 2020b), an environment characterized by variable soil fertility, periodic droughts, and significant temperature fluctuations. These ecological challenges necessitate a high level of resilience and adaptation. The ability of the plants to survive in such conditions suggests potential association with endophytic communities that may confer advantageous traits, such as enhanced drought tolerance, improved nutrient acquisition, and resistance to pathogens.

Aloe lettyae and *O. bullata* represent valuable resources for various industries due to their potential beneficial endophytic microorganisms and their ability to produce unique bioactive compounds. While the bioactive compounds of *O. bullata* are well-documented and utilized

for their therapeutic properties, the bioactive compounds of *A. lettyae* are rarely investigated, highlighting the need for further research into their potential applications. However, the exploitation of these plants through traditional methods, such as harvesting plant organs, may pose a threat to their long-term sustainability. To address these challenges and ensure the conservation of these endangered species, alternative approaches are necessary. One promising approach is the use of microbial technology, which offers innovative solutions for harnessing the potential of these plants without compromising their survival.

Microbial technology has applications in various industries, offering innovative solutions in agriculture, medicine, and environmental management (Ashraf et al., 2021). In agriculture, beneficial microbes such as *rhizobia* and *mycorrhizal* fungi are utilized to enhance plant growth and nutrient uptake, reducing the need for chemical fertilizers (Pierre et al., 2014, Mohammadi et al., 2011). In medicine, microbial fermentation processes are employed to produce antibiotics, vaccines, and other therapeutics that are essential for disease treatment and prevention (Rahman, 2013, Kapoor et al., 2020). Additionally, microbes play a pivotal role in bioremediation, where they are used to degrade pollutants and restore contaminated environments, showcasing their potential for sustainable environmental management (Abatenh et al., 2017). These diverse applications highlight the versatility and potential of microbial technology across different sectors. Within this context, the study of endophytic communities associated with endangered plant species offers a promising avenue for biotechnological innovation (Alsharif et al., 2020, Gao et al., 2022).

Furthermore, by understanding the interactions between these endophytes and their host plants, strategies to enhance plant stress tolerance and growth, contributing to conservation efforts and sustainable resource use can be developed. Harnessing the potential of endophytes from endangered species not only supports the conservation of these plants but also provides an opportunity to tap into a largely unexplored reservoir of bioactive compounds. Integrating traditional knowledge with modern biotechnological approaches could unlock the full potential of plant endophytic communities, offering innovative solutions to global challenges in agriculture, medicine, and environmental sustainability.

The goal of this literature review is to evaluate and highlight research gaps in the study of endophytic communities of endangered *O. bullata* and *A. lettyae*. By highlighting the significance of diverse endophytes and their potential as a substitute source for producing valuable bioactive compounds, we could uncover new endophytic strains within *O. bullata*

and *A. lettyae,* which might contribute to plant growth, stress resistance, or the synthesis of novel and important drugs. This approach not only allows us to harness the benefits offered by host plants before they are completely extinct but also offers a means to conserve the endangered plants by minimizing the need for large scale harvesting.

2.2: Identifying conservation gaps and potential research directions of *Ocotea bullata* and *Aloe lettyae*

The ecological and conservation status of *O. bullata* and *A. lettyae* deserves attention as both species are classified as endangered. A study has focused primarily on the response of *O. bullata* to flooding, which causes plant leaf drop and mortality (Lübbe and Mostert, 1991). Furthermore, studies on *A. lettyae* have examined its interactions with arthropods, particularly the role of honeybees in pollination (Kremer-Köhne et al., 2020b, Aslan et al., 2016). Despite these findings, there remains a significant gap in possible conservation measures and further evaluation of the medicinal and agricultural benefits of these species while minimizing risks to their existence. Table 2.1 provides a comprehensive overview of several endangered plant species, primarily belonging to the genera *Ocotea* and *Aloe*. The endangered plant species listed in Table 2.1 represent untapped reservoirs of endophytic microbial communities, which may harbour novel bioactive compounds or contribute to the ecological adaptability of the plants. However, very few studies have focused on the phytochemicals of the potential role of endophytic microbiome in these two genera, highlighting the need for further research to explore their medicinal and ecological significance (George et al., 2001, Jäger and Staden, 2005, Khumalo et al., 2023). The endophytes from these endangered species may have the potential to contribute to sustainable agriculture by enhancing plant growth, improving stress tolerance, and protecting against pathogens and pests and therefore, deserve exploration before they vanish forever. Additionally, each plant species faces unique threats and environmental pressures, which may influence the diversity and functionality of its endophytic microbiome. Investigating the endophytes from these species can provide insights into how microbial communities adapt to changing conditions and how their bioactive compounds can benefit both plant health and human well-being.

Traditional harvesting methods and habitat loss pose a significant threat (Table 2.1) to medicinal plants worldwide. Overexploitation of *O. bullata* for its bark used in traditional medicine has led to a decline in its population (Vermeulen et al., 2012). Similarly, the removal of native plants, including *O. bullata*, and their replacement with alien vegetation, as

well as the introduction of detergents into streams, impact *A. lettyae* habitat (Bamigboye, 2020). The lack of alternative sources of forest products and income-generating activities for local communities further exacerbates these threats (Zilihona et al., 1998). The physiological response of *O. bullata* to their bark harvesting, with smaller trees being more vulnerable, highlights the need for sustainable harvesting practices (Vermeulen et al., 2012). Therefore, it is crucial to address these threats by adopting sustainable harvesting methods and possibly conserving their natural habitats.

When medicinal plants are harvested for their therapeutic purposes, the ultimate targets are the bioactive compounds they contain. Previous research has extensively demonstrated that endophytes possess the capability to produce bioactive compounds comparable to that of their host with significant medicinal and agricultural importance (Gouda et al., 2016, Singh et al., 2017, Sharma et al., 2021). Therefore, utilizing endophytes for bioactive compounds production presents a far more sustainable approach compared to the direct harvesting of the entire plant organs. This approach not only mitigates the risk of plant extinction but also minimizes ecological impacts such as habitat destruction, soil degradation, and the disruption of local biodiversity. Even in cases where endophytes reside in non-sustainable parts such as the bark, isolating them requires only minimal samples, making it a minimally invasive and non-destructive alternative to collecting large amounts of plant material for metabolite production.

Table 2.1: An overview of some of the endangered *Ocotea* and *Aloe* species with their conservation status in South Africa and globally

Genus	Species	Use in trade	Conservation status	Endemism/distribution	Threats	Conservation plans	Reference
Ocotea	*O. rigens*	Unknown.	Endangered (IUCN)	Brazil	Deforestation and habitat destruction through conversion to agriculture and plantations.	This species occurs in at least one protected area.	de Kok, 2023
	O. rufovestita	Unknown.	Endangered (IUCN)	Madagascar	The major threats to this species are deforestation and habitat destruction through conversion to agriculture, grazing and plantations.	Unknown	de Kok, 2023
	O. bullata	*Ocotea bullata* has been logged for its timber, which was widely used for making furniture. Its	Endangered (SANBI)	South Africa	The main threats are timber logging in the past, and bark harvesting in the recent past, present and future.	This species was assessed as endangered in the Red List of South African Plants (Raimondo et al. 2009). The species is protected by South African	Williams et al., 2023

		bark is also harvested for traditional medicine.				Forestry Legislation as well as provincial Nature Conservation Legislation. There have been approved projects to grow the species and to provide a regular supply of bark to herbalists (Pooley 1998).	
	O. salvinii	Unknown	Endangered (IUCN)	Guatemala	The habitat of this species has been partially destroyed throughout its range due to agriculture, especially potato farming in Guatemala.	This species is not known to occur in any protected areas. This species is not kept in any ex situ collection (BGCI 2020).	de Kok and Hills, 2023.
	O. truncata	Unknown	Critically endangered (IUCN)	Mexico	The major threats to this species are deforestation and habitat destruction through conversion to agriculture and	This species is not maintained in any ex situ conservation collection (BGCI, 2020). It is found in the Lagunas de	Salas et al., 2023.

					plantations, as well as fires.	Montebello National Park, located in Chiapas, southern Mexico. The species was evaluated as endangered (EN) by the Red List of the Mexican Cloud Forest Trees (González-Espinosa et al., 2011).	
	A. pearsonii	Used as a laxative and to heal wounds.	Critically endangered (SANBI)	South Africa	Anthropogenic climate change is the primary threat to this species, with the increases in dry spell duration, mean and maximum temperatures in the Richtersveld having caused mass die-off in formerly large subpopulations of this species during	The species occurs within the protected area of Ais-Richtersveld Transfrontier Park. Unfortunately grazing occurs within the Richtersveld section of the park, furthermore the main threat which is drought related mortality cannot be	Raimondo et al.,2022.

					the extended drought that took place between 2015 and 2020.	mitigated by protection.	
Aloe	*A. francombei*	Unknown	Critically endangered (IUCN)	Kenya	The species is threatened by frequent fires which impacts seedling recruitment. Some living mature individuals were observed to be dying apically from the centre of the plant or side of the leaves in 2015, the cause of mortality is unknown. This species is also predated by game during periods of drought.	It occurs within a private conservancy (Laikipia Nature Conservancy/Ol Ari Nyiro Conservancy). In addition, a few individuals of the species are planted at the National Museums of Kenya (NMK) Botanic Garde n.	Matheka and Nyamai, 2022. 2024.
	A. babatiensis	It is used by local people	Critically endangered (IUCN)	Tanzania	This species is threatened by land	This species is recorded from	Mollel, 2022

		for medicinal purposes and for local brew flavouring. The species is grown by specialist collectors.			use change, where areas are being cleared for agriculture.	seven ex situ collections (BGCI 2022)	
	A. lettyae	Unknown	Endangered (SANBI)	South Africa	*Aloe lettyae* is endemic to the Critically Endangered Woodbush Granite Grassland, of which less than 30% remains intact, mainly due to extensive loss to timber plantations and agricultural expansion between 1950 and the late 1970s, and only about 10 relatively	Occurs in at least one protected area in Haenesburg	von Staden and Kremer-Köhne, 2015.

					undisturbed fragments remain, the rest being severely overgrazed and infested with unmanaged alien invasive plants. However, even relatively well-preserved fragments such as the Haenertsburg Townlands continue to be degraded as a result of inappropriate fire manag ement, which is possibly the cause of increasing encroachment by woody species.		
	A. conf usa	Used for dying cloth.	Endangered (IUCN)	Tanzania	There are no known species-specific threats other than minor collection by	This species is in many private collections/gardens in Tanzania but	Luke, 2022

					Aloe enthusiasts. The habitat on the southern slopes of Mt Kilimanjaro is threatened by rapid expansion of infrastructure and human settlement. These deep river gorges are subject to increasingly irregular water flows both as a result of water extraction and climate change. The scale of this impact is not known.	there are no known in situ conservation actions and it has not been recorded from any protected areas. It is recommended that targeted surveys are made for this species to see if it is found in other localities.	
	A. elgonica	Unknown	Endangered (IUCN)	Kenya and Uganda	This species is under pressure from degazettement of part of Mt Elgon. The locality at Kapsabet is likely to have been cleared by	This species does not occur in any protected areas.	Amani et al., 2022

					urbanisation. The locality on Eldoret Rd to Kitale is also suspected to have been cleared due to urbanisation.		
	A. ikiorum	Unkown	Critically endangered (IUCN)	Uganda	This species was collected close to agricultural land, by a pathway, and so is presumably tolerant to some disturbance. However, it is likely that there has already been some loss of individuals of this species due to clearing of land for agriculture. The population of Kaabong District has shown a sustained increase	Subjected to ex-situ conservation	Richards et al., 2022

					in recent years (Action Against Hunger & iRiS 2017) while it has been observed that, due to drought, cattle raids and hunger further north, some Ik communities have moved to the Timu area in re cent decades in search of greater security (Cole 2015, IPC 2021, Brinkhoff 2022).		
	A. lukeana	Nursery propagation of this aloe has allowed it to be traded as an ornamental; although it is not widely	Endangered (IUCN)	Uganda	Vegetation fires occur regularly on Mount Morungole near the borders of South Sudan but plants are not killed and regeneration after fire is evident from numerous	Subjected to ex-situ conservation	Richards et al., 2022

		sold it appears to be a desirable species for collectors.			seedlings observed (Cole 2015).		
	A. ngutwaensis	Aloes are known as important sources of medicine (for animals and humans), and cosmetic products, among other uses. Although there are no documented uses for Aloe ngutwaensis, it could be substituted or	Critically endangered (IUCN)	Kenya	The habitat is at risk of destruction for expansion of land for agriculture or timber plantations.	Three individuals of the species are planted at the Nairobi Botanic Garden, National Museums of Kenya.	Matheka, 2022.

		mistakenly used for one reason or another by the local community. The species is grown by specialist collectors.					
	A. pillansii	Ornamental	Critically endangered (SANBI)	South Africa	Livestock farming & ranching	Occurs in at least one protected area	Swart et al., 2022
	A. ramosissima	Sporadic illegal collection events have taken place in the past, but this species is not very popular in trade and collection levels are low.	Endangered (SANBI)	South Africa	Mining & quarrying. Droughts. Gathering terrestrial plants	Occurs in at least one protected area	Raimondo et al.,2022

2.3: Medicinal and economic importance of *Ocotea bullata* and *Aloe lettyae*

Ocotea bullata (Figure 2.1) is a plant of significant medicinal and economic importance. When it was still abundant between the 1980s and 1990s, it was among the top ten medicinal plants sold in traditional medicine markets in KwaZulu-Natal (Cunningham, 1991). The part of the matured plant mostly used for medicinal purposes is the bark (Zschocke et al., 2000). Traditionally, the bark is sniffed or burned and inhaled to relieve headaches (Zschocke et al., 2000). Though local use for urinary tract problems is less common, *O. bullata* bark is used traditionally for stomach problems and, in some cultural practices, as an emetic in the treatment of emotional or nervous disorders, presumably to "purge" or reset the individual (Hutchings and Scott, 1996). High demand resulting from the need of the growing population as well as destructive bark harvesting by unskilled foragers have led to the near extinction *of O. bullata* (Cunningham, 1991). Most research efforts on *O. bullata* focused primarily on the phytochemistry of this plant, where major compounds in the lipophilic bark extract were isolated and identified as the new neolignan ocobullenone and its structural isomer iso-ocobullenone (Sehlapelo et al., 1993, Drewes et al., 1997).

Figure 2.1: *Ocotea bullata* tree with enlarged sections of bark and leaves. Picture taken at the University of KwaZulu-Natal South Africa (29.616819 S, 30.394263 E) in July 2022.

The medicinal and economic importance of *O. bullata* underscores the urgency of investigating this important lesser-known and endangered plant species. Even though *O. bullata* has not been fully researched for its chemical components, it promises various applications. By studying and unlocking the potential of this endangered plant before it disappears, valuable resources that might otherwise be lost forever may be discovered. Similarly, *A. lettyae* is another lesser-known plant species listed as endangered. Similar to *O. bullata*, *A. lettyae* holds significant potential due to its unique properties, such as drought tolerance, ecological adaptations, and status as an endemic species; even though the microbial and phytochemical compositions of this species have not yet been explored.

Figure2.2: *Aloe lettyae* in the grassland of Haenertsburg in Limpopo Province

Aloe lettyae (Figure 2.2) has significant ecological and economic potential due to its adaptability to various environments. While A. *lettyae* itself has not yet been comprehensively studied for its medicinal potential, the *Aloe* genus is commercially important, with several species supporting various industries (Adlakha et al., 2022, Salehi et al., 2018, Grace et al., 2008). For example, *Aloe vera* is widely used in the cosmetic, pharmaceutical, and food industries due to its soothing and moisturizing properties (Eshun and He, 2004). *Aloe ferox* is another species known for its use in traditional medicine and skincare products, contributing significantly to the global aloe industry (Fox et al., 2014, Jia et al., 2008).

Aloe vera, a close relative of *A. lettyae*, has been identified as a suitable alternative plant for sustainable rural development in arid regions such as northern Mexico due to its ecological adaptation and high-water use efficiency (Golmohammadi, 2022). The *Aloe* genus, including *A. lettyae*, has strong development potential and prospects because of its ability to thrive in diverse environmental conditions, its potential for novel applications in pharmaceuticals and cosmetics, and its roles in promoting sustainable agricultural practices (O Lawal et al., 2021, Singha et al., 2024, Sharma et al., 2019).

However, considering the looming threat to the survival and genetic diversity of *A. lettyae* and *O. bullata*, bioprospecting the microorganisms, including bacteria, fungi, and actinomycetes, residing within the internal tissues of these plants through symbiotic association is a sustainable approach to further understand and leverage their benefits. A major group of these microorganisms is known as endophytes, which have demonstrated diverse adaptations and are recognised for their production of bioactive compounds, primarily contributing to the medicinal and other beneficial activities observed in plants (Pimentel et al., 2011, Strobel et al., 2004, Gouda et al., 2016).

2.4: Endophytic diversity and plant adaptation

Endophytic diversity includes the array of microorganisms inhabiting plant tissues without causing harm, comprising bacteria, fungi, and actinomycetes (Govindasamy et al., 2013, Basit et al., 2021, Hardoim et al., 2015). This diversity varies across plant species and different environment and it is influenced by various factors such as host genotype and microbial interactions (Brown et al., 2020). Figure 2.3 provides an overview of current research on microbial diversity, bioactivity, and plant growth promotion within the *Lauraceae* and *Asphodelaceae* families, with a focus on endangered species such as *O. bullata* and *A. lettyae*. The *Lauraceae* and *Asphodelaceae* families dominate the literature, with significant studies on microbial diversity, bioactivity, and growth promotion. In contrast, research on *O. bullata* is lacking in the area of microbial diversity, with only limited studies on bioactivity and no attention given to plant growth promotion. *Aloe lettyae* has the least

representation in published research on microbial diversity, bioactivity, and plant growth-promoting potential. This highlights notable research gaps for *O. bullata* and *A. lettyae*.

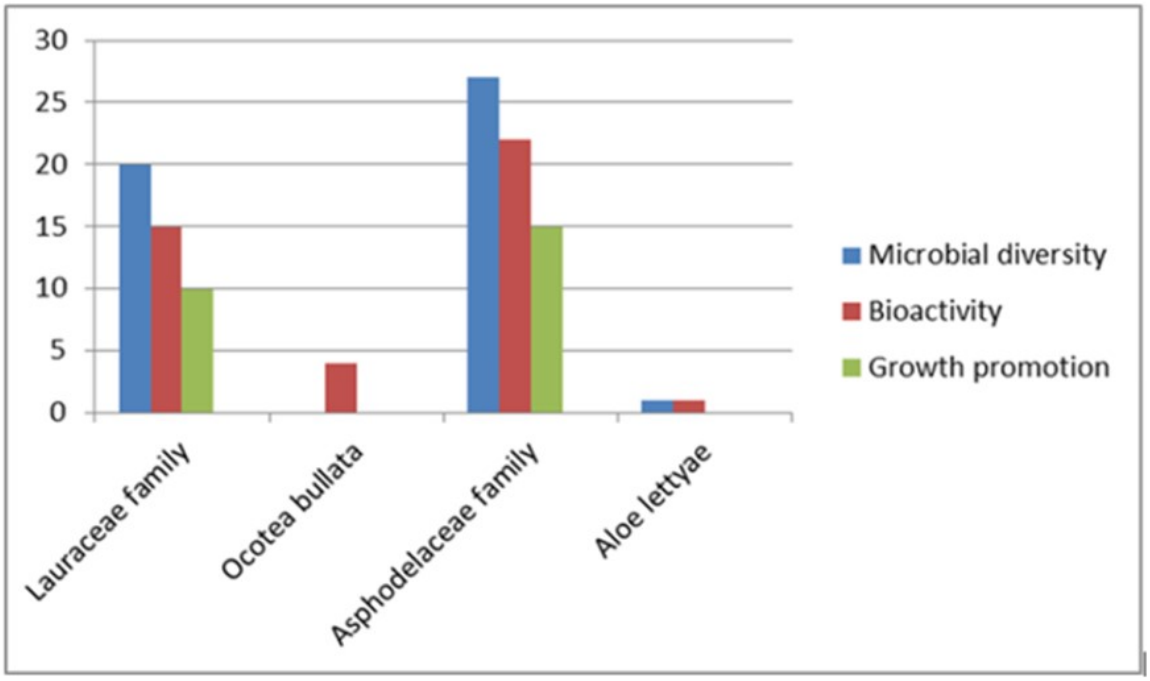

Figure2.3 Research coverage on microbial diversity, bioactivity, and growth promotion in *Lauraceae* and *Asphodelaceae* families, with specific emphasis on *O. bullata* and *A. lettyae*. Studies were categorized based on the following criteria: Microbial diversity includes research analyzing microbial communities (e.g., 16S rRNA sequencing); bioactivity covers studies identifying or analyzing bioactive compounds or metabolites; growth promotion involves studies showing microbial or plant growth benefits. Data were sourced from relevant literature databases (e.g., PubMed, Scopus, Web of Science) using specific query terms related to microbial diversity, bioactivity, and plant growth promotion in *Ocotea* and *Aloe* species. Studies were selected based on predefined inclusion and exclusion. Categories with no studies are not shown in the graph.

Understanding endophytic diversity is important for discovering their ecological functions and potential applications in agriculture, biotechnology, and medicine (Burragoni and Jeon, 2021). Endophytes enhance host plant resilience to environmental stressors and help plants adapt to changing conditions. They do this through several mechanisms of action which include the ability to assist the host plant with nutrient uptake through synthesis of enzymes that facilitate the absorption of minerals from soil or by nitrogen fixation, which is essential for plant growth (Rawat et al., 2018). In addition to improving nutrient availability, endophytes produce a range of bioactive compounds such as antimicrobial agents, antioxidants, and phytohormones (Solanki et al., 2022, Akhtar et al., 2019). These compounds not only help protect the plant against pathogens and

herbivores but also promote plant growth and stress tolerance (Anwar et al., 2016). In addition, many of these bioactive compounds, such as alkaloids, flavonoids, terpenoids, and phenolic acids, have been shown to possess significant medicinal properties. These include antimicrobial, anti-inflammatory, antioxidant, and anticancer activities, making them valuable sources for drug discovery and therapeutic applications (Alvin et al., 2014, Gouda et al., 2016). Endophytes, through the production of these compounds, contribute not only to the health of the host plant but also to the potential medicinal value that can be harnessed for human health and pharmaceutical development.

Another key mechanism by which endophytes enhance plant resilience is through the induction of systemic resistance (ISR) (Santoyo et al., 2022). When plants interact with beneficial endophytes, the microorganisms trigger the immune system of the plant, preparing it to respond more rapidly and effectively to environmental stresses or pathogen attacks (Mengistu, 2020). This priming effect enhances the plant's ability to withstand biotic and abiotic stress, such as drought, salinity, and pest infestations(Mengistu, 2020). By producing compounds like hydrogen cyanide or volatile organic compounds, endophytes can also suppress pathogen growth and create a more favourable environment for the growth of the host plant (Basit et al., 2021).Therefore, endophytes contribute to plant adaptation by improving nutrient uptake, producing protective compounds, and enhancing systemic resistance, making them valuable tools for improving plant health and resilience in both natural ecosystems and agricultural systems.

2.5: Rationale for screening endophytes for biotechnological use in drug discovery

In recent years, the potential of endophytes in biotechnology and drug discovery has gained significant attention due to their ability to synthesize a diverse array of bioactive compounds (Singh et al., 2021a, Raimi and Adeleke, 2021, Zotchev, 2024). The chemical components of the host plant play a critical role in shaping the metabolic capabilities of endophytes by providing a rich and complex chemical environment that influences their metabolic pathways (Hardoim et al., 2015, Sharma et al., 2023). These components include primary compounds like sugars and amino acids, which serve as nutrients for the endophytes, as well as secondary compounds such as alkaloids, flavonoids, and terpenoids, which can act as signalling molecules or precursors for endophyte-produced compounds (Nawrot-Chorabik et al., 2022). This intricate chemical interplay can induce endophytes to activate specific biosynthetic pathways, leading to the production of unique and potentially bioactive compounds that reflect the chemical profile of their host (Li et al., 2022). By adapting to the host's chemical environment, endophytes can develop specialized metabolic functions that enhance their ability to synthesize compounds of pharmaceutical interest through mechanisms such as gene expression modulation and horizontal gene transfer (Sharma et al., 2023,

Alam et al., 2021). This relationship underscores the importance of understanding the chemical ecology between plants and their endophytes in bioprospecting efforts.

Another fascinating aspect of endophyte research is the capacity for genetic exchange between host plants and their endophytes (Mishra et al., 2021a). This exchange can provide endophytes with traits that enable the production of compounds similar to those of their hosts (Ludwig-Müller, 2015, Mishra et al., 2021a). By leveraging the biosynthetic capabilities gained through horizontal gene transfer, endophytes can produce compounds that mimic or even enhance the biological activities of its host (Torres et al., 2012). Such genetic interactions not only increase the chemical diversity obtainable from endophytes but also open up possibilities for discovering compounds with superior pharmacological profiles (Staniek et al., 2008, Torres et al., 2012).

The production of comparable compounds by endophytes offers a sustainable, alternative, and cost-effective source for drug discovery (Gupta et al., 2020, Asogwa et al., 2024). Instead of relying on the extraction of bioactive compounds directly from plant materials, which can be resource-intensive and unsustainable, endophytes provide a renewable source that can be cultivated under controlled conditions. This approach reduces the pressure on wild plant populations and aligns with conservation efforts to protect endangered species. By leveraging endophytes as a source of bioactive compounds, we can contribute to biodiversity conservation while advancing the development of new, eco-friendly pharmaceuticals. This strategy not only meets the growing demand for sustainable and ethical drug discovery practices but also promotes the exploration of nature's untapped microbial chemical diversity for innovative therapeutic solutions.

Several findings have confirmed endophytes, including fungi and bacteria, as a promising reservoir of bioactive compounds with significant potential for drug discovery and diverse biotechnological applications (Raimi and Adeleke, 2021, Singh et al., 2021a, Abdel-Razek et al., 2020). Their bioactive compounds exhibit a spectrum of biological activities including antioxidant, antifungal, anti-malaria, anti-inflammatory, antidiabetic, anti-HIV and anticancer properties, underscoring their importance in the search for novel pharmaceutical agents (Aharwal et al., 2016, Khan et al., 2023, Dembitsky et al., 2021). Moreover, the endophytic fungus, *Aspergillus sydowii* has shown the ability to produce a compound with immunosuppressive activity, making it valuable in minimising organ rejection in transplant patients (Kaur et al., 2021a). However, further elucidation of the intricate dynamics governing endophytic communities and the mechanisms underlying their bioactive compound production remains important for unlocking their full potential (Mishra et al., 2021a).

The biotechnological potential of endophytes is further underscored by the type and attributes of their host plants, which include being medicinal and economically important species such as *Aloe vera, Moringa oleifera, Artemisia annua* and many more (Table 2.2). Screening endophytes from these plants for biotechnological use in drug discovery aligns with the rationale of leveraging natural biodiversity to discover novel drug candidates and therapeutic agents.

Table 2.2: Endophytic microorganisms and their biological activities in drug discovery

Endophyte	Microbe type	Host plant/Source of isolation	Bioactive compound	Bioactivity	References
Thielavia subthermophila	Fungus	*Hypericum perforatum*	Hypericin	Antimicrobial	(Kusari et al., 2009)
Bacillus subtilis	Bacterium	*Zea mays*	Bacilysocin	Anti-fungal	(Fiddaman and Rossall, 1993, San-Lang et al., 2002, Bolivar-Anillo et al., 2021)
Alternaria alternata	Fungus	*Jatropha heynei*	Kaempferol	Anticancer	(Ashoka and Shivanna, 2023)
Penicillium citrinum	Fungus	*Tragia involucrata*	Quercetin	Anticancer	(Danagoudar et al., 2018)
Pseudonocardia sp.	Actinomycete	*Artemisia annua*	Artemisinin	Anti-malaria	(Li et al., 2012)
Chaetomium globosum	Fungus	*Moringa oleifera*	Catechin, Chlorogenic acid, Coumaric acid, Kaempferol	Antioxidant	(Kaur and Arora, 2020)
Aspergillus nidulans	Fungus	*Ocimum basilicum*	Hexadecanoic acid	Antioxidant	(Sharaf et al., 2022)
Fusarium sp.	Fungus	*Mentha longifolia*	Fusaristerols B	Antiinflamatory	(Mohamed Ibrahim et al., 2018)
Streptomyces sp.	Actinomycete	*Bruguiera*	Xiamycin	Anti-HIV	(Liu et al.,

		gymnorhiza			2016)
Penicillium canescens	Fungus	*Juniperus polycarpos*	Methylxanthone	antidiabetic	(Malik et al., 2020)
Streptomyces sp.	Actinomycete	Unknown	Saadamycin	Anti-dermatophyte	(El-Gendy and El-Bondkly, 2010)
Nigrospora oryzae	Fungus	*Triticum sp*	Pipecolisporin	Anti-malaria	(Fernández-Pastor et al., 2021)
Aspergillus sydowii	Fungus	*Scapania ciliata*	Emodin	immunosuppresive	(Song et al., 2013)
Bacilus cereus	Bacterium	*Miquelia dentata Bedd*	Camptothecine	Anti-cancer	(Shweta et al., 2013)
Pseudomonas hibiscicola	Bacterium	*Aloe vera*	1,1-diphenyl-2-picrylhydrazyl (DPPH)	Antioxidant	(Akinsanya et al., 2015)
Pseudocercospore sp	Fungus	*Elaeocarpus sylvestris*	Terric acid	Antioxidant and antibiotic	(Prihantini and Tachibana, 2017)
Rhodiola spp	Plant associated	*Rhodiola rosea*	P-tyrosol, rosavins and salidrosides	Antioxidant, antidepresant	(Grech-Baran et al., 2015)

2.6: Rationale for screening endophytes for plant growth promotion

The rationale for screening endophytes for plant growth promotion lies in their mutualistic association with host plants, which provides a foundation for sustainable agricultural practices. Figure 2.4 below highlights the symbiotic relationship between endophytes and their host plants, where both parties cooperate for mutual survival and benefit (Saikkonen et al., 2004, Jha et al., 2018). This cooperation is particularly crucial for their continued existence under environmental stress conditions, where endophytes can help their hosts withstand adverse factors such as drought, salinity, and pathogen attacks (Pathak et al., 2022). By enhancing the plant's ability to adapt and thrive in various environments, endophytes contribute to improved crop yields and resilience,

making them valuable assets in agricultural systems (Omomowo and Babalola, 2019, Dey et al., 2018).

Another key aspect of this mutualistic relationship is the compatibility of endophytes with a wide range of plant species, which enhances the efficiency of inoculum applications. Endophytes have evolved to colonize diverse hosts, often establishing themselves in the plant's endosphere, including roots, stems, and leaves (Compant et al., 2021). This compatibility allows for the development of broad-spectrum biofertilizers and biostimulants that can be applied across different crops, thereby improving agricultural productivity on a larger scale (Sani and Yong, 2021). Additionally, the ability of rhizobacteria to colonize the plant endosphere underscores their role in forming a stable and beneficial plant-microbe interaction, further enhancing plant growth and health (Backer et al., 2018).

Furthermore, the comparison between gnotobiotic plants (those grown in sterile conditions) and plants in their natural environments highlights the importance of the host plant's microbiome. Gnotobiotic plants often exhibit less vigour and lower resistance to stress compared to their counterparts with a diverse microbiome, underscoring the critical role endophytes play in plant health and development (Molina et al., 2020). This understanding drives the need to identify and harness effective plant growth-promoting endophytes (PGPEs), as they offer a natural and sustainable means of enhancing crop performance and resilience in the face of growing agricultural challenges.

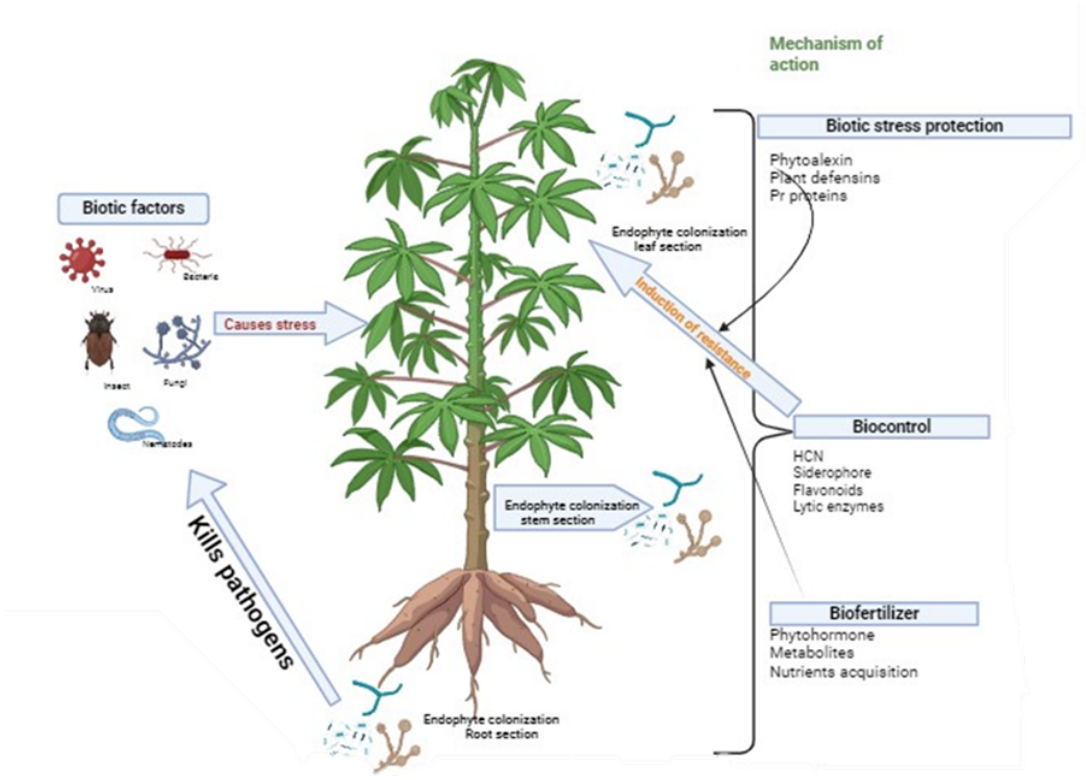

Figure 2.4: The role of endophytes in crops, encompassing functions such as bio-fertilization, bio-control, and biotic stress management.

Plant growth-promoting endophytes, particularly *Pseudomonas* and *Bacillus* species, are best considered the most promising candidates for indirect stimulation of plant growth (Santoyo et al., 2012). In addition, nitrogen conversion, increasing the bioavailability of phosphate, iron absorption, the development of specific enzymatic activity and plant protection against harmful pathogens through the production of antibiotics can also successfully improve the quality of crops in agriculture (Elnahal et al., 2022, Sharma et al., 2020, Meena et al., 2017). Therefore, based on mechanism of action, PGPEs can be classified into four categories: biofertilizers, phytostimulators, biopesticides, and biocontrol agents (e.g., biofungicides) (Riaz et al., 2021, El-Saadony et al., 2022, Pirttilä et al., 2021).

The classification of PGPEs is essential for understanding their different roles in agriculture. Table 2.3 provides a detailed breakdown of these classifications, highlighting their definitions, mechanisms of action, and specific examples of endophytes. Additionally, it includes information on registered products and the types of plants that benefit from these endophytes.

Table 2.3: Form of plant growth-promoting endophytes and their mechanism of action in stimulating plant growth.

PGPE form	Definition	Mechanism of action	Example of endophytes	Registered products	Plants grown	References
Biopesticide	Biological substances or organisms that promote plant growth by damaging, killing or rappelling organisms seen as plant pests.	Production of antibiotics, siderophore and hydrogen cyanide.	*Pseudomonas fluorescens, Bacillus subtilis*	Bio-save, Serenade,	Tomato, wheat, rice	(Sudakin, 2003, Liu et al., 2021, Fischer et al., 2013, Woo et al., 2014, Kulimushi et al., 2021, Commare et al., 2002, David et al., 2018)
Phytostimulator	A substance/compound that enhance the growth, development, or productivity of plants.	Production of phytohormones such as indole acetic acid, cytokinin, gibberellin, salicylic acid, auxin and ethylene.	*Azospirillum brasilense, Pseudomonas putida*	Plant Growth Promoting Rhizobacteria (PGPR) products	Maize, soybean, lettuce	(López-Hernández et al., 2023, Mangmang et al., 2015, Costa-Gutierrez et al., 2020, Pradebon et al., 2024, Yakhin et al., 2017)
Biofertilizer	A biofertilizer is a substance containing living microorganisms that, when applied to seeds, plant surfaces or soil, colonize the rhizosphere or interior of the plant and promote growth by	Utilization of insoluble phosphorus and biological nitrogen fixation.	*Rhizobium leguminosarum, Azotobacter chroococcum*	Mycorrhizal inoculants	Legumes, cereals, vegetables	(Chakraborty and Akhtar, 2021, Sekar and Karmegam, 2010, Ibrahim and El-Sawah, 2022, Faye et al., 2013)

	increasing the supply or availability of primary nutrients to the host plant					
Biocontrol agents	Organisms that control plant pathogens and pests, enhancing plant health and growth.	Production of lytic enzymes, competition for space and nutrients, induced systemic resistance	*Trichoderma harzianum*, *Bacillus amyloliquefaciens*	Biofungicides like *Trichoderma spp*.	Grapes, strawberries, ornamental plants	(Labuschagne et al., 2011, Egamberdieva et al., 2023, Abdullah et al., 2008, Yao et al., 2023, Woo et al., 2014)

2.7: Mechanisms of endophyte-mediated plant growth promotion and bioactive compound production

Beyond growth promotion, endophytes contribute to plant resilience by inducing systemic resistance (ISR) and enhancing abiotic stress tolerance (Tsotetsi et al., 2022). The ISR primes the plant's immune system, allowing it to mount a quicker defense against pathogens, while osmoprotectants and antioxidant enzymes produced by endophytes help the plant endure environmental stresses like drought and salinity (Pieterse and Van Wees, 2015, Phurailatpam and Mishra, 2020). Furthermore, many endophytes modulate plant metabolic pathways and are capable of producing bioactive compounds, including phenolics, alkaloids, and terpenoids, which are similar to the bioactive compounds synthesized by the host plants (Ogbe et al., 2020, Alurappa et al., 2018, Singh et al., 2017). These compounds not only play a key role in plant defense but also have significant pharmaceutical and biotechnological potential, such as antimicrobial, antioxidant, or anticancer compounds (Savi et al., 2015).

2.8: Comparing sampling methods: Endophyte isolation vs. bioactive compound extraction from endangered plant species

To be utilised for biotechnological applications, endophytes must first be effectively isolated, identified, and characterised. This process is essential to link microbial identity with functional traits such as bioactivity, plant growth promotion, or stress tolerance. However, when working with endangered plant species, methodological choices must balance scientific objectives with conservation ethics. In comparing the methodologies for endophyte isolation and the direct

extraction of bioactive compounds, it is important to address the specific challenges and limitations inherent to each approach (Table 2.4).

Table 2.4 highlights the principles underlying culture-dependent and culture-independent methods, focusing on their relevance to microbial studies. While culture-dependent methods involve isolating and studying microorganisms by growing them on laboratory media, culture-independent methods analyze microbial DNA directly from environmental or plant samples without cultivation (Stefani et al., 2015). These methods can significantly influence the detection and extraction of bioactive compounds, as culture-dependent approaches may overlook non-culturable microorganisms that could harbour valuable bioactive molecules. Conversely, culture-independent methods allow access to the genetic information of all microbial community members, offering a broader perspective on potential bioactive compound producers(Culligan et al., 2014). Leveraging both approaches can help achieve a more comprehensive understanding of the microbial communities associated with endangered plants. However, regardless of the method used, certain preliminary steps, such as surface sterilization, are crucial for isolating endophytes effectively and minimizing contamination.

Surface sterilization is a foundational step in the process of isolating endophytes. This step aims to remove external microbial communities and contaminants while preserving the internal microbial communities (Anjum and Chandra, 2015). The choice of sterilizing agents, such as ethanol or sodium hypochlorite, and the duration of sterilization are critical factors for successful sterilization. Over-sterilization may cause loss of endophytes, while under-sterilization can result in contamination by external microorganisms or non-endophytes, thereby complicating the isolation process, suggesting the need for process optimisation to obtain the precise time and concentrations of reagents required for an efficient sterilisation (Waheeda and Shyam, 2017)

The selection of growth media further influences the success of endophyte isolation. Media such as Potato Dextrose Agar (PDA), which is selective for fungi and general-purpose media like Luria-Bertani (LB), and Nutrient Agar (NA) are frequently employed. While PDA specifically supports fungal growth, LB and NA are non-selective and allow the growth of a wide range of microorganisms, including both endophytes and non-endophytes (Rat et al., 2021). This can be beneficial for capturing a broad diversity of endophytes but may also result in the growth of contaminants or non-target organisms (Rat et al., 2021). In contrast , selective media are designed to favour the growth of specific groups of microorganisms by incorporating certain nutrients, inhibitors, or antibiotics that suppress unwanted microbes while promoting the growth of the target endophytes (Karwehl and Stadler, 2016). While this can be advantageous for isolating particular strains of interest, it also introduces a bias, as only those endophytes that can thrive under such

selective conditions are likely to be detected. This selective pressure can lead to an underrepresentation of the full diversity of endophytes present within the plant tissue, skewing the overall community profile (Johnston-Monje et al., 2014). Therefore, the decision to use selective or non-selective media during endophyte isolation plays a crucial role in shaping the diversity of endophytes that are ultimately recovered.

Furthermore, sampling different plant organs such as leaves stems, flowers and roots for endophyte isolation add another layer of complexity due to the inherent variability in endophyte communities across these tissues. Each plant organ provides a distinct microenvironment, influenced by factors such as nutrient availability, moisture content, and the presence of specific plant compounds (Amitrano et al., 2021, Reichstein et al., 2014, Ehlers et al., 2020). These variations can lead to significant differences in the composition and diversity of endophyte populations inhabiting each organ (Reichstein et al., 2014). Endophytes in roots may be more adapted to soil-associated conditions, such as lower oxygen levels and higher microbial competition, whereas those in leaves may have evolved to cope with higher levels of light, radiation, and different chemical defences produced by the plants (Belesky and West, 2009, Arnold and Lewis, 2005)

The organ-specific distribution of endophytes necessitates careful consideration of which tissues or organs to sample, as the choice can profoundly impact the isolation outcomes. Sampling only one organ might result in a biased view of the overall endophyte community, potentially overlooking important species that reside in other parts of the plant (Hardoim et al., 2015). To obtain a comprehensive understanding of the endophyte diversity within a plant species, it is often necessary to sample multiple organs, which increases the complexity of the isolation process and the subsequent analysis of the recovered endophytes. However, this may not be feasible in the case of rare or endangered plant species, where sampling of unsustainable plant organs like bark and roots is not advisable to avoid further threatening the plant.

To address the challenges of potentially missing important endophytes when only a sustainable plant organ is collected from an endangered species, it may be necessary to employ advanced, non-invasive techniques such as metagenomics or metatranscriptomics (Hawkes et al., 2021, Esiobu et al., 2022). These methods allow for the characterization of endophyte communities without the need for extensive tissue collection (Esiobu et al., 2022). Alternatively, in cases where minimal root or bark sampling is unavoidable, a careful balance of the need for comprehensive endophyte data with the imperative to conserve the endangered species is necessary, possibly by employing micro-sampling techniques that minimize damage to the plant(Schilling et al., 2022, Smith and Wang, 2014).

In contrast, the extraction of bioactive compounds from endangered plant species has its own set of challenges. The selection of solvents, based on their polarity (e.g., methanol, ethanol, water), directly influences the range of compounds that can be extracted from plant tissues. Methanol, for example, is a polar solvent commonly used to extract a wide range of polar compounds, including phenolics, flavonoids, and alkaloids, which are known for their antioxidant, anti-inflammatory, and antimicrobial properties (Truong et al., 2019). Ethanol, which is slightly less polar than methanol, is also effective at extracting these types of compounds, but it is often favoured for its lower toxicity and greater compatibility with food and pharmaceutical applications (Duval et al., 2016). Water, as a highly polar solvent, is primarily used to extract hydrophilic compounds such as sugars, proteins, glycosides, and certain alkaloids, which are important for understanding the nutritional and medicinal properties of plants (Muhamad et al., 2017).

These solvents are not the only options available; however, they are commonly used due to their effectiveness, availability, and relatively low toxicity, which make them suitable for a wide range of applications in both research and industry (Plaskova and Mlcek, 2023, Liu et al., 2009, Gallina et al., 2022). Other solvents, such as acetone, hexane, or chloroform, can be used to target non-polar or semi-polar compounds like lipids, terpenoids, and sterols, depending on the specific objectives of the extraction (Daud et al., 2022). However, methanol, ethanol, and water are often preferred in initial extractions due to their ability to extract a broad spectrum of bioactive compounds, providing a comprehensive profile of the plant's chemical constituents (Altemimi et al., 2017). Moreover, the efficiency and yield of bioactive compounds are further determined by the extraction method used, be it Soxhlet extraction, maceration, or ultrasonic-assisted extraction (Abbas et al., 2021, Vieitez et al., 2018, Alara et al., 2018). Each method has specific advantages and limitations that must be carefully weighed based on the study's objectives.

To accurately characterize the bioactive compounds extracted, advanced analytical techniques such as Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS), and Nuclear Magnetic Resonance (NMR) are employed (Donno et al., 2020, Rawat et al., 2023, Ingle et al., 2017, Araujo et al., 2020). These techniques provide detailed insights into the chemical profiles of plant extracts, offering a comprehensive understanding of their bioactive potential (Rawat et al., 2023). Each technique contributes uniquely to the identification and quantification of compounds, making them essential processes in phytochemical research.

Furthermore, when exploring the endophytes (potential plant partners in producing bioactive compounds) within their host plants, molecular techniques are crucial to distinguish the endophytes from other host’s microbes. The use of conserved genetic regions, such as the 16S rRNA gene for

bacterial endophytes and the Internal Transcribed Spacer (ITS) region for fungal endophytes, allows for precise identification and classification (Mishra et al., 2017, Moronta-Barrios et al., 2018). These molecular markers are commonly used to identify microorganisms at the taxonomic level but cannot conclusively distinguish endophytes from external contaminants or non-endophytic microbes. Additional validation steps are often required to confirm the endophytic nature of isolated microorganisms (Pinski et al., 2019, Kumar et al., 2019). A detailed comparison of endophyte isolation and bioactive compound extraction methodologies reveals the unique challenges and considerations associated with each approach. These insights are important for optimizing the methods used in studying endangered plant species, ensuring the reliability and relevance of the findings in both ecological and commercial contexts.

Table 2.4: Methods for studying microbial communities: culture-dependent and culture-independent approaches

Criteria	Culture dependent method	Culture independent method
Principle	Isolating and studying microorganisms by culturing them in laboratory media.	Analyzing microbial DNA directly from environmental or plant samples without the need for cultivation.
Process	Collection of samples (e.g., roots, stem, leaves). Culturing on selective media. Incubation and colony growth. Morphological identification and further screening.	Collection of samples (e.g., roots, stem, leaves). DNA extraction from the sample. PCR amplification of specific genes (e.g., 16S rRNA, ITS). High-throughput sequencing. Bioinformatics analysis.
Microbial diversity captured	Limited to only culturable microbes, which may not represent the full microbial diversity.	Captures both culturable and unculturable microbes, offering a more comprehensive view of microbial diversity.
Detection of microbial functions	Allows for functional analysis like bioactivity, plant growth promotion, or biodegradation through cultured strains.	Does not directly measure microbial functions (such as bioactivity) but reveals genetic diversity and composition.
Speed	Slower due to the time required for culturing and colony formation.	Faster, as DNA sequencing and bioinformatics analysis can be done without cultivation.
Cost	Generally less expensive in terms of equipment but can be labor-intensive.	More expensive due to the cost of sequencing technologies and bioinformatics tools.

Biases	Potential bias due to the growth conditions and media used, which may not support the growth of all microbes.	No bias from culturing conditions, as the DNA is directly extracted from the sample.
Ideal for	Studying specific isolated strains and screening them for various applications (e.g., agriculture, biotechnology).	Studying microbial communities as a whole, especially for unculturable microbes and rare species.
Advantages	Simple and well-established. Can obtain pure cultures for detailed study. Provides functional data (growth, metabolites, etc.).	Provides a complete microbial profile, including unculturable species. Can explore microbial diversity in natural environments. Does not require culturing, avoiding bias from media limitations.
Disadvantages	Not all microbes are culturable, leading to incomplete data. Time consuming and requires careful handling of cultures. May not reflect the full microbial community.	Does not directly provide functional data (e.g. Bioactivity, growth promotion). Requires complex sequencing and bioinformatics analysis, which can be expensive. Interpretation of data can be challenging due to the vast diversity.

2.8: Importance of investigating the endophytic communities in *Ocotea bullata* and *Aloe lettyae*

The study of endophytes within endangered plant species like *O. bullata* and *A. lettyae* holds great significance for multiple reasons. From a conservation perspective, these microbial communities can provide an alternative approach to preserving these endangered species, reducing the pressure on destructive harvesting practices that have threatened their survival (Ashraf et al., 2012, Bothe et al., 2010, Pandey et al., 2018). The endophyte *Taxomyces andreanae* isolated from *Taxus brevifolia*, have been shown to produce paclitaxel, a compound originally derived from the host plant and used in cancer treatment (Cheng et al., 2022). While specific studies on endophytes producing bioactive compounds in *O. bullata and A. lettyae* are limited, such findings underscore the potential for endophytes within this species to synthesize similar bioactive molecules, offering a sustainable alternative to direct plant harvesting.

Ecosystem protection is another crucial aspect, as healthy populations of endangered species are vital to maintaining biodiversity. By supporting plant health, endophytes help these species endure

environmental stresses and resist pathogens, promoting their long-term survival in their natural habitats (Eid et al., 2019, Chaudhary et al., 2022). This in turn ensures that these species continue to fulfil their ecological roles, such as supporting other flora and fauna that depend on them (Fischer et al., 2006).

In addition to conservation and ecosystem benefits, the potential of endophytes to produce bioactive compounds should not be overlooked. The bioactive compounds produced by endophytes often mimic or enhance those found in the host plants, offering a sustainable and scalable source of bioactive compounds with medicinal and biotechnological applications (Akhtar et al., 2019, Bhaskar et al., 2024). By exploring the endophytes of *O. bullata* and *A. lettyae*, we do not only safeguard the future of these endangered species but also open up new opportunities for the sustainable production of valuable metabolites, further extending the potential benefits of these plants beyond their natural ecosystems.

2. 9: Conclusion

This review has emphasized the vast potential of the microbial world within plants for advancing medicine, biotechnology, and sustainable agriculture. Endophytes are capable of producing a wide range of bioactive compounds with antidiabetic, antioxidant, anticancer, and antimicrobial properties. Although research on bioactive compound production from endophytes is still in its early stages, existing studies highlight the significant role these microorganisms play in various applications. Investigating the endophytic communities within endangered plant species such *as O. bullata* and *A. lettyae* is crucial, as these plants hold both ecological and medicinal importance. In addition to contributing to the preservation of these species, such research offers promising benefits in agriculture, medicine, and biotechnology. To ensure that the study of endophytes from endangered plants is both sustainable and ethical, policies and guidelines should focus on minimizing plant disturbance through non-invasive sampling techniques and ensuring the responsible use of permits for collection. In addition to permits for collection, equitable access to the discoveries made from these plants should be ensured, particularly by involving local communities or traditional knowledge holders, who can be key stakeholders in both conservation and research efforts. While databases like the SANBI Red List track endangered species, creating specialized regional databases focused on microbial research could further enhance coordination and conservation. Capacity-building initiatives should involve local communities, integrating traditional knowledge with scientific advancements. The urgency of this research is heightened by the threat of extinction facing these plants, making swift action essential. By exploring the

microbial worlds within these species, we not only promote conservation but also open the door to ground-breaking discoveries that could provide significant benefits to humanity.

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Chapter3

Metabolomic profiling of two *Aloe* species and 16S rRNA metabarcoding of their endophytes revealed diverse metabolites

[a] Cynthia K. Marokane-Radebe, [a] Adekunle R. Raimi, [a. b] Stephen O. Amoo, [a] Rasheed A. Adeleke[*] ***AMB Express (2024) 14:122https://doi.org/10.1186/s13568-024-01784-3***

[a] Unit of Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa

[b] Agricultural Research Council - Vegetables, Industrial and Medicinal Plants, Roodeplaat, Pretoria, 0001. South Africa.

Abstract

Aloe species are often used interchangeably for medicinal and cosmeceutical applications, presenting a challenge to the biological efficacy consistency of some herbal preparations. Sustainable production of high-quality commonly used medicinal plants remains a limitation for commercialisation. Thus, this study investigated the potential for plant substitution by examining bacterial endophytes capable of producing similar host plant secondary metabolites. The metabolite profiles and endophytic bacterial communities of endangered *Aloe lettyae* were compared with those of *Aloe longibracteata* using nuclear magnetic resonance spectroscopy and 16S rRNA gene sequencing. Only 15 metabolites differed significantly between *A. lettyae* and *A. longibracteata* based on concentrations. However, both plants' functionality and potential application remain comparable. Phytohormones, including indole-3-acetate and 5-hydroxyindole-3-acetate, were more concentrated in *A. lettyae* than *A. longibracteata*. Metabolites such as tyrosine, allantoin, and myo-inositol, were annotated in both species. *Aloe lettyae* harboured a phylogenetically diverse bacteria community compared to *A. longibracteata*, with a higher richness of bacterial species, indicating a likelihood of diverse metabolic capabilities among the bacteria. Dominant endophytes, including *Bacillus, Comamonas,* and *Pseudomonas*, possess enzymes contributing to various metabolic pathways, potentially influencing the host plant's overall metabolic composition. This study does not advocate the medicinal use of *A. lettyae* or *A. longibracteata* without further safety evaluation. Instead, it emphasizes endophyte roles in metabolite production, informing future applications in regulated contexts. Thus, this study supports the interchangeability of *A. lettyae* and *A. longibracteata* due to their similar metabolite profiles, and diverse endophytic communities, highlighting the potential of their endophytes as sources of secondary metabolites.

3.1 Introduction

In medicine, biotechnology and pharmacology, there is a controversy about the nature and type of bioprospecting approach that can be adopted for medicinal plants to guide drug discovery (Saslis-Lagoudakis et al. 2012). Efficacy and sustainable production of traditionally used medicinal plants are two of the most important concepts driving research interests in the bioprospecting of medicinal plants. For instance, perceived efficacy is strongly influenced by cultural biases linked to ethnobotanical data, especially when similar plants are used for the same purpose in different regions (Saslis-Lagoudakis et al., 2011). However, over-reliance on ethnobotanical data may not present complete information about the potential benefits and bioactivity of certain medicinal plants (Saslis-Lagoudakis et al., 2011). This means ethnobotanical data comparisons of medicinal plants could only be used as a guide in bioprospecting if accompanied by relevant data, especially the nature and quantity of metabolite production by the medicinal plants. Interestingly, recent developments in drug discovery have expanded the focus beyond plant metabolites and there is an increasing interest in the plant endosphere that harbours the endophytes. Endophytes are known to have the capability to produce similar categories of metabolites as their host plants (Ludwig-Müller, 2015, Kusari et al., 2013). In addition, endophytes can produce unique and novel bioactive compounds of biotechnological importance (Gouda et al., 2016, Christina et al., 2013, Uzma et al., 2019). A powerful but underutilised approach to address challenges associated with the sustainable production of high-quality bioactive medicinal plants could be through an unbiased comparison of plants used for the same medicinal purposes. This could be achieved by focusing on the metabolites from closely related plant species and their native microbiome, especially the endophytes.

The genus *Aloe* comprises over 500 species of succulent plants renowned for their medicinal properties and widespread use in traditional medicine (Grace et al., 2008). Among these, *Aloe lettyae* and *Aloe longibracteata* are closely related species native to South Africa (Smith and Klopper, 2022). They belong to the group of spotted *Aloes*, a group with species not easily distinguishable, and taxonomically difficult to identify at the vegetative phase (Van Wyk and Smith 2014) However, *A. lettyae* is classified as an endangered species due to habitat loss, abiotic disturbances such as fire and the effect of the abundance of interacting species (Kremer-Köhne et al., 2020b). Therefore, sustainable production of endangered plant species such as *A. lettyae* is necessary to protect the plants from extinction. In addition, associated microbiomes, especially the microbial endophytes, known to possess the ability to influence the production of secondary metabolites and yield in *Aloe* sp. should be investigated.

Endophytic microbes could be applied to improve plant biomass and/or the quantities of metabolites produced by host plants. In addition, plant growth-promoting endophytes could help protect the

plants against some negative effects of abiotic and biotic factors, thereby improving their resilience and growth under suboptimal growing conditions. Furthermore, the isolation and characterisation of endophytes associated with medicinal plants may provide insights into their capabilities to synthesise secondary metabolites similar to that of their host plants (Pimentel et al., 2011).

Plant metabolites play crucial roles in various physiological processes, defence mechanisms and ecological interactions (Pichersky and Gang, 2000). Metabolomic approaches have been employed to characterise the chemical diversity of *Aloe* species, revealing the presence of diverse metabolites such as anthraquinones, chromones, and polysaccharides (Cock, 2015, Sahu et al., 2013). In addition to their intrinsic metabolic capabilities, *Aloe* plants harbour diverse microbial communities, including endophytic bacteria that inhabit their internal tissues without causing apparent harm (Hardoim et al., 2015). These endophytic bacteria contribute to plant growth and development, through nutrient acquisition, phytohormone production (Raimi and Adeleke, 2023, Santoyo et al., 2016). Endophytes have been shown to influence plant secondary metabolite synthesis, leading to the production of unique and analogous metabolites with potential bioactivities (Kumari et al., 2023). Thus, insight into the endophytic bacterial communities of *Aloe* species may improve our knowledge of the correlations between the metabolites of the host plant and its associated endophytic bacteria.

Despite studies about metabolite production and associated endophytic bacterial communities of *Aloe* species, results have had limited impacts on potential biotechnological applications (Swati et al., 2022, Silva et al., 2020). If *A. lettyae* and *A. longibracteata* harbour bacterial endophytes capable of synthesising metabolites analogous to those of the host plant, there might be less need to rely on the harvesting of plant material for metabolite sourcing, particularly in the case of the endangered *A. lettyae*. Thus, this chapter hypothesized that plants from the same lineages utilized for the same purpose produce similar sets of metabolites and harbors similar types of bacterial endophytes. Furthermore, the chapter hypothesized that such endophytes have the potential to produce similar metabolites as that of their host plants. If these hypotheses are correct, then such plant groups should be strong candidates for bioprospecting. To our knowledge, this is the first study that aims to gain insight into the correlation between the differential metabolites and endophytic bacterial communities of *A. lettyae* and *A. longibracteata.* The results may provide baseline information for the unique utilisation of specific *Aloe* species and improve microbial technology applications, especially in the production of bioactive compounds using endophytic microbes.

3.2 Materials and methods

3.2.1 Plant sampling area

Aloe lettyae and *Aloe longibracteata* (family *Asphodelaceae)* were collected from Haenersburg (23° 55' 59.99" S 29°56'59.99" E) and Makapanstad (*25° 14′ 35″ S, 28° 7′ 18″ E*) respectively, in Limpopo Province, South Africa. Six individual plants from each species were collected per site (approximately 200 m apart). The plant samples were carefully uprooted and placed in sterile polyethylene bags and brought to the laboratory in a portable cooler maintained at 4ºC using ice packs. The formal identification of the plant specimens (Figure 3.1) was carried out at the herbarium of the Botany Department at North-West University, where plant herbarium speci mens were deposited.

Figure3.1: Sampled *Aloe* species. (a) *Aloe lettyae* and (b) *Aloe longibracteata* collected from Haenersburg and Makapanstad villages in Limpopo province, South Africa

3.2.2 Plant extraction and NMR analysis

Following the method of (Kim et al., 2010) with modifications, the freeze-dried powdered leaf samples (50 mg) of the *Aloe lettyae* and *Aloe longibracteata* (Fig. 1) were respectively extracted with 750 µL methanol-d_4 (CH_3OH-d_4) and 750 µL potassium dihydrogen phosphate (KH_2PO_4) buffer in deuterium water (D_2O) (pH 6.0) containing 0.01% (w/w) trimethylsilanepropionic acid (TSP). The mixture was vortexed for 1 min, ultrasonicated for 20 min, and then centrifuged for 20 min (at 10,000 rpm. Samples were then filtered through a 0.22 µm syringe filter and 500 µL of the

filtrates were transferred to 5 mm Norell standard NMR tubes. All the proton NMR spectra were acquired using a 600 MHz NMR spectrometer (Varian Inc, CA, USA). Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisitions. All spectra were Fourier-transformed, and phase and baseline were corrected manually.

3.2.3 Multivariate data analysis

Data analysis and processing were performed using MestReNova software (10.0.1, Mestrelab Research, Spain), and the correction of phasing and baseline, normalisation and peak alignment was done manually on the 1H NMR spectrum. All processed data were divided into 0.04 ppm bins, representing 0 – 10 ppm and converted to Excel comma-separated values (CSV) file format for pattern recognition multivariate data analysis. Transformed data were statistically analysed, and all the imported data were Pareto scaled in the soft independent modelling of class analogy (SIMCA) software (SIMCA, Version 14.0, Umetrics, Umeå, Sweden). Similar and non-similar samples were statistically discriminated using the Principal Component Analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA). Annotations of compounds were performed using the Chenomx software (NMR suite, version 8.3) and published NMR data.

3.2.4 Endophytic bacterial community composition of *A. lettyae* and *A. longibracteata* as revealed by high-throughput sequencing of the 16S rRNA gene.

3.2.4.1 Library preparation

Genomic DNA was extracted from the two *Aloe* species leaves using the Qiagen DNeasy Plant kits (QIAGEN®, Hilden, Germany), following the manufacturer's instructions. The DNA was quantified with a Nanodrop™-1000 (Nanodrop lnc., Wilmington, USA), and the integrity and size were checked with electrophoresis using 1% agarose gel. The 16S rRNA gene was amplified in a polymerase chain reaction (PCR) using Illumina barcoded primers, 341F (5′-CC TACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) targeting V3-V4 region (Klindworth et al., 2013). The library preparation was performed according to (Van Wyk et al., 2017) and sequenced as paired-end (250 bp) on Illumina MiSeq sequencer using Nextera v3 kit (Illumina Inc., San Diego, USA) at the Novogene Sequencing facility, Singapore.

3.2.4.2 Bioinformatics analysis

The read quality was checked with FastQC v. 0.11.9 (Babraham Bioinformatics, UK) and trimmed using Trimmomatic software v. 0.38 (Bolger et al., 2014) after demultiplexing. Removal of chimeras, correction for errors and clustering of the sequences at 100% similarities to amplicon sequence variance (ASVs) were done in QIIME2 using the DADA2 plugin. Phylogenetic relationship, and taxonomic classification, alpha and beta diversity analyses were performed in

QIIME2 and R software v. 4.3.2 (R Development Core Team 2018). The ASV table was rarefied to an even depth and the community diversity, richness, and uniformity were analysed using alpha diversity based on Observed ASVs, Chao1, Shannon, Simpson and Pielou evenness. The rarefaction curve was used to analyse the sequencing depth adequacy. Endophytic bacterial community composition and complexity were calculated and compared between the different species of *Aloe* using beta diversity, based on weighted and unweighted unifrac distances with nonmetric multidimensional scaling (NMDS) in R studio. A Venn diagram was drawn using the ggvenn package in R to show the presence and distribution of core and shared species between the *Aloe* species. Core species are the 'stable' part of the community exhibiting key functions that impact host physiological performance (Alibrandi et al., 2020).

3.2.4.3 Endophytic bacteria community functional profiling

Phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2) pipeline (version 2.3.0) was employed to compute the functional metabolic profile of the endophytic bacterial communities (Douglas et al., 2020). The pipeline was performed in QIIME2 using the ASV sequence and abundance table generated against the SILVA rRNA (138) database as the input. The output files included gene family and pathway abundances (Douglas et al., 2020). The impacts of plant species on the endophytic bacterial community functions were assessed using the NMDS. A subset of essential pathways involved in the production of key metabolites of ecological importance were analysed.

3.2.4.4 Statistical analyses

The statistical analyses were performed using R software version 4.3.2 (R Development Core Team 2018) with a statistically significant level of $P < 0.05$. Data normality was examined with the Shapiro-Wilk test and non-normally distributed data were transformed using log function. The parametric and non-parametric tests were adopted for normally and non-normally distributed data, respectively. The Mantel test was used to assess correlations between bacterial community composition and unique metabolic pathways. Bacterial community structure in multivariate space was visualised with a non-metric multidimensional scaling (NMDS) and an unweighted pair-group method with arithmetic mean (UPGMA) using the vegan and dendextend (v. 1.12.0) packages in R. Differences between groups were established with weighted and unweighted unifrac distances dissimilarity using permutational multivariate analysis of variance (PERMANOVA) and Permutational test for homogeneity of multivariate dispersions (PERMDISP). The Linear Discriminant Analysis (LDA) Effect size (LEfSe) was used to detect the most differentially abundant community and pathways (Mann-Whitney U test, $P < 0.05$, LDA score > 2.0) between sample groups. The differentially abundant bacterial communities that are statistically significant

were visualised as bar plots using the web-based Microbiome Analyst tool (www.microbiomeanalyst.ca).

3.2.4.5 Data availability

The 16S rRNA gene sequences have been submitted to the sequence read archives (SRA) of the National Centre for Biotechnological Information as part of a BioProject under the SRA accession number PRJNA1098075 (http://www.ncbi.nlm.nih.gov/bioproject/1098075).

3.3 Results

3.3.1 Metabolomic analysis of the metabolites produced by *Aloe* species.

The 1H NMR spectra of both *A. longibracteata* and *A. lettyae* revealed a complex array of signals, suggesting the presence of numerous metabolites ranging from amino, fatty and organic acids in the aliphatic region, carbohydrates in the sugar region and phenolic metabolites in the aromatic region (Figure 3.2). In the spectrum for *A. longibracteata*, there is a notable high intensity of signals across sugar and aliphatic regions (Figure 3.2). In the sugar region (3-5.5ppm), the strong signal suggests a higher concentration of glycosylated metabolites and free sugars in *A. longibracteata* compared to *A. lettyae.* Similarly, the aliphatic region (0-3ppm) indicated a higher prevalence of metabolites such as fatty acids, organic acids and other aliphatic metabolites in *A. longibracteata* as compared *to A. lettyae.* Despite the differences in the intensities of the metabolites in the sugar and aliphatic region of *A. longibracteata* and *A. lettyae*, these two *Aloe* species show similar signals in the aromatic region (6-8 ppm), indicating the presence of aromatic compounds like phenolics or aromatic amino acids in both species. Overall the visual inspection of *A. longibracteata* and *A. lettyae* spectra indicate that *A. longibracteata* has a more diverse and abundant metabolite profile, particularly in terms of sugars and aliphatic metabolites compared to *A. lettyae*.

Based on the NMR profiles, the relative differences between *A. lettyae* and *A. longibracteata* were visualised by plotting the scores of a Principal Component Analysis (PCA).

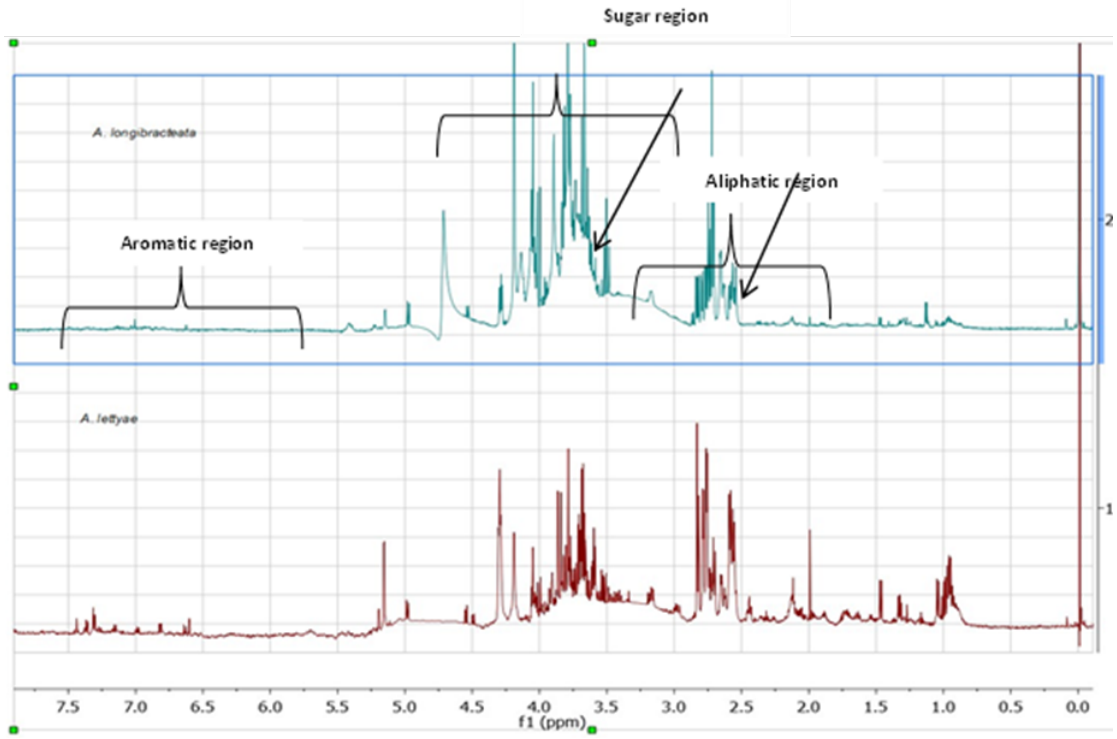

Figure3.2: Stacked 1H NMR spectra of *Aloe longibracteata* and *Aloe lettyae.* The arrows show similar occurrences of metabolites between *A. longibracteata* (blue) and *A. lettyae* (red).

The unsupervised pattern recognition analysis using PCA was applied to give an overview of the dimensions of the samples (Figure 3.3a). The OPLS-DA, which is the supervised recognition analysis that allows the algorithm to expose discrimination between the groups, was applied to the data set (Figure 3.3b). The variance of 95% and coefficient R2X = 0.92 and Q2 = 0.72 were used to validate the goodness and predictability of the model. Observations of both the PCA scores plot (Figure 3.3a) and the OPLS-DA score plot (Figure 3.3b) showed a clear separation among the samples. Furthermore, the HCA dendrograms (Figure 3.3c and d) grouped samples with similar features into three clusters, which suggests the distinction amongst the samples and reveals underlying patterns of the data.

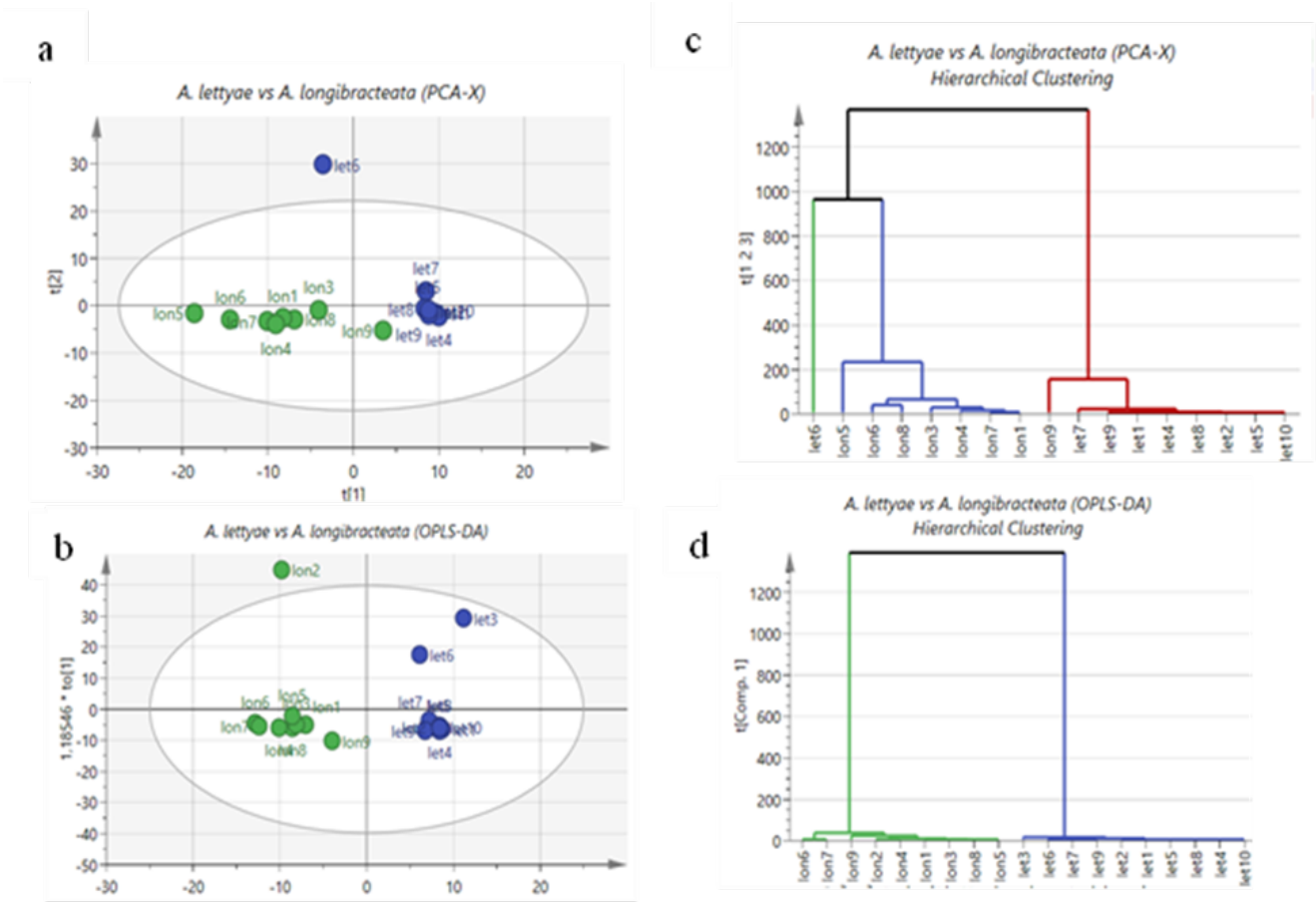

Figure3.3: Unsupervised recognition analysis using (a) PCA and (b) OPLS-DA scores of *A. lettyae* and *A. longibracteata* aqueous methanol extract with HCA dendrograms derived from the (c) PCA and (d) OPLS-DA showing metabolic relativity of the samples, with green and blue circles representing *A. longibracteata* and *A. lettyae* samples, respectively.

The statistical interference analysis was used to further validate the built OPLS-DA discriminant model. One hundred and twenty permutation tests were performed to interactively analyse the predictor variable Y based on the known measured data variable X and obtain statistics on the variables (Figure 3.4a). By examining the intercept of the fitting line formed by the calculated values R2X and Q2 corresponding to all samples on the Y coordinate axis, the reliability of the model and the degree of overfitting were determined. The larger the value of Q2, the better the predictive ability of the model, and the larger the value of R2X, the stronger the explanatory ability (Westerhuis et al., 2008). The intercept of the Q2 regression line was negative, indicating that although there was a clear difference in predictability, the OPLS-DA discriminant model established was not over-fitted and had a good predictive ability. The Receiver Operated Characteristic (ROC) which calculates the area under the curve (AUC) was plotted (Figure 3.4b) and the cross-validated predictive residual was performed (CV-ANOVA, p-value < 0.05).

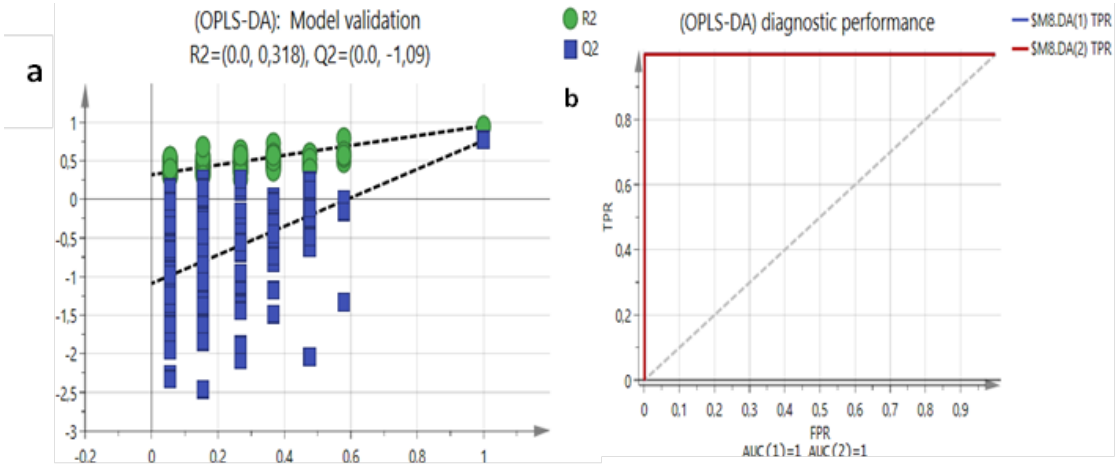

Figure: 3.4 Statistical validation (a) of the OPLS-DA model by permutation testing (n = 120 permutations) and diagnostic performance through ROC (AUC=0.999) (b) analysis. Q2 intercept of -1.09 is less than 0.05 indicating a valid model.

Adopted from the supervised OPLS-DA results, the contribution plot (Figure 3.5) revealed regions that are positively associated with the clustering of the *A. lettyae* and *A. longibracteata* samples. The sugar region in the peaks in the positive bars (*A. longibracteata)* of the contribution plot is more abundant followed by the aliphatic region with little intensity in the aromatic region. However, with *A. lettyae* (negative bars of contribution plot), the sugar and aromatic regions are more abundant than the aliphatic region. From the multivariate data analysis, a visible discrimination factor between the two *Aloe* plants lies between the aliphatic and the aromatic regions, while the sugar region is the major source of the similarities in metabolites, although most chemical shifts remain unknown. Using Chenomx, the metabolites from *A. lettyae* and *A. longibracteata* were annotated in Table 3.1.

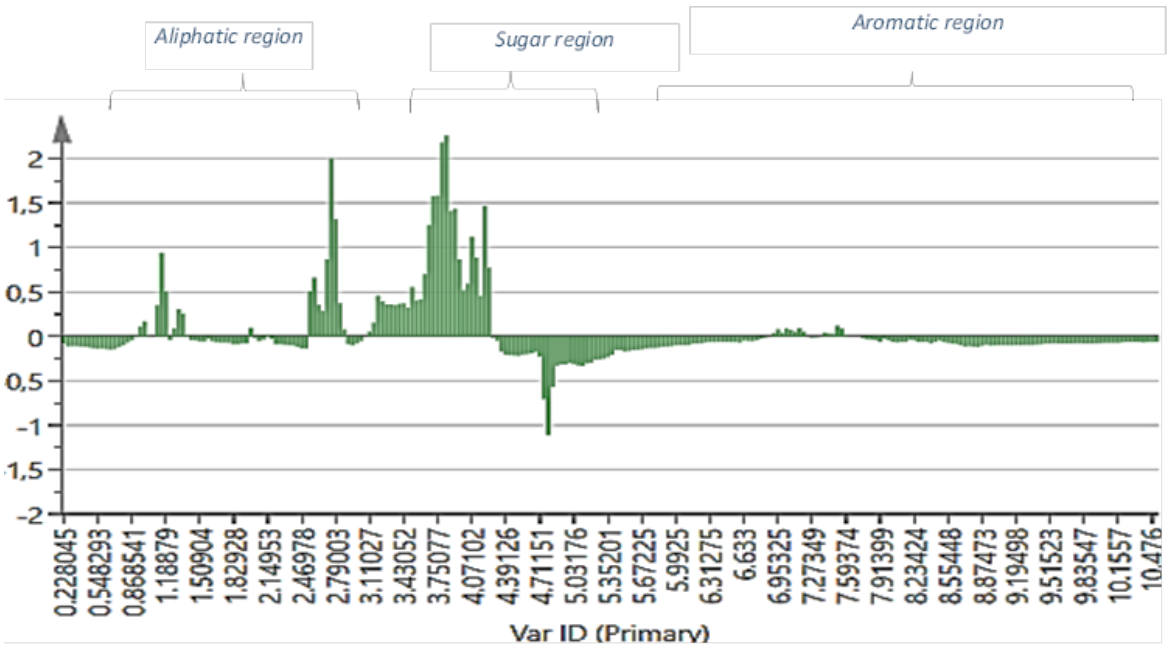

Figure3.5: A contribution plots showing significant 1H NMR spectral regions responsible for the separation of *A. lettyae* from *A. longibracteata*. Positive scores are regions that are positively associated with the separation of the *A. longibracteata* samples from the *A. lettyae* samples.

The Variable Important in Projection (VIP) scores (Supplementary figure S1) are used to effectively identify the key metabolites that are most responsible for chemical diversity and uniqueness between two *Aloe*. The VIP scores indicate the significance of each metabolite in distinguishing between the two *Aloe* based on their chemical composition. Higher VIP scores signify a greater influence of a specific metabolite in differentiating the two species. 3-hydroxykynurenine has the highest VIP score (Supplementary figure S1) indicating the abundance of the metabolite varies significantly between the two species and is a major discriminating factor in their metabolite compositions. Compounds such as N-phenylacetyl, Salicylurate, Xanthurenate, and NADPH also have relatively high VIP scores, suggesting their levels or structural features contribute substantially to differentiating the chemical profiles of the two species. Metabolites with lower VIP scores such as tyrosine, N-acetylglutamine, and IAA are less influential in distinguishing between the plant species based on their metabolite data.

Table 3.1: Chenomx assisted annotations of differentially produced metabolites of *A. lettyae* and A. *longibracteata.*

Plant species	Metabolites	Chemical shift (ppm)	Concentration(mM)
Aloe lettyae	4-hydroxybenzoate	7.8 (d), 6.9(d)	0.019
	Adenine	8.2(s)	0.020
	Cadaverine	3.0(t), 1.7(m), 1.5(m)	0.053
	Putrescine	3.2(t), 1.8(m)	0.030
	Allantoin	8.0(s),7.3(s),6.0(s),5.4(s)	1.021
	Myo-inositol	3.6 (t), 4.1(t), 3.5(dd)	0.533
	Indole-3-acetate	7.6 (d), 7.5(d), 7.2(m), 3.6(d)	0.131
	Cis-Aconitate	5.7 (t), 3.1 (d)	0.034
	Erythritol	3.8(dd), 3.7 (m)	0.764
	Tyrosine	7.16(d),6.87(d),3.92(dd)	0.124
Aloe longibracteata	Aspartate	3.9(dd), 2.8 (dd), 2.7 (dd)	0.911
	Betaine	3.9 (s)	0.083
	Biotin	6.5 (s), 6.4(d)	0.908
	Chlorogenate	7.6(s), 7.2(d), 6.9(d), 4.3(q), 3.9 (q)	0.518
	Citrate	2.7(d), 2.5 (d)	0.377
	Tyrosine	7.16(d),6.87(d),3.92(dd)	0.387
	5-hydroxy indole-3-acetate	7.4(d), 7.2(s),7.0(d),6.8 (dd),	0.076
	Arabinitol	3.9(m), 3.8(dd), 3.6(dd)	0.984
	Myo-inositol	3.6 (t), 4.1(t), 3.5(dd)	0.475
	Allantoin	8.0(s),7.3(s),6.0(s),5.4(s)	0.023
	Glycerate	4.0(dd), 3.8(dd), 3.7 (dd)	2.145
	β-Alanine	3.2(t), 2.6(t)	0.090

Peak multiplicity (s=singlet; d= doublet; t= triplet; dd= doublet of doublet; q= quartet; m = multiplet).

3.3.2 Endophytic bacterial community distribution in the *Aloe* species

The alpha diversity indices including Observed ASVs, Chao1, Shannon, Simpson, phylogenetic diversity, and core abundance were significantly different (Mann-Whitney U test, $P < 0.05$) across the *Aloe* species (Figure 3.6). The *A. lettyae* species had a higher relative abundance and greater richness of bacterial endophytic communities compared to *A. longibracteata* (Figure 3.6a-e). The higher phylogenetically diverse index in *A. lettyae* indicates higher richness and diversity compared to the *A. longibracteata.* However, relative proportion of core species was higher in the *A. longibracteata* than in *A. lettyae,* as shown in Figure 3.6f.

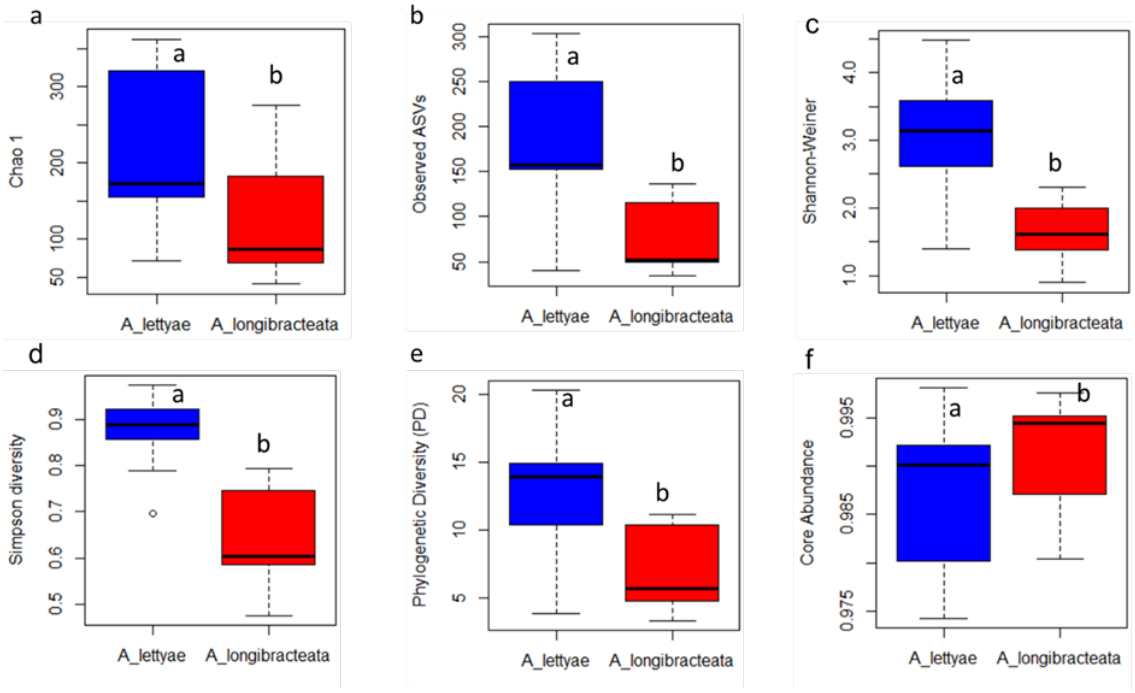

Figure 3.6: Alpha diversity measures across *A. lettyae* and *A. longibracteata*. A) Chao1 (B) Observed ASVs (C) Shannon-Weiner diversity (D) Simpson diversity (E) Phylogenetic diversity (PD) (F) Core abundance

3.3.2.1 Core and shared bacterial communities between the *Aloe* species

The *A. lettyae* and *A. longibracteata* species differ in their number of unique and shared bacterial communities (Supplementary figure S2). The highest number of unique ASVs (40%) was observed in *A. lettyae* compared to *A. longibracteata* 28%, while both plant species shared 31% of the total bacterial communities. The shared and unique ASVs are taxonomically diverse with no dominant taxa identified between the *Aloe* species.

3.3.2.2 Endophytic bacterial community structure between the *Aloe* species

The NMDS ordination analysis reveals that the endophytic bacterial communities of the *Aloe* plants are well differentiated between the species (Figure 3.7a). The bacterial communities of *A. lettyae* cluster separately from that of *A. longibracteata*. A similar trend was observed for the dendrogram plot (Figure 3.7b). The PERMANOVA analysis suggests that the plant species had significant effects on the bacterial community composition (PERMANOVA; $P < 0.05$), and the dispersion test (PERMDISP, $P > 0.05$, Supplementary table S1) was not significant, which infer the assumption of homogeneity was met. The non-significant PERMDISP indicates the dispersion between the plant species types may not have affected the observed variations in the bacterial communities of the species.

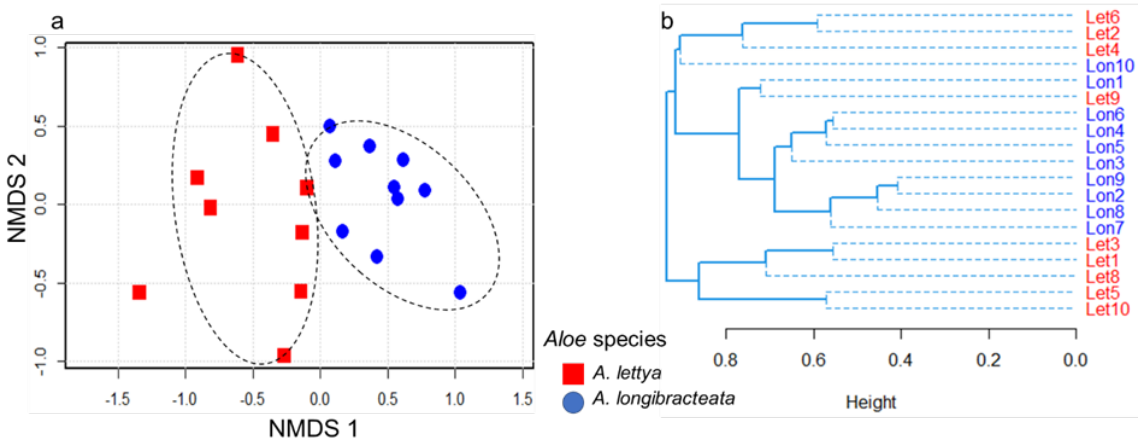

Figure 3.7: Bray-Curtis's distance dissimilarity between endophytic bacterial communities of *Aloe* species (a) Nonmetric multidimensional scaling plots and (b) cluster dendrogram. The dotted lines show the distances between species communities. The stress plot run was at 0.1941 (Supplementary figure. S5).

3.3.2.3: Relative abundance of endophytic bacteria across the *Aloe* species

The filtered reads were clustered into 1930 ASVs and the sequencing depth was confirmed adequate using the rarefaction curve (Supplementary figure S6). The dominant ASVs as shown by the taxonomic classification belong majorly to three phyla and seven genera (Figure 3.8a and b), with *Proteobacteria* and *Firmicutes* being the most relatively abundant across the *Aloe* species, representing over 80% of the total endophytic bacterial communities at the phyla level. Actinobacteria and *Bacteroidota* were more dominant in *A. lettyae. Pseudomonas*, *Weisella*, *Enterobacter*, *Lactococcus, Sphingomonas*, *Ruminococcus, Streptococcus* and *Prevotella* genera were the most relatively abundant bacterial communities across both species (figure 3.8b and c). However, their relative abundance differs with *A. lettyae* having the highest bacterial community richness. *A. longibracteata* had more abundance of *Pseudomonas*, *Weisella*, and *Lactococcus* compared to *A. lettyae*.

LEfSe analyses showed taxa differences from phylum to species with the absolute LDA score value > 2.76. *Enterobacterales, Clostridia, Lachnospirale* and *Oscillospirales* were discriminant in *A. lettyae*, while *Pseudomonas*, *Lactobacillales, Bacilli* and *Weisella* were discriminant in *A. longibracteata* (figure 3.8c).

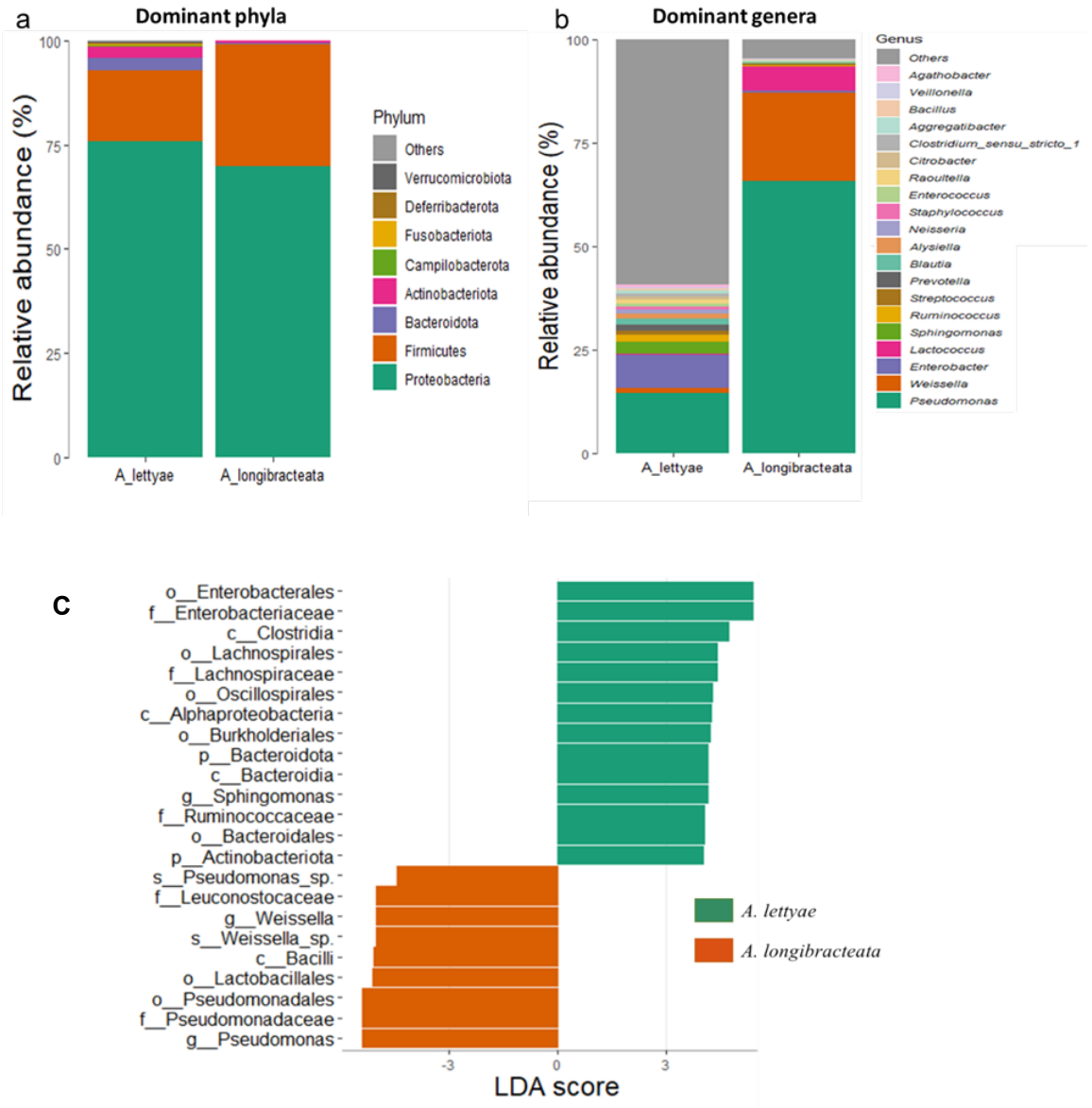

Figure 3.8: Relative abundance of endophytic bacterial community (>1%) in *Aloe* spp. (a) Dominant phyla (b) Dominant genera (c) Bar plot from LEfSe analysis comparing the discriminant features between *Aloe* species. The taxa level Phylum p_ to genus g_ is shown.

3.3.2.4 Correlation between important metabolites and endophytic bacterial communities of *Aloe* species

The correlation analysis showed a clear separation between *A. lettyae* and *A. longibracteata*, with some of the metabolites exhibiting strong positive correlations with the microbial taxa (Figure 3.9). The RDA signifies that the plant metabolites contributed a negligible part of the variations (3%, R-squared adjusted value) observed in the bacterial community structure. Importantly, indole-3-acetate (N) and N-acetylserotonin (O) greatly impact the bacterial communities of *A. lettyea* and are

largely associated with *Citrobacter, Micrococcus* and *Pantoea*. The L*;* 5-Hydroxyindole-3-acetate, and M*;* Xanthurenate drive the endophytic bacterial communities in *A. longibracteata*, with high correlation with *Comamonas* and *Bacteroides* while the other metabolites (A-K cluster) were highly associated with *Bacillus*, *Comamonas* and *Bacteroides.* Metabolites of *A. lettyae* cluster more with many of the endophytic communities compared to the *A. longibracteata* and metabolites such as xanthurenate (M), N-acetylserotonin (O) and indole-3-acetate (N) have key human health importance.

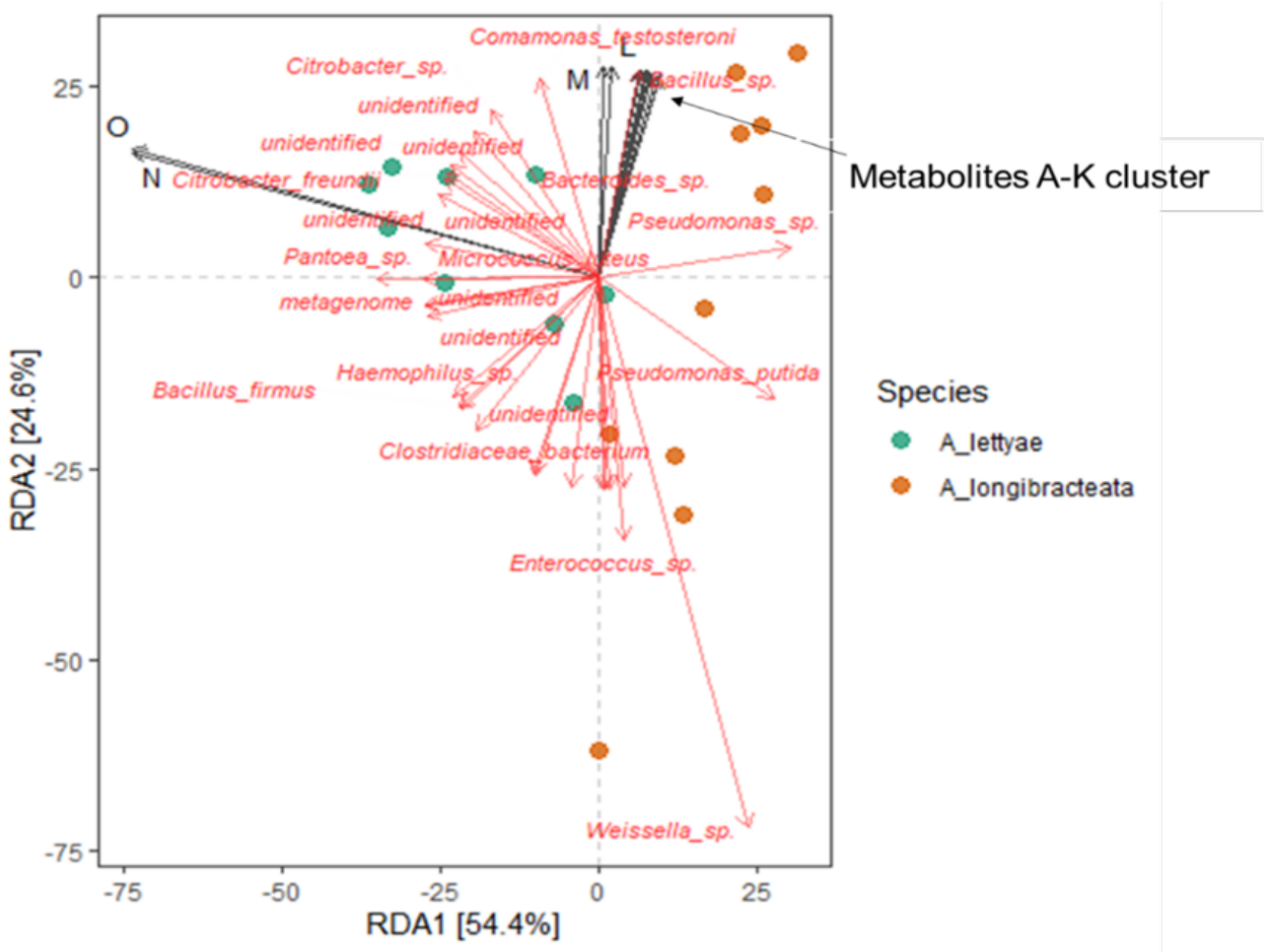

Figure 3.9: Redundancy analysis showing the correlation between endophytic bacterial communities and important metabolites of *Aloe* plants. A; N-Acetylglutamine, B; 3-Hydroxyphenylacetate, C; Tyrosine, D; 3-Hydroxymandelate, E; NADPH, F; Salicylurate, G; 3-Hydroxykynurenine, H; N-Phenylacetylphenylalanine, I; N-Acetyltyrosine, J; 4-Hydroxyphenylacetate, K; Agmatine, L; 5-Hydroxyindole-3-acetate, M; Xanthurenate, N; Indole-3-Acetate, O; N-Acetylserotonin.

Furthermore, the correlation analysis showed that 14 ASVs had a positive correlation with at least one of the differential metabolites while 36 ASVs were negatively correlated. Of note are ASV851 (*Staphylococcus*), ASV161 (*Pseudomonas*), ASV1503 (*Pseudomonas*), ASV340 (*Enterobacteriaceae*), ASV569 (*Weissella*) and ASV 731 (*Enterobacteriaceae*) which had a strong positive correlation with indol-3-acetate and N-acetylserotonin (Supplementary Fig. S4). These ASVs in addition to ASV1128 (Uncultured species), ASV1230 (*Weissella*), and ASV446 (*Pseudomonas*) were positively correlated with the other differential metabolites. The ASV1230 and ASV446, had a significant ($P < 0.05$) correlation with all the differential metabolites except for indole-3-acetic acid and N-acetylserotonin while ASV731, ASV340 and ASV997 in the family *(Enterobacteriaceae*), and ASV161 (*Pseudomonas*), had a significant correlation with all the metabolites, Similarly, ASV1128 had a significant ($P < 0.05$) correlation with all metabolites except 5-hydroxyindole-3-acetate, Xanthurenate, indole-3-acetate and N-acetylserotonin (Supplementary figure S4).

3.3.2.5 Metabolic profiling of endophytic bacterial communities in *Aloe* species

The PICRUSt2 predicted metabolic profiling of the endophytic bacteria in the *Aloe* plants revealed 99.9% of the ASVs mapped with the KEGG with less than 0.6% of the gene sequences having above the maximum NSTI cut-off of 2. The total metabolic pathways and enzyme classification (EC) metabolic functions inferred were 395 and 2,064, respectively. A total of 6560 KEGG orthology (KO) was also predicted from all the ASVs. Predicted metabolic functions above 5% frequency at subclass 1 are cofactor, carrier and vitamin biosynthesis (15.44%), amino acid biosynthesis (9.11%), aromatic compound degradation (8.86%), nucleoside and nucleotide biosynthesis (7.59%) carbohydrate biosynthesis (5.32%) and carbohydrate degradation (5.06%) (Supplementary table S2). *A. lettyae* had the highest relative abundance of carbohydrate degradation, fatty-acid and lipid degradation, fermentation and glycolysis compared to *A. longibracteata* (Supplementary figure S3). The rest metabolic functions were higher for *A. longibracteata.*

The endophytic bacterial communities of the *Aloe* species were found to have the potential to produce metabolites analogous to those of the host plants. This is evident in their diverse enzymes contributing to different pathways in the degradation and biosynthesis of important host plant metabolites (Figure 3.10). Enzymes involved in the biosynthesis of metabolites such as adenine, aspartate and biotin were more predicted in *A. lettyae* compared to *A. longibracteata*, which had a higher relative abundance of enzymes for Cis-aconitate, myo-inositol, betaine, 5-hydroxyindoleacetic acid and 4-hydroxybenzoate (Figure 3.10).

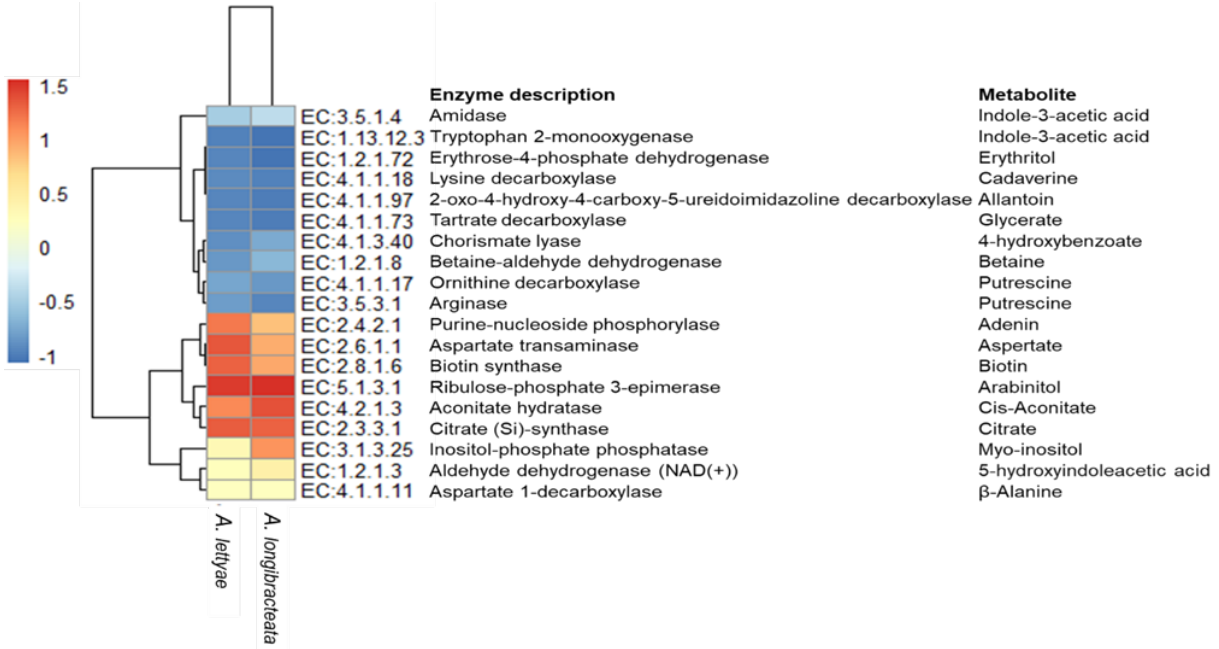

Figure 3.10: Variation of bacterial metabolic functional profile between *A. lettyae* and *A. longibracteata* showing enzymes involved in the pathways of some important metabolites of the endophytic bacteria in the *Aloe* species.

The presence of these enzymes in some of the endophytic bacteria may contribute to the host plant metabolite production. Similar to what was obtained for the plant metabolites, the enzyme participating in putrescine biosynthesis was highly predicted among endophytic bacteria of *A. lettyae*. Some of the endophytic bacteria found in this study, including *Pseudomonas, Bacillus, Lactococcus, Streptococcus* and *Enterobacter* possess various enzymes involved in the synthesis of some of the metabolites reported in the host plant. *Pseudomonas* and *Bacillus* contribute to cadaverine and cis-aconitate production through the enzyme lysine decarboxylase and aconitate hydrolase, respectively. In addition, the genus *Streptococcus* could synthesise the enzyme ribulose-phosphate 3-epimerase in the pathway of arabinitol production.

3.4 Discussion

Bioprocessing of medicinal plants has been a major strategy for sourcing metabolites used in drug synthesis and biotechnological applications (Sahoo et al., 2017). However, as some of the important plants are becoming extinct and classified as endangered species, it is important to find sustainable alternative sources of their metabolites and gain insight into their genetic components, including endophytic microbial communities before they are lost (Raimi and Adeleke, 2021). Thus, this chapter evaluates the differential metabolites of endangered and unendangered *Aloe* species and further correlates the results with the potential metabolic profile of the plant's endophytic bacterial communities. The NMR spectroscopy was used to evaluate the variations in the metabolite composition of *Aloe* species. NMR has been a major method for efficient analyses of plant extracts

(Bilia, 2006). Unlike HPLC, HPTLC, and capillary GC with specific detectors such as coupled systems (Mass Spectrophotometer), NMR is less time-consuming for simple and rapid chromatographic separation and can detect unknown metabolites that contribute to biological activity (Bilia, 2006). In addition, the high-throughput sequencing employed in this study provided an effective way to analyse all microbes present in a sample, allowing for easy annotation and classification of diverse and numerous microorganisms to obtain a complete composition of microbial communities (Kulski, 2016).

Aloe plants are widely used for various purposes due to their secondary metabolites (Bajpai, 2018). The metabolite annotation in this study revealed important phytohormone (indole-3-acetate and 5-hydroxyindole-3-acetate) and phytochemicals (4-hydroxybenzoate, adenine, chlorogenate, biotin and citrate) from the *Aloe* species. Previous studies have identified and established the biological activities of the metabolites tyrosine, allantoin, putrescine, indole-5-acetic acid and alanine, which were also annotated in this study (Botes et al., 2008, Kaur et al., 2021b, Kim et al., 2013, Ahluwalia et al., 2021, Zapata et al., 2013, Beppu et al., 2004, Chelu et al., 2023, Martínez-Sánchez et al., 2020). The results also show that both *Aloe* spp. contain some analogous metabolites (Table 3.1), although in different concentrations, highlighting the potential feasibility of using *A. longibracteata* as a replacement for *A. lettyae* due to its greater availability and lower conservation concerns. In this context, the study's hypothesis that the use of *A. longibracteata* should be promoted over the endangered *A. lettyae* is validated, representing an interesting avenue for sustainable resource management. The interchangeable use of *A. lettyae* and *A. longibracteata* is plausible due to their common metabolites, including allantoin, tyrosine, and myo-inositol. Extensively studied in other *Aloe* species, these metabolites are associated with various uses in cosmeceuticals and human health management. Allantoin, recognized for its moisturizing properties, finds common application in skincare products and pharmaceutical formulations (Talakoub et al., 2009). Tyrosine, functioning as a precursor for neurotransmitters and thyroid hormones, plays crucial roles in mood regulation, cognitive function, and metabolism (Wang et al., 2012). Myo-inositol has been investigated for its potential therapeutic effects on conditions like depression, anxiety, and metabolic disorders (Vadnal et al., 1997). Due to the similarities in the morphological structures of most *Aloe* plants, it is possible for collectors to accidentally collect one species instead of the other. However, because of the shared biochemical chemistry of most *Aloe* species, the intended uses are still realized. When considering the possibility of this substitution, it is important to consider factors beyond the presence of metabolites, such as bioactivity, quantity, and market demand. It is necessary to further investigate the qualitative and quantitative differences in metabolite profiles between the *Aloe* species to comprehensively assess their suitability for industrial applications and conservation initiatives.

Although *A. lettyae* and *A. longibracteata* exhibit common metabolites, they also showed some distinct differences in their metabolic profiles due to various factors, reflecting the complexity of their metabolic pathways and the influence of environmental and genetic determinants. In our study, a clear distinction was observed in the endophytic bacterial community between *A. lettyae* and *A. longibracteata* (Figure 3.7), which can significantly impact metabolite profiles (Woźniak et al., 2022, Zhang et al., 2020, Chevrette et al., 2022). Different bacterial strains possess unique enzymatic capabilities and metabolic interactions with their host plants, influencing the synthesis, modification, or degradation of specific metabolites (Narayanan and Glick, 2022). As a result, the observed variations in microbial composition could have contributed to the differences in metabolite profiles between *A. lettyae* and *A. longibracteata*. Additionally, environmental factors such as soil composition, climate, and habitat conditions can exert selective pressures that shape metabolic adaptations in each species over time, influencing the production of specialized metabolites (Sampaio et al., 2016). Although not established in this study, the relationship between microbial and environmental factors underscores the complexity of metabolite production in *Aloe* species, emphasizing the need for more comprehensive studies to elucidate the underlying mechanism driving metabolic diversity under different ecological factors.

In addition, the *Aloes* produced key metabolites with human health importance, such as 4-hydroxybenzoate and chlorogenate (Table 3.1) produced by *A. lettyae* and *A. longibracteata,* respectively. In a study by (Kosová et al., 2015), 4-hydroxybenzoate was demonstrated to have comparable inhibitory activity to commercially used parabens against various pathogens, such as *Staphylococcus aureus* ATCC 6538*, Escherichia coli* ATCC 700728*, Saccharomyces cerevisiae* DMF 1017 and *Fusarium culmorum* DMF 0103. This metabolite may also exhibit neuroprotective effects against Alzheimer's disease (Winter et al., 2017). These dual properties underpin the significance of further investigating the therapeutic potential of 4-hydroxybenzoate both as an antimicrobial agent and as a candidate for neuroprotection against Alzheimer's disease. Adenine, another metabolite with human health benefit annotated from *A. lettyae*, is an essential component of nucleic acids (DNA and RNA) and adenosine triphosphate (ATP), playing a critical role in cellular energy metabolism and genetic processes (Matsumoto et al., 1980). The deficiency of adenine may contribute to severe combined immunodeficiency disorder (SCID) (Flinn and Gennery, 2018). Chlorogenate, biotin and citrate are differentially abundant metabolites in *A. longibracteata*. Previous studies have revealed the antiviral effect of chlorogenic acid, a chlorogenate type metabolite against influenza A viruses on MDCK cells: A/puertoRico/8/1934(H1N1) and A/Beijing/32/92(H3N2) viruses with EC_{50} values of 44.87 μM and 62.33 μM, respectively (Ding et al., 2017). Biotin is an essential vitamin involved in various physiological processes such as energy metabolism, gene regulation, and histone modifications

(Zempleni et al., 2008). Its deficiency can lead to metabolic disorders, skin rashes, and neurological symptoms (Mock, 2017). The energy metabolism, bone health, and acid-base balance of citrate, as outlined by (Costello et al., 2012), suggest that it is an anticoagulant and chelating agent for the treatment of certain diseases, which further underscores its potential therapeutic applications. Collectively, the findings of these studies reveal that *A. lettyae* and *A. longibracteata* species possess a rich reservoir of secondary metabolites with promising applications for human health and disease management.

Furthermore, some of the annotated metabolites, including indole-3-acetate and its derivative 5-hydroxyindole-3-acetate are phytohormones, which play key roles in plant growth promotion (Raimi et al., 2017). These phytohormones are naturally occurring auxins in plants, which enhance cell elongation, apical dominance, root initiation, vascular tissue differentiation, and plants' ability to acquire water and nutrients from the soil (Fu et al., 2015). This growth-promotion property suggests that the metabolites of unendangered *Aloe* species can be harnessed to enhance the yield and biomass of endangered *Aloe* species. Interestingly, microbial endophytes have been reported to produce these plant hormones (Raimi and Adeleke, 2023), emphasising the need for further research into the endophytic microbial communities of *Aloe* species and their abilities to synthesise host metabolites. Such microbial communities with similar metabolite production as their host hold huge promise in microbial formulation. Thus, *Aloe* species and their microbial endophytes are useful in the conservation strategy of endangered *Aloe* species and in increasing metabolite synthesis through plant biomass production and plant health management.

The NMR-based metabolomics showed that there are 15 differential metabolites between the two *Aloes* (Supplementary Figure S1). Thirteen out of 15 metabolites from *A. longibracteata* had a significantly higher concentration compared to that of *A. lettyae*. On the other hand, the microbial community analysis indicated that *A. lettya*e harbours a more phylogenetically diverse bacterial community compared to *A. longibracteata,* suggesting higher diversity may not necessarily translate to more differential metabolites. It may be necessary to further study the *A. lettyae* and its complete microbiome before it disappears from the face of the earth, depriving us of the opportunity to leverage its unique bacterial community for beneficial purposes. The correlation analysis (figure 3.9) showed a high association between the metabolites and endophytic bacteria, providing additional evidence that the microbes could play a pivotal role as primary drivers of secondary metabolite production in the host plant (Prakash et al., 2019, Hiruma, 2019, Singh et al., 2019). (Apine and Jadhav, 2011) demonstrated IAA production by *Pantoea agglomerans*, a bacterium associated with plants. These findings align with the result of this study, where the metabolite N-acetylserotonin and IAA exhibited a notable correlation with both *Pantoea* and *Citrobacter*,

suggesting the endophytes of *Aloe* species can influence the production of notable metabolites of the host plant.

Although there is currently no prior documented study on the bacterial community of *A. lettyae* and *A. longibracteata* species, the endophytic bacterial communities of *Aloe vera*, a commonly known *Aloe* species, have been extensively investigated, revealing a diverse spectrum of bacteria with the potential to promote plant growth and produce bioactive compounds (Joshi et al., 2018, da Silva et al., 2022, Swati et al., 2022). (Silva et al., 2020, Akinsanya et al., 2015) identified *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria* as the dominant phyla, with specific genera such as *Pseudomonas, Bacillus* and *Enterobacter* being predominant. These bacteria have been reported to produce siderophores and antimicrobial compounds, indicating their potential in plant growth promotion and biocontrol of pathogens, especially of endangered *Aloe* species (Jha et al., 2011, Sivasakthi et al., 2014, Nutaratat et al., 2017, Saranraj et al., 2022). These endophytic bacteria can be employed in the production of bacterial inoculum to improve the growth, yield and productivity of *Aloe* plants (Raimi and Adeleke, 2023), thereby increasing the availability of *Aloe* biomass for biotechnological applications. There were high numbers of unclassified taxa (data not shown), which may suggest that the endophytic bacterial community of *Aloe* species are still underexplored with several unculturable species, presenting a source of potential novel isolates. In addition, our findings suggest that *Aloe* species harbour microbes, which may act as partners with the host plants in metabolite production (Sharma et al., 2021). However, further study is necessary to validate this claim, particularly to substantiate the possibility of utilizing endophytes for metabolite production rather than relying solely on plant tissues may offer a sustainable approach to utilizing plant resources (Mishra et al., 2021b).

The significance of correlating differential metabolites with dominant endophytic bacteria lies in unravelling the complex interplay between plant metabolism and microbial communities within the host. *Pseudomonas aeruginosa* has been found to produce agmatine, a metabolite of arginine, through the aguBA operon (Williams et al., 2010). This production is said to be induced during the stationary phase of growth and biofilm formation (Williams et al., 2010). The agmatine deiminase pathway, involved in the metabolism of agmatine and microbial putrescine biosynthesis, is characteristic of *Pseudomonas* species (Stalon and Mercenier, 1984), a dominant genus reported in the *Aloe* species in this study. Moreover, the use of C4-dicarboxylic acids, such as malate, by Bacteroides in N-fixation is regulated by amino acid excretion and polyhydroxybutyrate biosynthesis, with glutamate being an overflow product (Poole and Allaway, 2000). *Comamonas testosteroni* has been found to produce tyrosine, a key amino acid involved in various biological processes (Brooks and Benisek, 1994). This ability is possibly due to the presence of a distinct tyrosinase (TyrA), which is involved in melanin production in the related genus *Aeromonas* (Wan et

al., 2009). The unique ability of endophytes to produce similar metabolites as the host suggests their potential as viable resources in microbial technology for the synthesis of various bioactive compounds.

The robust alignment between our findings and those of the referenced studies underscores a compelling consistency in the identification of dominant endophytic bacteria across the two *Aloe* species. The observed correlation between the bacterial communities and the differential presence of key metabolites such as agmatine, tyrosine, and IAA strengthens the significance of plant-microbe interactions. These results not only enhance the reliability of our findings but also highlight the potential implications for leveraging the endophytic microbial communities in various fields, ranging from agriculture to pharmaceuticals.

Very few studies have explored the biosynthetic pathways of secondary metabolites in *Aloe* species to date (Choudhri et al., 2018, Kim et al., 2021, Ushasree et al., 2024). However, research on the endophytic communities of *Aloe* species has revealed a diverse range of bacterial species including *Bacillus, Pseudomonas*, and *Enterobacter* being the dominant genera that contribute to various metabolic pathways (Akinsanya et al., 2015), suggesting their application in microbial technology for the production of unique metabolites. These bacteria were found to produce bioactive compounds with medicinal importance (Akinsanya et al., 2015). *Bacteroides fragilis* and other species from this genus have been demonstrated to metabolize tryptophan via the kynurenine pathway, resulting in the synthesis of xanthurenate alongside other tryptophan-derived metabolites (Agus et al., 2018, Bear, 2023). These findings are consistent with the outcomes of our study, where xanthurenate was reported amongst the key metabolites in the *Aloe* species.

As we have established the biochemical similarities between *A. lettyae* and *A. longibracteata*, the next logical step is to investigate the microbial interactions within these species, focusing on plant growth-promoting bacteria. Understanding how these bacteria enhance plant growth and contribute to resilience is essential. In the following chapter, we will examine the plant growth-promoting bacteria associated with *A. lettyae* and *A. longibracteata*, aiming to uncover their potential for supporting *A. lettyae* in re-establishment efforts. This exploration will deepen our understanding of their interactions and potential applications in sustainable conservation practices.

3.5 Conclusion

This study presents the first comprehensive investigation into the metabolite profiles and endophytic bacterial communities of *A. lettyae* (endangered) and *A. longibracteata*. The identification of important metabolites, including phytohormones with plant growth-promoting potential and bioactive compounds with health benefits, underscores the value of these *Aloe* species in sustainable agriculture and drug production. The presence of phylogenetically diverse endophytic bacterial communities in *A. lettyae* may further suggest the communities have varied functional profiles with the potential for biotechnological applications and conservation use. The observed differences in metabolite composition and endophytic community structures between the Aloe species highlight the need for a deeper exploration of their unique biochemical and ecological characteristics. Future studies should focus on elucidating the specific roles of these metabolites and their endophytic bacteria in agriculture, medicine, and bioremediation. Given the endangered status of *A. lettyae*, the findings of this study emphasize the urgency of preserving this unique species and its associated microbial communities. Additionally, the identification of shared metabolites and endophytic bacterial communities between the *Aloe* species could facilitate the use of *A. longibracteata* instead of *A. lettyae,* thereby leading to the conservation and sustainable utilization of the unique biochemical resources of the endangered species.

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Chapter 4

In vitro evaluation and genomic analyses of endophytes of *Aloe* species for their potential to enhance plant growth and stress tolerance: A unique strategy for plant conservation

This chapter is intended for submission to the Journal Microbial Ecology.

[a] Cynthia K. Marokane-Radebe, [a]Kazeem A. Alayande, [a.b] Stephen O. Amoo, [a] Rasheed A. Adeleke*

[a] Unit of Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa

[b] Agricultural Research Council - Vegetables, Industrial and Medicinal Plants, Roodeplaat, Pretoria, 0001. South Africa.

Abstract

The critically endangered *Aloe lettyae* faces significant habitat loss, necessitating innovative conservation approaches. This chapter explored the plant growth-promoting (PGP) potential of endophytic bacteria associated with *A. lettyae* and *Aloe longibracteata*, a closely related, non-threatened species. A total of 17 bacterial endophytes were isolated and functionally screened for key Plant Growth Promotion traits. Most isolates demonstrated nitrogen fixation and siderophore production, while six exhibited phosphate-solubilizing ability. Two isolates identified through 16S rRNA sequencing as *Bacillus velezensis* and *Bacillus safensis* were selected for further testing based on high IAA production and strong enzymatic activity. These strains also showed moderate tolerance to salinity and fluctuating temperatures in vitro, highlighting their potential for promoting plant resilience. Genome annotation confirmed the presence of genes linked to auxin biosynthesis (ipdc), phosphate solubilisation (pho family), nitrogen metabolism (nifU), and siderophore production (pvd), aligning with observed phenotypic traits. Although limited to *in vitro* analyses due to slow aloe growth rates and lack of commercially available *A. lettyae* seeds, the results underscore the value of microbiome-assisted conservation. This research provides a foundational step toward using beneficial endophytes to support the survival and restoration of *A. lettyae* in degraded or novel environments.

4.1. Introduction

Aloe lettyae (*A. lettyae*), an endemic species of South Africa's critically endangered Woodbush Granite Grassland, is facing significant threats due to habitat destruction (Kremer-Köhne et al., 2020a). Historically, this unique grassland ecosystem has suffered extensive degradation, with less than 30% of the original habitat remaining intact (Kremer-Köhne et al., 2022, Raimondo et al., 2015). Key drivers of this loss include agricultural expansion, the spread of timber plantations, and overgrazing by livestock (Raimondo et al., 2015). In addition, the invasion of unmanaged alien plant species and the mismanagement of fire regimes have further intensified the decline of this ecosystem (Moshobane et al., 2022, Raimondo et al., 2015). As a result, *A. lettyae* populations have become increasingly fragmented and vulnerable, with only a few relatively undisturbed areas still available for the species to thrive (Kremer-Köhne et al., 2020b). It is therefore pertinent to identify an environmentally friendly approach that could be used to restore the population. One focus that is becoming increasingly popular is the use of biological approach such as endophytes.

Endophytic bacteria, microorganisms that live symbiotically within plant tissues have gained attention for their ability to enhance plant growth and resilience in stressful environments (Kamran et al., 2022, Vaishnav et al., 2019, Jasim et al., 2013). These beneficial microbes can promote nutrient acquisition, boost tolerance to abiotic stressors such as drought and salinity, and even help plants fend off pathogens (Verma et al., 2021, Ullah et al., 2019, Oukala et al., 2021). Most studies have demonstrated the growth-promoting potential of endophytes isolated from the same plant species (Afzal et al., 2019). However, there is evidence suggesting that endophytic bacteria can also enhance the growth of non-host plants (Sessitsch et al., 2005). The question of host specificity in endophytes has produced contrasting reports. This specificity may be influenced by factors such as plant secondary metabolites, root exudates, and tissue structure, which affect microbial colonization and compatibility (Hardoim, 2015). On the other hand, several researchers have documented instances where endophytes promote the growth of a wide range of plant species, beyond their original host (Ma et al., 2011, Sessitsch et al., 2005). Such broader host range supports the potential transfer of endophytes between related, yet distinct, plant species. This adaptability underscores the value of endophytes as a powerful tool for promoting plant growth in conservation efforts, especially for endangered plant species such as *A. lettyae*.

The vulnerability of *A. lettyae* is primarily due to habitat destruction rather than an inherent difficulty in its cultivation. This opens the possibility of reintroducing the species to new environments outside its native range, provided that its growth and stress tolerance can be improved. Reintroducing endangered species like *A. lettyae* into non-native or degraded habitats

presents a unique challenge, as these environments often subject plants to stressors. Overcoming these challenges requires innovative approaches to ensure plant survival and establishment in such conditions. By investigating the *in vitro* plant growth-promoting potential of endophytic bacteria isolated from *A. longibracteata*, this study provides a foundation for using these beneficial endophytes from *A. longibracteata* in conservation strategies aimed at enhancing the adaptation and re-establishment of *A. lettyae* in degraded or novel habitats.

The ecological compatibility between *A. lettyae* and *A. longibracteata* further supports the potential for endophyte transfer between these species. *Aloe lettyae* thrives at altitudes between 860 and 1800 meters above sea level (asl), while *A. longibracteata* is found at similar elevations, approximately 1524 to 1829 meters asl (5000–6000 ft) (Kremer-Köhne et al., 2020b, Grace, 2009). This overlap in altitude suggests that both species are adapted to comparable mid- to high-altitude climates, which often include similar environmental conditions such as temperature, humidity, and solar exposure. Additionally, *A. lettyae* inhabits Woodbush Granite Grassland, while *A. longibracteata* grows in rocky outcrop grassland(Kremer-Köhne et al., 2020b, Smith and Klopper, 2022). These environments share similarities in terms of open exposure, rocky or nutrient-poor soils, and potential water scarcity, suggesting that both species are adapted to surviving under harsh conditions. The endophytes of *A. longibracteata* have likely evolved mechanisms to help the plant cope with stressors such as drought, poor soil nutrition, and temperature extremes traits that could prove advantageous for *A. lettyae*. By investigating the in vitro plant growth-promoting potential of these endophytes, this study provides a foundation for their eventual use in field applications aimed at conservation.

Through improving *A. lettyae* resilience and adaptability, the ultimate goal of this approach is to increase the population of *A. lettyae* through cross-biofertilization using beneficial endophytic communities from A. longibracteata. Success in this approach would not only enhance its conservation status but may also lead to its removal from the endangered species list. It is therefore hypothesized that, *A. lettyae* and *A. longibracteata* harbour similar culturable bacterial endophytes with plant growth promotion potential and the introduction of endophytic bacteria from *A. longibracteata* will significantly improve the growth and stress tolerance of *A. lettyae* in unfavourable environmental conditions, thereby facilitating its successful re-establishment in degraded or alternative habitats.

4.2 Materials and Methods

Due to the unavailability of *A. lettyae* seeds, it was not possible to conduct greenhouse or field trials at this stage of the study. As such, the research was confined to *in vitro* experiments, which allowed

for an initial exploration of the potential for endophytic bacteria from *A. longibracteata* for direct and indirect plant growth promotion. *In vitro* conditions provide a controlled environment to assess plant endophyte interactions without the complexities and variables associated with field settings.

4.2.1 Isolation of bacterial endophytes

From each *Aloe* species, one leaf was excised from the shoot tip and washed under running tap water. Sampling was conducted using a non-destructive approach that ensured minimal harm to the parent plant, with only a single leaf collected per individual. All necessary ethical guidelines for working with endangered plant species were strictly followed. A collection permit was obtained from the Limpopo Department of Economic Development, Environment and Tourism (permit number:ZA/LP/109633) for *Aloe* species, which were sampled from their natural habitats. The surface sterilization of the aloe samples closely follows the approach outlined by Sahu et al. (2022), with slight modifications. Briefly, leaf tissues were treated in a series of baths containing sterile distilled water for 1 min, 70% ethanol for 30 s, and 2.5% sodium hypochlorite for 4 min. Finally, the leaf was rinsed thrice in sterile distilled water. A 0.1 mL aliquot of the final rinse water was plated onto nutrient agar plates to confirm the success of surface sterilization.

The sterilized plant leaves were then cut into approximately 5 mm segments under sterile conditions, and 20 leaf segments per individual plant were placed in four Petri dishes (9 cm; five segments/plate) containing nutrient agar and incubated in the dark at 35 ± 2 ºC (Arora and Ramawat, 2017). The culture plates were regularly observed for bacterial growth, for a period of 48 h. Bacteria growing from the plates were streaked on fresh nutrient agar plates to obtain single colonies and stored at 4 ºC until further analyses.

4. 2.2. Pathogenicity screening of endophytes using haemolysis assay

All pure culture isolates of the bacterial endophytes from the aloes were evaluated for potential pathogenicity through a haemolysis assay, as described by Amaria et al. (2023). The isolates were spot inoculated onto sheep blood agar plates and incubated at 30 °C for 48 hours. Hemolytic reactions were determined based on the presence or absence of discoloration or clear zones around the colonies.

Alpha (α) haemolysis was indicated by a greenish or dark halo surrounding the colonies, reflecting partial lysis of red blood cells. Beta (β) haemolysis was confirmed by the formation of clear zones, indicating complete lysis. In contrast, gamma (γ) haemolysis, signifying non-hemolytic activity, showed no visible changes in the agar surrounding the colonies. Only isolates exhibiting gamma haemolysis were considered non-pathogenic and were subsequently selected for molecular

identification and evaluation of plant growth-promoting (PGP) traits, including nitrogen fixation, phosphate solubilisation, indole-3-acetic acid (IAA) and siderophore production, as well as hydrolytic enzyme activity.

4.2.3 Molecular identification of culturable endophytes

The genomic DNA of the isolated organisms was extracted using a ZymoBIOMICS™ DNA extraction kit (Zymo Research) according to the manufacturer's protocol. In the final step for DNA elution, the volume of the elution buffer was made to 50 µL to increase the DNA concentration. 2 µL DNA sample was used as a template in 25 µL PCR 16S rRNA gene amplification. The remaining DNA was stored at -23 °C for further test including whole genome sequencing. Using the bacterial universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction contained: 12 µL master mix, 7 µL PCR buffer, 1 µL of each primer and 2µL of bacterial genomic DNA. The PCR cycling conditions were 94ºC for 3 min, followed by 30 cycles of 94ºC for 0.5 min, 55ºC for 0.5 min, and 72ºC for 1 min, followed by a final extension performed at 72ºC for 10 min. The PCR products were sent to Inqaba Biotech, a commercial sequencing service provider based in South Africa, for Sanger sequencing. The 16S rRNA sequences generated in this study were compared against the Genbank database using the National Center for Biotechnology Information (NCBI) BLAST nucleotide search and then deposited in Genbank. A multiple sequence alignment was constructed on approximately 600 bp of the 16S rRNA gene fragments using the ClustalX (version 2.0) software (http://www.clustal.org/clustal2) and a phylogenetic tree was constructed using the neighbour-joining method in the MEGA v11.0.13 software (www.megasoftware.net), with confidence tested by bootstrap analysis (1000 repeats).

4.2.4 *In vitro* screening for plant growth-promoting (PGP) traits

4.2.4.1 Phosphate solubilisationsolubilisation

The isolated bacterial endophytes were screened for their phosphate solubilisation potential. Pikovskaya medium (10 g/L glucose; 2.5 g/L Ca_3 $(PO4)_2$; $(NH_4)_2SO_4$ 0.5 g/L; NaCl 0.2 g/L; $MgSO_4.7H_2O$ 0.1 g/L; KCl 0.2 g/L; $FeSO_4.7H_2O$ 0.002 g/L; yeast extract 0.5 g/L; $MnSO_4.2H_2O$ 0.002 g/L; agar 15 g/L; and 1 L dis. H_2O) was prepared and bromophenol blue was added as an indicator. The medium was inoculated with endophytic isolates and incubated for 48 h at 30ºC. The Pikovskaya medium without bacterial inoculation was used as a control. The formation of clear zones around the colony, due to the utilization of tricalcium phosphate, was measured to assess the ability of endophytes to solubilise phosphate(Jasim et al., 2013). All phosphate solubilisation

screens were performed in triplicates. Their phosphate solubilising efficiency (PSE) and solubilising index were calculated using the following formulae:

$$PSE(\%) = \frac{Z - C}{C} \times 100$$

Where,

Z=Solubilisation zone (mm)

C=Colony diameter (mm)

$$Solubilization\ index(SI) - \frac{Solubilization\ zone + Colony\ diameter}{Colony\ diameter}$$

4.2.4.2 Nitrogen fixation

The nitrogenase activity of the isolates was determined by the growth on a nitrogen-free medium, the Burks Agar (HiMedia, India) according to Burk (1941). Pure bacterial colonies were streaked on the Burks Agar and incubated for 5–7 days at 28 ± 2°C. All tests were done in triplicate. The appearance of the bacterial colonies indicated a positive test.

4.2.4.3 Siderophore production

Siderophore production was evaluated on universal Chrome Azurol S (CAS) agar (Schwyn and Neilands, 1987). Each bacterial strain was first pre-cultured on nutrient agar and incubated at 37 °C for 24 h. A single colony was then transferred into nutrient broth and incubated at 37 °C with shaking for 12hrs. The bacterial suspension was adjusted to a McFarland standard of 0.5 using a spectrophotometer at 600 nm. A 10 µL aliquot of the standardized bacterial suspension was then spot-inoculated at the center of the CAS agar plate under aseptic conditions. The plates were incubated at 28 °C for 72 h. The appearance of an orange halo surrounding the bacterial colony indicated siderophore production. The assay was performed in triplicates to ensure accuracy and reproducibility.

4.2.4.4 Enzymes

The enzyme activity of endophytes isolated from *A. longibracteata* and *A. lettyae* was qualitatively analysed for their ability to hydrolyse starch, cellulose, casein, and lipids. Amylase activity was assessed using starch agar medium containing 0.2% soluble starch and 1.5% agar, where bacterial endophytes were spot-inoculated and incubated at 25ºC for 7 days; the plates were then flooded with Gram's iodine solution to reveal zones of inhibition. Protease activity was analysed on casein

agar with 10% skim milk powder and 1.5% agar, which was autoclaved separately before mixing and plating; bacterial endophytes were point-inoculated and incubated at 25ºC for 7 days, and enzyme activity was indicated by clear zones around the inoculation sites. For cellulase analysis, nutrient agar with 1% cellulose powder was used, with bacterial endophytes spot-inoculated and incubated at 25ºC for 7 days, after which Gram's iodine solution was added to visualise zones of inhibition. Lipase activity was assessed by inoculating endophytes onto nutrient agar supplemented with 1% filtered Tween 20 after autoclaving, with plates incubated at 25ºC and checked regularly over 7 days for zones of inhibition indicating lipase activity. All tests were performed in triplicates.

4.2.4.5 Indole-3-acetic acid (IAA) production

Indole acetic acid production was quantitatively measured according to Gordon and Weber (1951). Bacterial cultures were grown in test tubes, each containing 5 mL nutrient broth (Merck Millipore, UK) amended with 0.1% (w/v) tryptophan, the bacterial cultures were also allowed to grow in a tryptophan-free nutrient broth, and incubated at 28 ± 2°C for 48 h. Then, the cultures medium was centrifuged at 10,000 rpm for 10 min. A total of 1 mL supernatant was mixed with 2 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid). Tubes were incubated in the dark at room temperature for 25 min. The development of pink colour indicates high production of IAA and the intensity of pink colour was read at 530 nm wavelength in a spectrophotometer. The concentration of IAA produced was then extrapolated from a standard curve, which was plotted using known concentrations of pure IAA as the standard (Gordon and Weber, 1951)

4.2.5.1 Salt tolerance test

Bacterial isolates were evaluated for salt tolerance by culturing them in nutrient broth (NB) supplemented with different concentrations of sodium chloride (NaCl; 5%, 7.5%, and 10% w/v) and magnesium chloride ($MgCl_2$; 5%, 7.5%, and 10% w/v). Cultures were inoculated into 96-well microplates and incubated at 28 ± 2 °C for 24 hours. Nutrient broth without any added NaCl or $MgCl_2$ served as the control to assess normal bacterial growth. The growth of the isolates under salt stress was determined by measuring the optical density at 600 nm (OD_{600}) using a microplate reader. All tests were conducted in triplicate, and the mean ± standard deviation (SD) of the OD values was calculated to compare growth under different salt concentrations (Zahra et al., 2023).

4.2.5.2 Temperature tolerance test

The bacterial isolates were assessed for their cold temperature tolerance by growing them on nutrient agar plates incubated at varying temperatures (4°C, 10°C, 25°C, 30 °C and 37°C) for seven days. After incubation, the viability of the isolates was confirmed by streaking a portion of the

bacterial growth from each temperature condition onto fresh nutrient agar plates, incubated at optimal temperature (30ºC) and observing colony formation. All tests were performed in triplicates.

4.2.6 Preparation of the genome assembly and annotation

Total reads generated for the isolates was 4,285,401 (2x150-bp) paired-ends. The reads were filtered to remove adapter sequence and low-quality fragments using Trimmomatic v0.36 (Bolger et al., 2014) with a minimum quality score of 15 and a minimum sequence length of 70. The adapter regions were clipped using a mismatch value of 2, a palindrome clip threshold of 30, and a simple clip threshold of 10. The assembly was performed with the quality reads using SPAdes v3.15.3 (Bankevich et al., 2012; Prjibelski et al., 2020). Completeness of the assembled genome and presence of undesired sequence fragments were assessed using CheckM v1.0.18. The genome assembly was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v6.7 (Li et al., 2021) and Rapid Annotations using Subsystems Technology (RAST) with SEED viewer v. 2.0 (Overbeek et al., 2014). Mapping of the entire circular genome was visualised using Proksee (Grant et al., 2023) while the CRISPR array and Cas clusters was determined by CRISPR/Cas finder, and CARD resistance genes Identifier was used to identify antimicrobial resistance genes within the genome.

4.2.7 Data analysis

Plant growth-promoting parameters (e.g., optical density, enzyme activity) were analysed using one-way ANOVA followed by Tukey's post-hoc test ($P < 0.05$) to compare differences across bacterial strains.

For IAA production, a two-way ANOVA (factors: strain × tryptophan treatment) was applied, with Bonferroni's post-hoc test (adjusted $P < 0.05$) to identify specific effects of tryptophan within each strain. Significant differences are denoted as: ****$P < 0.0001$, **$P = 0.0001–0.0004$, *$P = 0.0031$, $P \leq 0.0366$. All analyses were performed using GraphPad Prism version 8.0.2. Data are presented as means ± SE (n = 3)

4.3 Results

A total of 62 endophytic bacterial isolates were obtained, with 33 isolates from *Aloe lettyae* and 29 from *Aloe longibracteata*. Following haemolysis screening for potential pathogenicity, only 17 non-pathogenic isolates were selected for further analysis. These selected isolates were subjected to molecular identification using 16S rRNA gene sequencing and evaluated for plant growth-promoting (PGP) traits and other functional characteristics. The endophytic bacteria were identified

as members of the genera *Bacillus* and *Enterobacter* (Table 4.3.1). Specifically, *A. lettyae* harboured four *Bacillus* species and four *Enterobacter* species, while *A. longibracteata* contained five *Bacillus* and four *Enterobacter* species. The 16S rRNA gene sequences of isolates from *A. lettyae* (AL samples) showed 97–99% similarity to reference sequences of *Bacillus xiamenensis*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter sp.*, *Bacillus sp*., *Bacillus pumilus*, and *Bacillus safensis* (Figure 4.3.1; Table 4.3.1). In contrast, isolates from *A. longibracteata* (Alon samples) exhibited 95–99% sequence similarity to *Enterobacter asburiae, Enterobacter hormaechei*, *Enterobacter cloacae*, *Enterobacter sp*., *Bacillus australimaris*, *Bacillus velezensis*, *Bacillus pumilus*, and *Bacillus siamensis*.

Table 4.3.1: The 16S rRNA sequences identification of endophytic bacterial strains from *A. lettyae* and *A. longibracteata*

Plant species	Bacterial strain code	Homologue sequences (sequence identity %)	NCBI Accession Numbers
Aloe lettyae	AL7	*Bacillus xiamenensis* (99%)	OR991825
	AL9	*Enterobacter asburiae* (97%)	OR991826
	AL10	*Enterobacter cloacae* (99%)	OR991827
	AL13	*Enterobacter sp* (98%)	OR991828
	AL22	*Bacillus sp* (99%)	OR991829
	AL24	*Enterobacter asburiae* (99%)	OR991830
	AL27	*Bacillus pumilu* (97%)	OR991832
	AL33	*Bacillus safensis* (99%)	OR991831
Aloe longibracteata	Alon1	*Enterobacter asburiae* (98%)	OR991817
	Alon4	*Enterobacter hormaechei* (95%)	OR991818
	Alon6	*Bacillus australimaris* (99%)	OR991819
	Alon9	*Bacillus velezensis* (99%)	OR991820
	Alon13	*Enterobacter sp.* (97%)	OR991821
	Alon14	*Enterobacter cloacae* (99%)	OR991822
	Alon18	*Bacillus velezensis* (99%)	OR991816
	Alon20	*Bacillus siamensis*(99%)	OR991823
	Alon22	*Bacillus pumilus* (98%)	OR991824

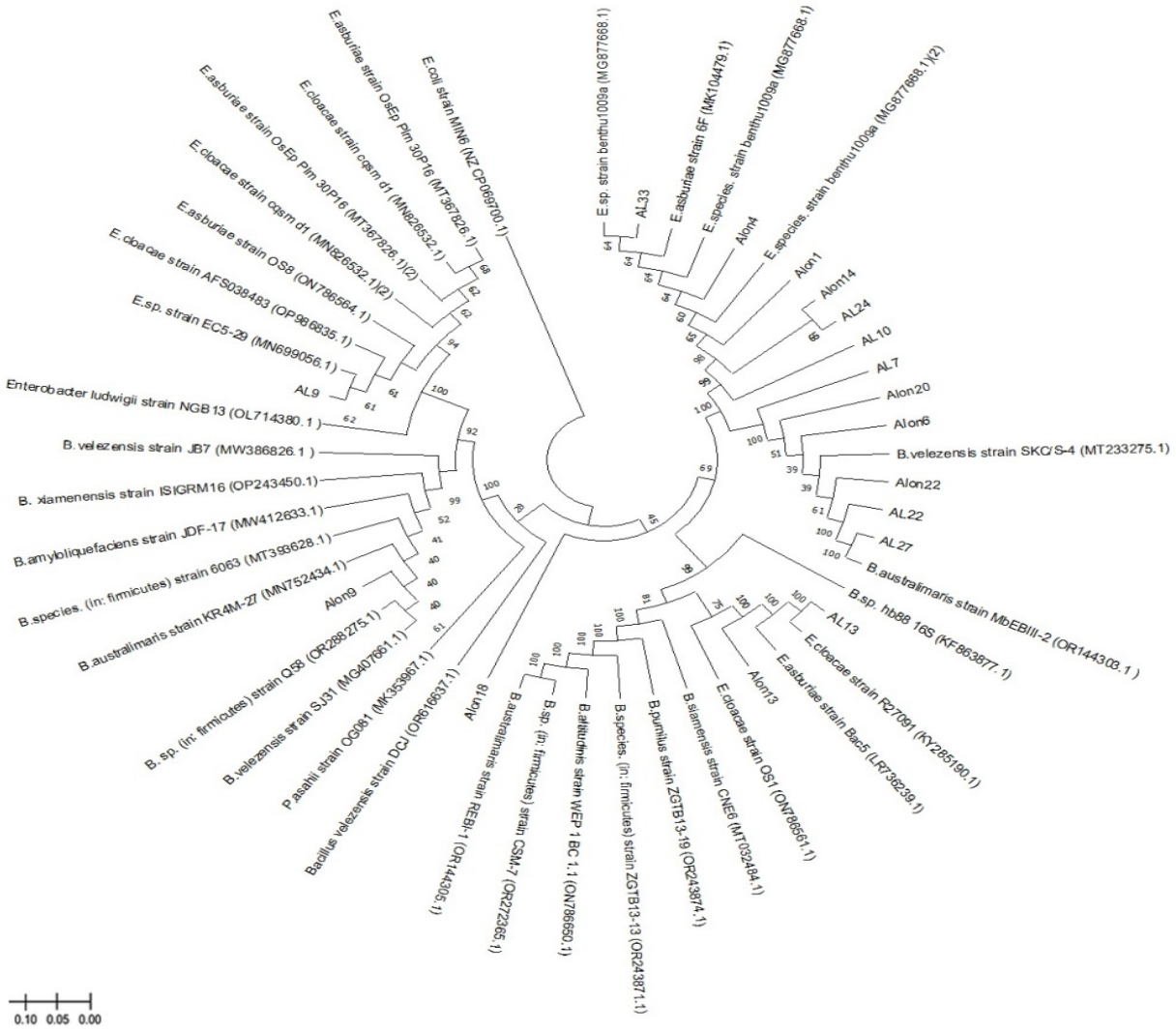


Figure 4.3.1: Phylogenetic analysis of 16S rRNA sequences of bacterial strains with reference sequences from NCBI. ALon refers to bacteria isolated from *Aloe longibracteata* plants, whereas AL refers to sequences from *Aloe lettyae* isolates. The identity of the bacterial isolates is provided in Table 2. A multiple sequence alignment of approximately 1200 bp of the 16S rRNA gene fragments was performed using ClustalX 2.0 (http://www.clustal.org/clustal2). The evolutionary distances were calculated using the p-distance method (Nei and Kumar, 2000), which was preferred due to the high sequence similarity and computational simplicity. These distances were then used to construct a neighbour-joining tree in MEGA v11.0.13 (www.megasoftware.net), with confidence levels assessed by bootstrap analysis with 1000 replicates (Felsenstein, 1985; Tamura et al., 2021)..

The plant growth-promoting potential of the 17 endophytic bacterial isolates was investigated and it was observed that five isolates, including Alon9, Alon13, Alon1, Alon18 and Alon20 were the only phosphate solubilisers from *A. longibracteata* while *A. lettyae* had only one isolate that can solubilise phosphate (AL33) (Figure 4.3.2). However, 80% of both the *A. lettyae* and *A. longibracteata* isolates were able to produce the iron-chelating compound, siderophore (Figure 4.3.2). All the isolates from both *A. lettyae* and *A. longibracteata* showed the ability to fix nitrogen,

with the highest activity observed in Alon4, Alon14, Alon18, Alon20 and AL13 (in order of decreasing ability) (Figure 4.3.2).

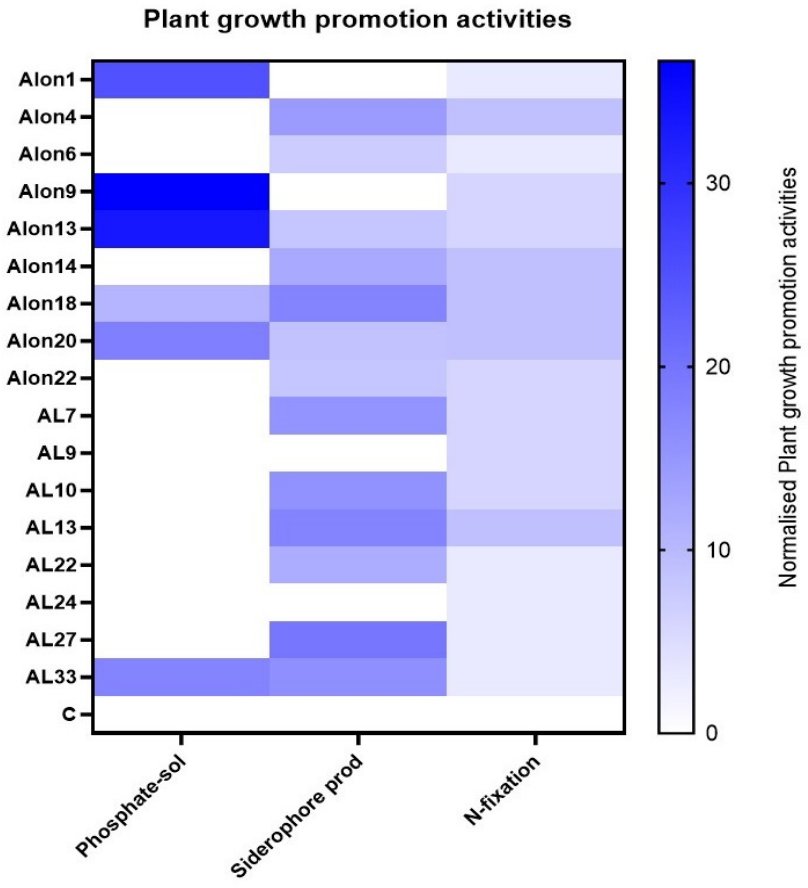


Figure 4.3.2: Heat map showing phosphate solubilisation, siderophore production and nitrogen fixation activity of endophytic bacteria from *A. lettyae* and *A. longibracteata*.

All bacterial endophytes except AL22 and AL24 displayed enzymatic activity for at least one enzyme, with varying degrees of activity across protease, lipase, amylase, and cellulase assays (Figure 4.3.3). Notably, the highest cellulase activity was observed in isolates AL33 and AL9, as indicated by the darkest, largest bubble in the cellulase column (Figure 4.3.3). For lipase activity, isolate AL7 exhibited the highest level, demonstrated by a distinct, large blue bubble. Amylase activity was notably high in isolates Alon13 and Alon20, both displaying larger, darker bubbles in the amylase column. Protease activity, although present across several isolates, was generally lower in intensity, with Alon4, Alon13, and Alon18 showing moderate activity (Figure 4.3.3). This variability in enzymatic activities suggests that these endophytes may possess unique functional roles, with certain isolates excelling in specific enzyme production, potentially contributing to plant growth or adaptation by degrading or modifying different substrates.

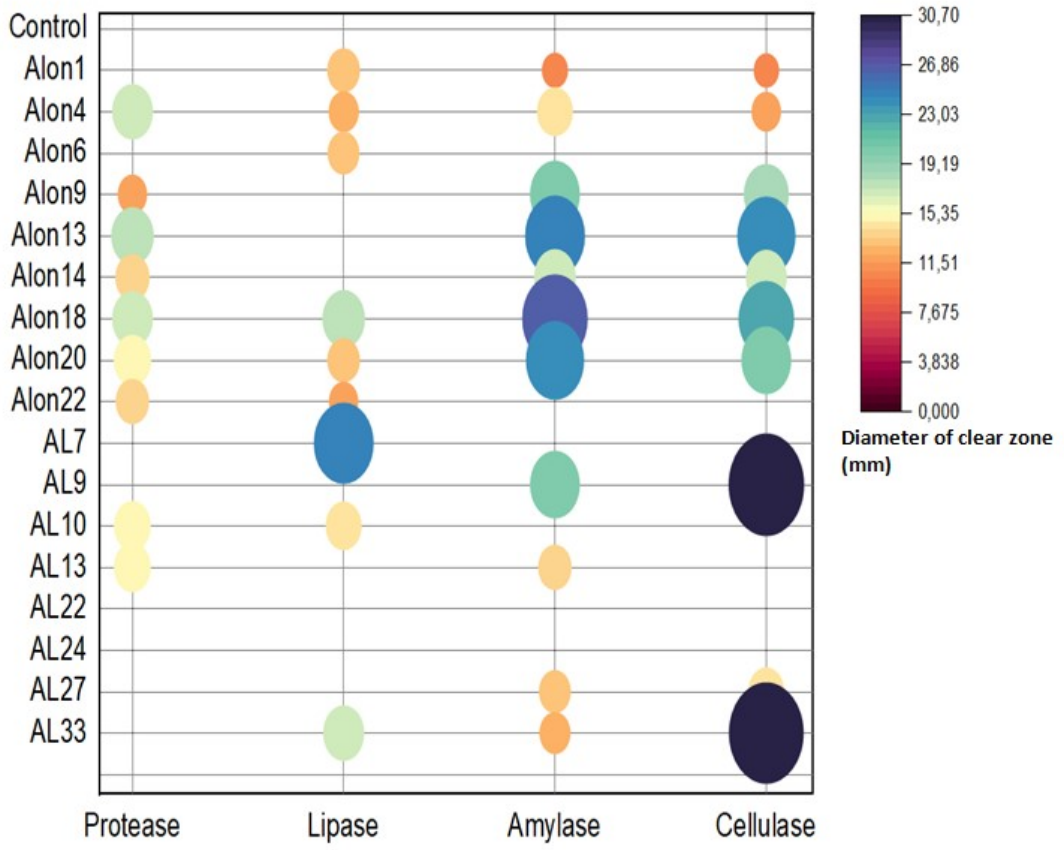


Figure 4.3.3: Extracellular enzymatic activity of bacterial endophytes isolated from *A. lettyae* and *A. longibracteata*

The bar graph below presents the IAA concentrations (µg/mL) produced by different endophytic bacterial isolates under two conditions; with and without supplementation of 0.1% tryptophan. In most cases, the presence of tryptophan significantly enhanced IAA production, indicating a tryptophan-dependent IAA biosynthetic pathway in many isolates. Notably, isolate Alon18 exhibited the highest IAA concentration (~80 µg/mL) with tryptophan, suggesting strong IAA-producing potential. Several other isolates, including Alon4, Alon6, Alon13, Alon20, Alon22, and AL33, also demonstrated significant increases ($p < 0.0001$) in IAA levels when grown with tryptophan. In contrast, a few isolates such as Alon1, Alon14, AL24, and AL27 showed no statistically significant difference (ns) between the two conditions, suggesting possible tryptophan-independent IAA production. The results highlight both the variability in IAA synthesis among isolates and the stimulatory effect of tryptophan on auxin production in many endophytic bacteria.

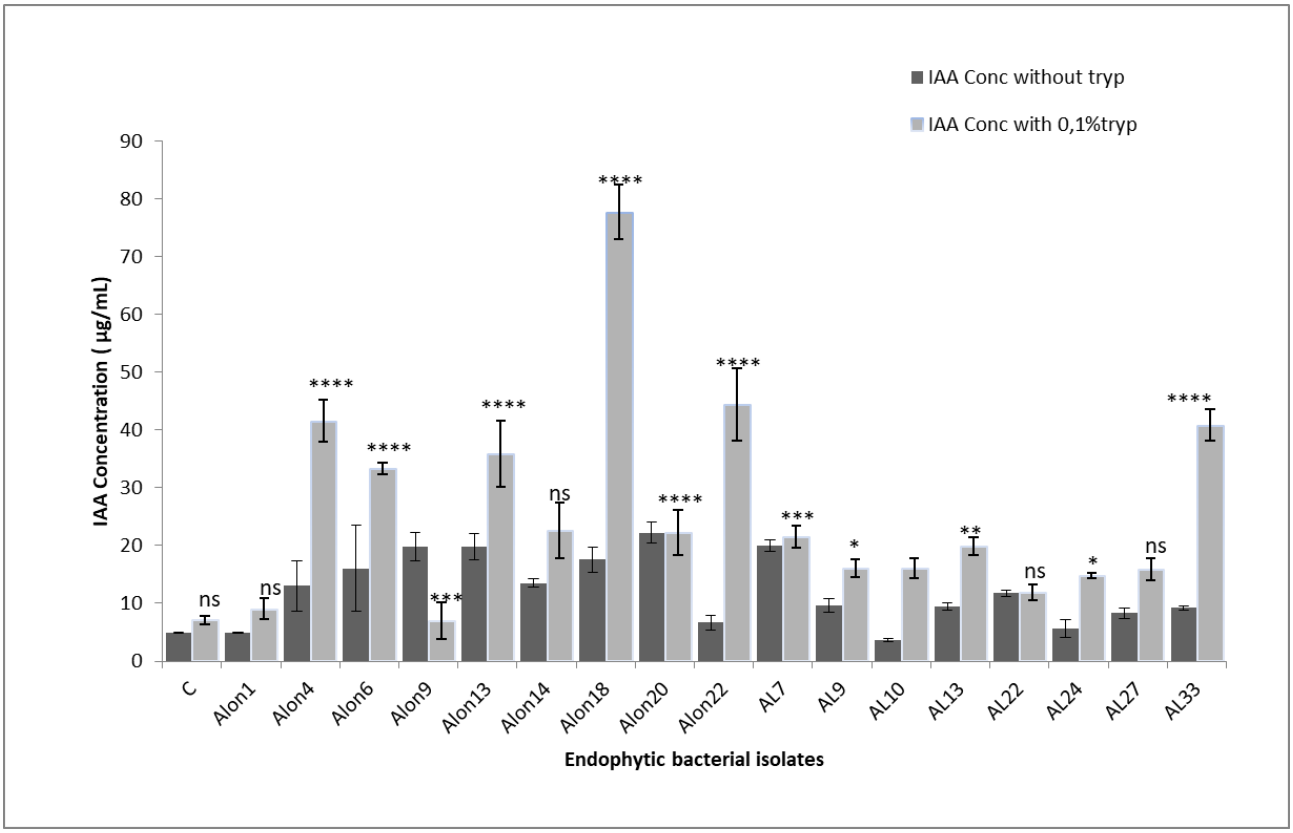


Figure 4.3.4: Quantitative production of Indole Acetic Acid (IAA) by endophytic bacterial strains with and without tryptophan. C, controls without bacterial inoculation. Identity of the bacterial isolates is available in Table 4.3.1. Data were analysed by Bonferroni's multiple comparisons test (α = 0.05); error bars represent means ± SE (n = 3). Significant differences between treatments (with vs. without tryptophan) for each strain are denoted by asterisks (**P < 0.0001, ***P = 0.0001–0.0004, **P = 0.0031, *P ≤ 0.0366) and non-significant comparisons (ns: P > 0.05).**

Out of the 17 endophytic bacterial isolates, 2 were tested for the ability to tolerate NaCl and KCL, the two salts mostly responsible for salinity in soils (Srivastava et al., 2019). The selection of the two isolates was based on the highest plant growth-promotion screening and IAA production from the *A. lettyae* and *A. longibracteata*. At different concentrations, the two isolates (Alon 18 and AL33) appeared to be resistant to inhibition by KCl, particularly at a concentration of 5%, where the bacterial growth was comparable to that of the control for both isolates (Figure 4.3.5). However, Isolate AL33 exhibited reduced growth under NaCl stress, with optical densities (OD_{600}) remaining below 0.7 across all tested concentrations (5%, 7.5%, and 10%). Isolate Alon18 could not tolerate NaCl at 10%, however, at 7.5% the strain formed a diauxic pattern which may be an indication of some form of defence against the salt concentration.

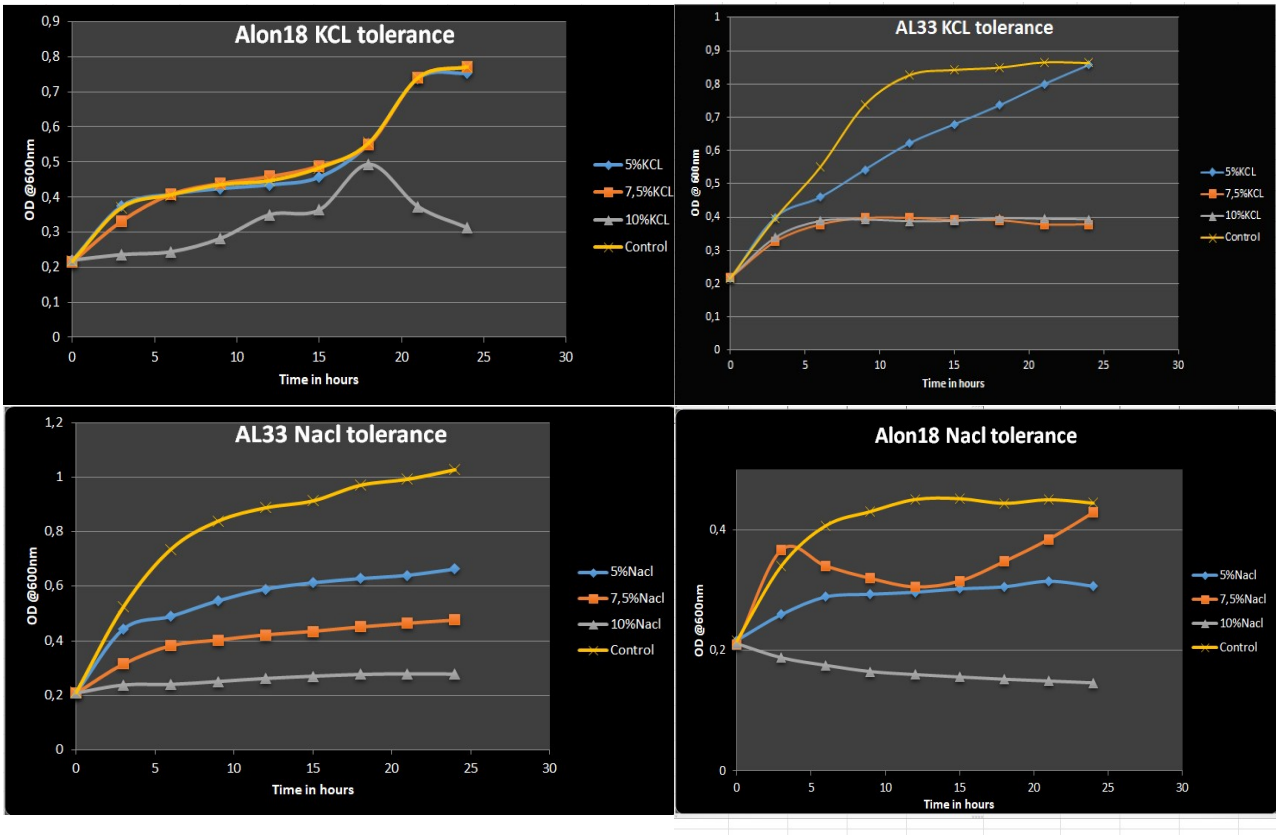


Figure 4.3.5: Effect of selected salt concentrations on the growth of *Bacillus velezensis* (Alon 18) and *Enterobacter sp*. (AL 33)

At different temperatures, the two isolates (Alon18 and AL33) exhibited varying growth responses. Isolate Alon18 showed robust growth at 10ºC, 25ºC, and 30ºC, with growth reaching a peak at 30ºC, although growth at 25ºC stopped after day 5 (Table 4.3.2). Notably, at 37ºC, growth was initially strong but diminished significantly after day 3, suggesting that Alon18 has a limited tolerance for higher temperatures. In contrast, isolate AL33 demonstrated a more consistent growth pattern across temperatures, with peak growth observed at 30ºC, where it maintained a growth throughout the 7 days. At 37ºC, however, the growth of AL33 decreased after day 4, indicating a similar temperature tolerance limit as Alon18. At 4ºC, both isolates showed delayed or minimal growth, with Alon18 starting to show growth on day 3 and AL33 demonstrating only slight growth after day 2. These results suggest that while AL33 is more resilient to temperature variation, both isolates show clear preferences for moderate temperatures, with temperatures above 30°C being unfavourable for their growth.

Table 4.3.2: Temperature tolerance of *Bacillus velezensis* (Alon18) and *Bacillus safensis* AL33)

Isolate	Temperature(°C)	Day1	Day2	Day3	Day4	Day5	Day6	Day7
Alon18	4	-	-	+	+	-	-	-
Alon18	10	-	++	++	++	++	-	-
Alon18	25	+	+	++	+++	+++	-	-
Alon18	30	+++	+++	+++	+++	+++	+++	+++
Alon18	37	+++	+++	+++	-	-	-	-
AL33	4	-	+	-	-	-	-	-
AL33	10	+	+	++	-	-	-	-
AL33	25	++	++	+++	+++	+++	+++	-
AL33	30	+++	+++	+++	+++	+++	+++	-
AL33	37	+++	+++	+++	+++	-	-	-

Parameters: - (No visible growth); - (No visible change); + (Low growth); ++ (Moderate growth); +++ (Very dense growth)

The genome analysis of *B. velezensis* KCN2 (Alon18) and *B. safensis* KCN5 (AL33) revealed differences in genome size, GC content, and assembly statistics (Table 4.3.3). The genome of *B. velezensis* KCN2 was slightly larger (3,835,038 bp) compared to *B. safensis* KCN5 (3,697,479 bp). Both genomes were assembled into 16 contigs, with *B. velezensis* exhibiting a higher N50 value (999,144 bp) than *B. safensis* (623,808 bp), indicating a more contiguous assembly. The GC content of *B. velezensis* (46.5%) was higher than that of *B. safensis* (41.6%).

Both strains harboured genes associated with plant growth promotion (PGP) (Table 4.3.3). The presence of IPyA and ipdc suggests the potential for indole-3-pyruvate (IpyA) pathway-mediated auxin biosynthesis, which may enhance root development. Genes such as PatA and fusE play a role in cellular processes that could contribute to stress tolerance and overall plant-microbe interactions. The pvd gene, involved in siderophore biosynthesis, suggests iron acquisition capabilities that can enhance plant nutrient availability. Additionally, the presence of nifU implies potential involvement in nitrogen metabolism. The phosphate solubilisationsolubilisation genes (phoA, phoD, phoE, and

phoR) indicate the ability of both strains to mobilize inorganic phosphate, potentially improving plant phosphorus uptake.

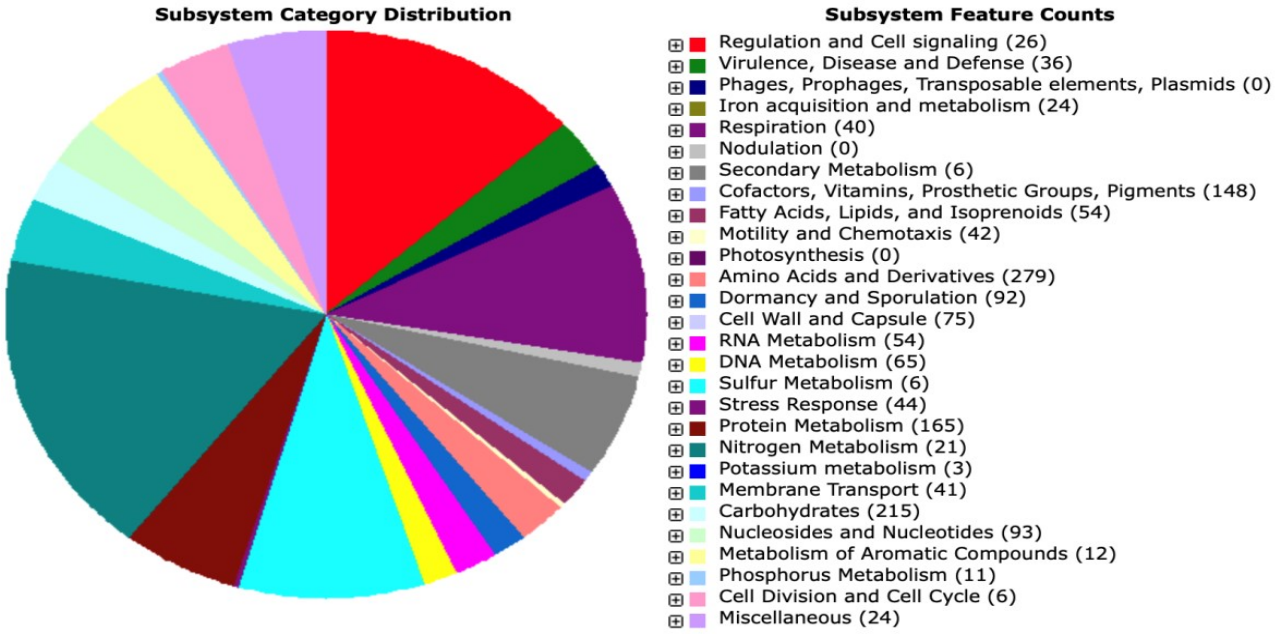
These genomic features suggest that both *B. velezensis* KCN2 and *B. safensis* KCN5 possess genetic traits needed for plant growth promotion, though differences in genome size and GC content may influence their ecological adaptations and interactions with host plants

Table 4.3.3: Whole-genome features of *Bacillus velezensis KCN2* and *Bacillus safensis KCN5*

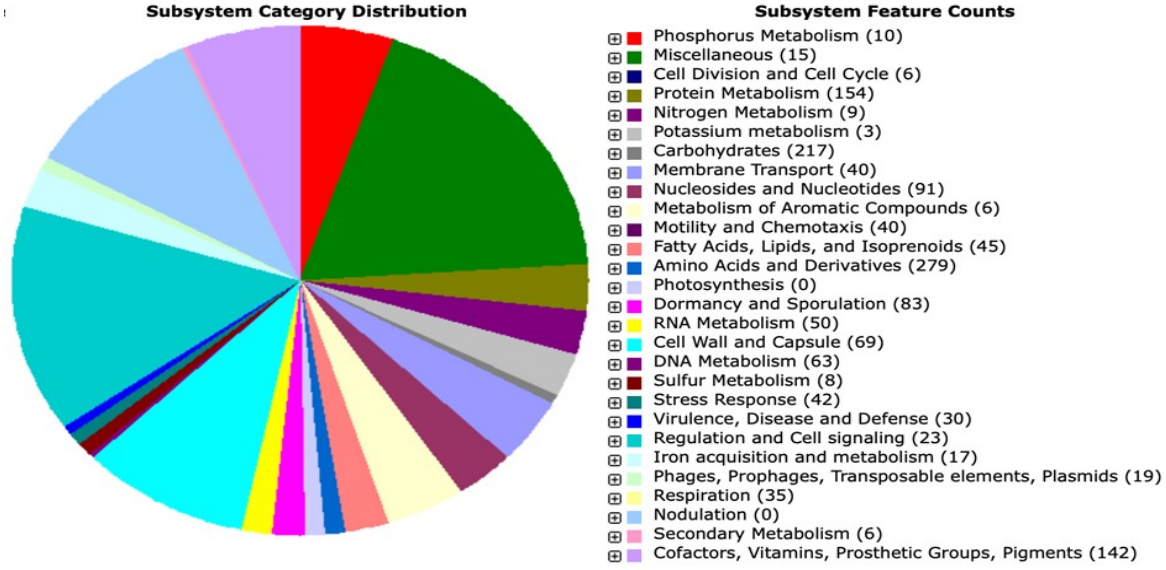
Feature	***Bacillus velezensis* KCN2**	***Bacillus safensis* KCN5**
Genome size(bp)	3,835,038	3,697,479
Contigs	16	16
GC content (%)	46,5	41,6
N50 (bp)	999,144	623,808
L50	2	3
PGP genes identified	IPyA, ipdc, PatA, fusE, pvd, nifU, phoA, phoD, phoE, phoR	IPyA, ipdc, PatA, fusE, pvd, nifU, phoA, phoD, phoE, phoR
Overall functions of genes identified in *B. velezensis* and *B. safensis*	**IPyA** (Indole-3-pyruvate decarboxylase) converts indole-3-pyruvate to indole-3-acetaldehyde, a key step in IAA biosynthesis, promoting root elongation (Imada et al., 2017).	
	ipdc (Indolepyruvate decarboxylase) is involved in the indole-3-pyruvate pathway for auxin production, enhancing plant growth(Shah et al., 2022).	
	PatA (Peptidyl-tRNA hydrolase) aids protein synthesis and bacterial stress adaptation(VanDrisse and Escalante-Semerena, 2019)	
	fusE (FusA elongation factor G) is essential for bacterial protein translation and adaptation to environmental stress(Guo et al., 2012).	
	pvd (Pyoverdine biosynthesis genes) is involved in siderophore production, improving iron uptake and plant health(Visca et al., 1993)	
	nifU (Nitrogen fixation protein) is crucial for nitrogen fixation by assembling iron-sulfur clusters for nitrogenase function(Barahona et al., 2024)	

	phoA, phoD, phoE, phoR (Phosphate metabolism genes) is involved in phosphate solubilisation, uptake, and regulation, enhancing soil phosphorus availability(Marzan and Shimizu, 2011).

Subsystem category distribution analysis (Figure 4.3.6) highlights similarities and distinctions between the two strains. Both genomes show significant representation in essential metabolic pathways, including protein metabolism, carbohydrate metabolism, and amino acid derivatives, indicating their potential roles in nutrient cycling and adaptation to diverse environments (Figure 4.3.6). *Bacillus velezensis* exhibits a higher count in cofactors, vitamins, and prosthetic groups, suggesting a broader enzymatic functionality, while *B. safensis* has a higher representation in amino acid metabolism, indicating possible differences in biosynthetic or degradation pathways (Figure 4.3.6). These variations in subsystem feature counts provide insights into their distinct metabolic potentials, which may influence their ecological roles and biotechnological applications.

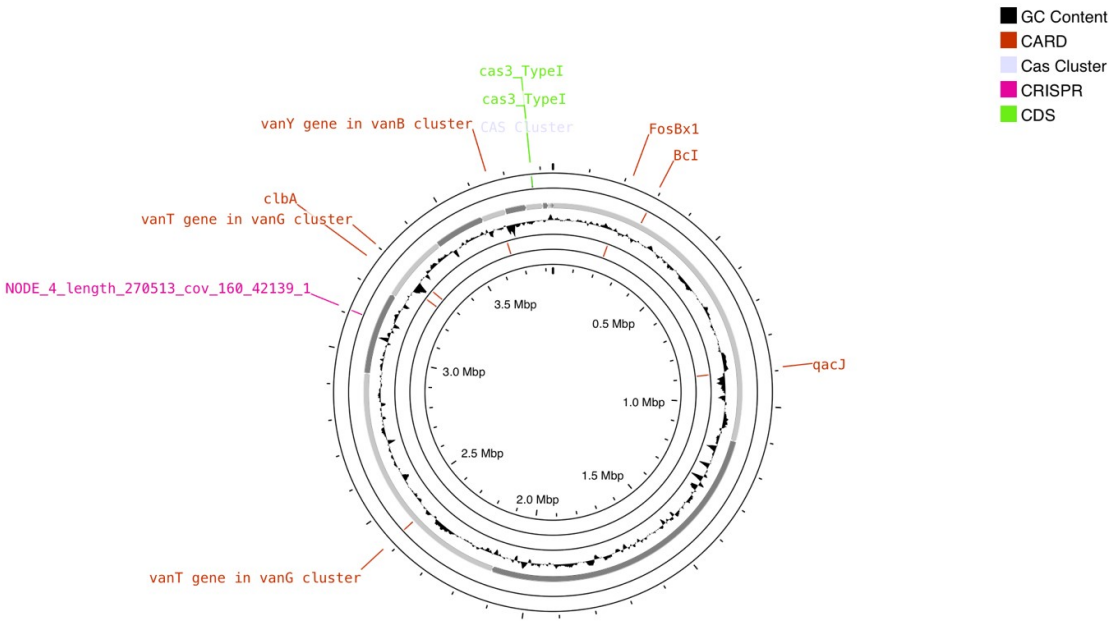


A: ***Bacillus velezensis***

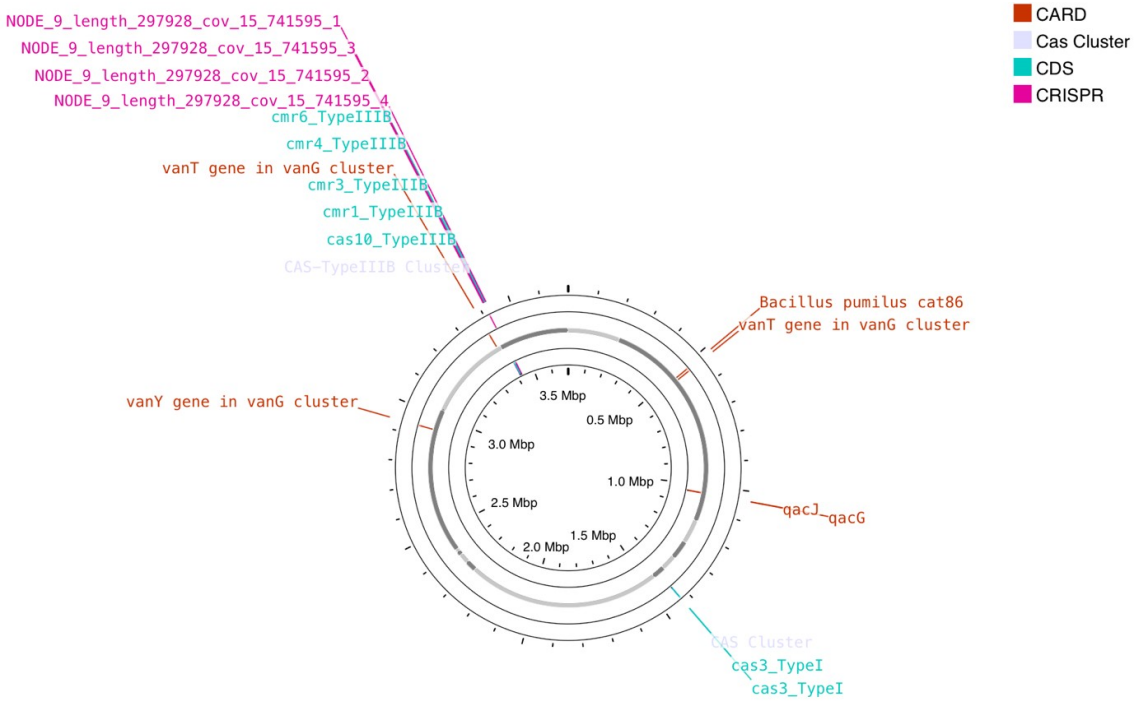


B: ***Bacillus safensis***

Figure 4.3.6: Subsystem category distribution of annotated genes in *Bacillus velezensis* (A) and *Bacillus safensis* (B). The pie charts illustrate the functional classification of genes based on various metabolic and cellular processes, including amino acid metabolism, carbohydrate metabolism, stress response, nitrogen metabolism, and phosphorus metabolism, among others. The differences in subsystem feature counts highlight the metabolic capabilities and potential ecological roles of each strain.



A: ***Bacillus velezensis***



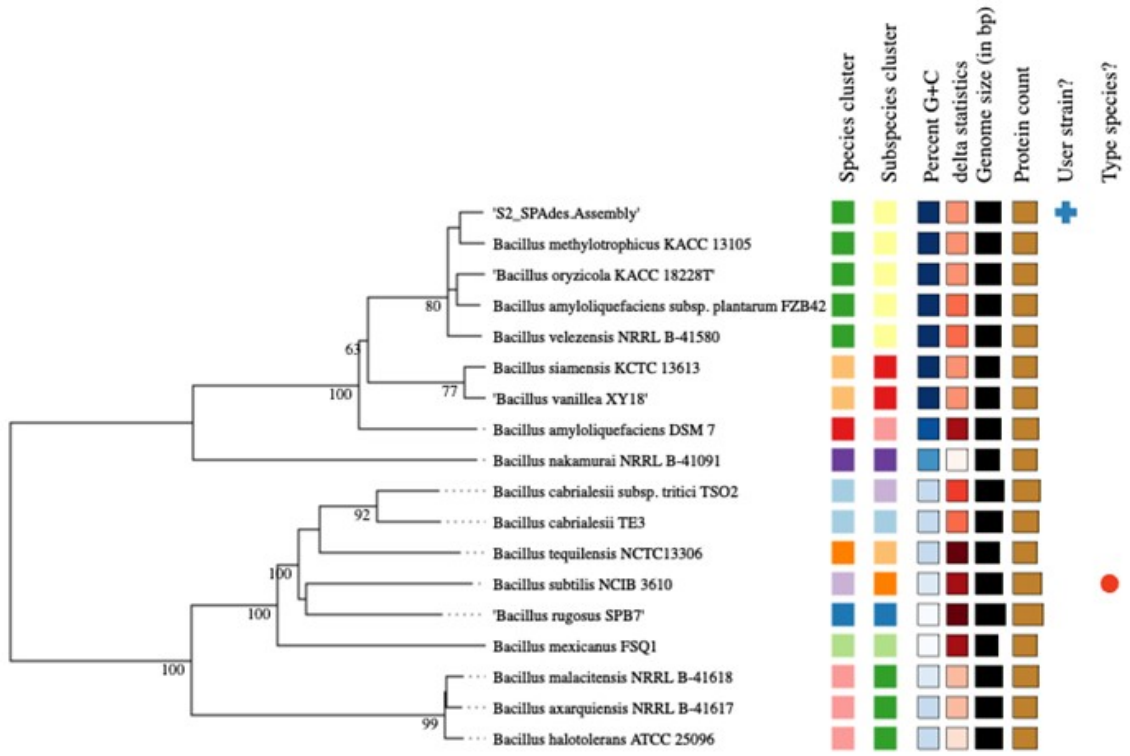
B: ***Bacillus safensis***

Figure 4.3.7: Circular genome maps of *Bacillus velezensis* (A) and *Bacillus safensis* (B) showing the distribution of genomic features. The maps highlight the presence of antimicrobial resistance genes (CARD, orange), CRISPR-associated sequences (blue), Cas clusters (pink), and coding sequences (CDS, green). Specific genes, including vancomycin resistance genes (*vanY*, *vanT*), CRISPR-associated proteins (*cas3_TypeI*, *cas10_TypeIIIB*), and other notable genetic elements (*qacJ*, *FosBx1*), are annotated. These features highlight the potential adaptive mechanisms and genomic traits of the two bacterial strains.

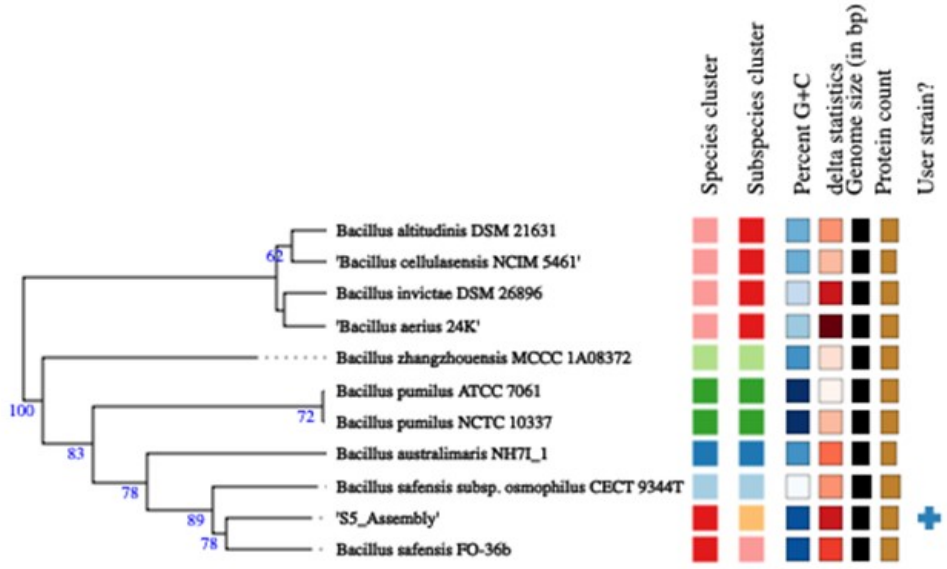
The genomic analysis of *B. velezensis* and *B. safensis* showed key genetic features related to antibiotic resistance, CRISPR-Cas systems, and virulence-associated genes (Figure 4.3.7 A and B). *Bacillus velezensis* possesses multiple resistance-associated genes, including vanY and vanT in the vanG cluster, which are involved in vancomycin resistance. Additionally, resistance determinants such as FosBx1 and qacJ are present, suggesting resistance to fosfomycin and quaternary ammonium compounds, respectively (Figure 4.3.7A). The presence of cas3 Type I and cas3 Type III CRISPR-Cas systems in *B. velezensis* highlights the capability of the strains for adaptive immunity against foreign genetic elements (Figure 4.3.7A). The genome also includes a BcI gene, potentially contributing to bacteriocin production, which may play a role in microbial competition.

Bacillus safensis genome exhibited a diverse set of resistance genes, with multiple copies of vanT in the vanG cluster and additional cmr genes encoding multidrug efflux pumps (Figure 4.3.7B). The CRISPR-Cas system in this strain is more complex, with Type I and Type III variants (cas3 Type I, cas10 Type IIIB), potentially enhancing bacterial defense against phage infections and horizontal gene transfer. The presence of qacJ-qacG suggests additional resistance to antiseptic agents. Interestingly, a genetic link to *Bacillus pumilus* (cat86) is noted, which may indicate horizontal gene transfer events contributing to environmental adaptability. These genomic features suggest that both strains possess robust defense mechanisms, with *B. safensis* displaying a broader spectrum of antimicrobial resistance elements.

The phylogenetic trees below illustrate the relationships between different *Bacillus* species and subspecies, with a focus on *B. velezensis* (Figure 4.3.8A) and *B. safensis* (Figure 4.3.8B). *Bacillus velezensis* is shown in relation to several closely related species, including *B. amyloliquefaciens*, *B. siamensis*, and *B. methylotrophicus*. The clustering patterns suggest high genomic similarity within subspecies groups, particularly among *B. amyloliquefaciens* strains. Notably, bootstrap values of 100 at key nodes indicate strong phylogenetic support for certain branches. Similarly, in Figure 4.3.8B, *B. safensis* forms a well-supported clade, with its close relatives, such as *B. pumilus* and *B. altitudinis*. The metadata columns provide additional insights into genomic differences, reinforcing the distinct taxonomic placement of strains. These phylogenies contribute to understanding the evolutionary relationships within *Bacillus* and have implications for taxonomy, industrial applications, and ecological studies.



A: ***Bacillus velezensis***



B: ***Bacillus safensis***

Figure 4.3.8: Phylogenetic relationships of *Bacillus velezensis* (A) and *Bacillus safensis* (B) strains based on whole-genome comparisons. The phylogenetic trees display bootstrap values at the nodes, indicating the confidence levels of the clustering. Accompanying heatmaps show genome characteristics, including species and subspecies clusters, GC content, genome size, protein count, and delta statistics.

4.4 Discussion

The isolation and identification of endophytic bacteria from *A. lettyae* and *A. longibracteata* revealed the microbial diversity associated with these plants. Both plants hosted endophytes primarily from the genera *Bacillus* and *Enterobacter*, known for their growth-promoting and stress-tolerant traits, which can be beneficial in plant conservation and adaptation efforts (Ahmed et al., 2021, Kaur and Karnwal, 2023). The identification of *Bacillus* and *Enterobacter* species in both *Aloe* species suggests a selective association between these bacterial genera and the host plants. *Bacillus* species are known for their ability to form endospores, allowing them to survive within plant tissues under diverse environmental conditions (Hong et al., 2009). Similarly, *Enterobacter* species are facultative anaerobes capable of colonizing various plant tissues, often contributing to nutrient acquisition through nitrogen fixation and phytohormone production (Jha et al., 2011, Bhat et al., 2015). This overlap in genera but variation in species across the two aloes highlights a shared core microbiome that may serve similar ecological roles, while species-specific strains could indicate adaptation to the particular needs of each aloe (Hao and Xiao, 2015; Trivedi et al., 2020).

Although *A. lettyae* and *A. longibracteata* were collected from distinct regions (Haenersburg and Makapanstad, respectively) in Limpopo, both were found in elevated, chilly grassland areas. Climatic conditions, along with other environmental factors, can influence the types of endophytes that successfully colonize plant tissues. While a detailed community comparison was conducted using NGS in Chapter 3, the culture-based approach in this chapter primarily revealed members of the genera *Bacillus* and *Enterobacter*. This discrepancy is likely due to the inherent limitations of culture-dependent methods, which capture only a subset of the microbial community capable of growing under the selected laboratory conditions, whereas NGS can detect a broader range of both culturable and non-culturable microorganisms. These genera are widely recognized for their roles in enhancing plant stress tolerance and promoting growth under challenging conditions. Their presence in both *Aloe* species suggests a functional significance, potentially aiding the host plants in coping with abiotic stress through mechanisms such as nitrogen fixation, phytohormone production, and resistance to oxidative damage.

The plant growth-promoting properties of the endophytic isolates from *A. lettyae* and *A. longibracteata* as observed in Figure 4.3.2 suggest a strong potential for supporting plant health and resilience, particularly for conservation efforts aimed at re-establishing *A. lettyae* populations in new environments. This is evidenced by the abilities of isolates to solubilise phosphate, produce siderophores, and fix nitrogen, these are key mechanisms that improve nutrient availability and stress tolerance in host plants (Rawat et al., 2021, Timofeeva et al., 2023). Specifically, five isolates

from *A. longibracteata* demonstrated phosphate-solubilizing capabilities, which is crucial for promoting growth in nutrient-poor soils (Bargaz et al., 2021).

Bacillus velezensis, which represents three of the best phosphate-solubilizing isolates (Figure 4.3.2), is widely recognized for its plant growth-promoting capabilities in different crops, including phosphate solubilisation, siderophore production, and nitrogen fixation (Mosela et al., 2022, Vitorino et al., 2024, Wang et al., 2022). Chen et al. (2020) demonstrated that *B. velezensis* contributes to increased biomass and stress resilience in crops such as wheat, showing its versatility in different soil and plant types. This aligns with its potential to support the growth and resilience of *A. lettyae* in new habitats because, similar to wheat, *A. lettyae* might benefit from the stress tolerance mechanisms conferred by *Bacillus velezensis*. Specifically, *B. velezensis* has been shown to enhance plant resilience under various environmental stress conditions, which could be critical for promoting the growth and survival of *A. lettyae* when reintroduced to different or less than ideal environments. The shared ability of *B. velezensis* to adapt to diverse ecological conditions strengthens the argument that this bacterium could play a role in improving the establishment and resilience of *A. lettyae* in newly established habitats.

The enzymatic activities observed in the bacterial endophytes from *A. lettyae and A. longibracteata* highlight their potential contributions to plant growth and adaptability. Enzyme production, particularly cellulase, lipase, amylase, and protease activities, is often linked to the capacity of bacteria to aid in nutrient cycling, promote plant health, and increase stress resilience (Neemisha and Sharma, 2022, Abdul Rahman et al., 2021). The variation in enzyme activity across isolates from *A. lettyae* and *A. longibracteata* suggests diverse functional roles that these endophytes may play in the ecological interactions with their host plants, potentially influencing their growth and survival.

The high cellulase activity observed in *B. safensis* and *E. asburiae* (isolates AL33 and AL9), both isolated from *A. lettyae*, is in agreement with studies done on other plant species which found that cellulase-producing *Enterobacter* and *Bacillus* species can break down plant cell walls, enhancing nutrient release and availability to the host plant (Juturu and Wu, 2014, Panicker and Sayyed, 2022, Jadhav et al., 2017). This suggests that the cellulase activity of *B. safensis* and *E. asburiae* in *A. lettyae* may similarly support the plant's nutrient acquisition, potentially aiding in its growth and resilience in challenging environments.

Bacillus xiamenensis (isolate AL7) isolated from *A. lettyae* showed the highest lipase activity highlighting the capacity of this endophyte for lipid breakdown, contributing to nutrient cycling in soil environments (Yousuf et al., 2022). *Bacillus xiamenensis* is known to produce lipases that

degrade complex lipids, transforming them into simpler nutrient forms that plants can readily absorb (Ehis-Eriakha and Adetunji, 2022, Mohapatra et al., 2019). Such functionality is particularly valuable in nutrient-poor or challenging settings, where efficient resource mobilization can significantly enhance plant growth and resilience, supporting the adaptability of *A. lettyae* and other host plants in novel environments.

The highest amylase producing endophyte, *Enterobacter sp*. and *Bacillus velezensis* isolated from *A. longibracteata* (isolates Alon13 and Alon18), further supports the potential of these endophytes in plant growth promotion. Amylase producing bacteria, especial those from *Enterobacter* and *Bacillus* species have often been linked to improved plant growth, particularly in the context of root colonization and the breakdown of complex sugars that may otherwise be inaccessible to the plant(Fathima et al., 2023, Luang-In et al., 2019, Fasiku et al., 2020). These bacteria could help enhance the resilience of *A. lettyae* in new habitats by improving its nutrient uptake and overall growth rate.

The relatively moderate protease activity observed across isolates, especially *Enterobacter hormaechei, Enterobacter sp*. and *Bacillus velezensis* (Alon4, Alon13, and Alon18), suggests that these bacteria may play a role in protein degradation and nitrogen mineralization in the soil, which could further support the nitrogen needs of *A. lettyae* when introduced to certain environmental conditions (Schaller, 2004, Olajuyigbe and Ajele, 2005, Tian et al., 2017, Fajingbesi et al., 2018). While protease activities detected was low, they still show the potential of these bacteria to contribute to the nitrogen cycle, an important factor for plant growth, particularly under nutrient-limited conditions.

Overall, the enzymatic activity observed in these bacterial endophytes isolated from both *A. lettyae* and *A. longibracteata* suggests that they may offer a range of functional benefits to *A. lettyae,* potentially contributing to its growth, stress resilience, and adaptation to new environments. The ability of these bacteria to degrade various substrates, enhance nutrient availability, and promote plant health supports their potential use in conservation efforts, especially for the re-establishment of *A. lettyae* populations in new or altered habitats.

The ability of endophytic bacterial isolates from the two *Aloe* species to produce IAA, particularly with increased production upon tryptophan supplementation, further underscores their potential role in promoting plant growth. Indole-3-acetic acid, a key plant hormone, influences root elongation, cell division, and differentiation, directly impacting root architecture and nutrient uptake (Etesami and Glick, 2024, Khadr et al., 2020). In this study*, B. australimaris, B. velezensis, and B. pumilus* (isolates Alon6, Alon18, and Alon22), isolated from *A. longibracteata,* demonstrated heightened

IAA production, a trait closely linked to enhanced root growth and adaptation under various environmental stresses (Zhang et al., 2022). Previous studies support these findings, reporting that *B. australimaris, B. velezensis,* and *B. pumilus* are notable IAA producers (Barros-Rodríguez et al., 2024, SUKMAWATI et al., 2022, Salwan et al., 2021). The ability of these endophytes to synthesize IAA is recognized as beneficial for plant development, as it promotes root system expansion and nutrient uptake, ultimately aiding plants in adapting to and thriving in diverse or stressful environments.

Moreover, the observed IAA production in endophytes from both *Aloe* species without tryptophan and enhanced levels with tryptophan (Figure 4.3.4), aligns with findings that many bacterial endophytes produce IAA in response to available tryptophan, a precursor commonly released from plant roots (Naveed et al., 2015, Etesami and Glick, 2024). This suggests an adaptable mutualistic interaction that could be harnessed for reintroducing certain endangered plant species such as *A. lettyae* to challenging environments.

Following plant growth promotion screenings of the 17 endophytic isolates, Alon18 (*B. velezensis*) and AL33 (*B. safensis*) were selected for their strong plant growth-promoting properties and high IAA production to evaluate their tolerance to NaCl and KCl, which are key contributors to soil salinity (Chen et al., 2008, Srivastava et al., 2019). It was shown that both isolates display differential tolerance to salt stress, with Alon18 exhibiting a robust response to KCl and NaCl, and AL33 showing limited tolerance to NaCl at higher concentrations.

Bacillus velezensis, as observed in Alon18, is well-known for its resilience under saline conditions(Mahdi et al., 2022). Previous studies have shown that *B. velezensis* can produce a range of stress-related metabolites, including osmoprotectants, which help the bacteria and their host plants cope with salt stress (Valencia-Marin et al., 2023, Krishnamoorthy et al., 2022). Additionally, *B. velezensis* has been linked to the promotion of plant growth under saline conditions, making it a beneficial endophyte for plants in salt-affected soils (Bai et al., 2023, Masmoudi et al., 2021). On the other hand, *B. safensis* (AL33), while exhibiting tolerance to salt environments, showed a decreased tolerance to NaCl in this study, aligning with some reports where *B. safensis* demonstrated limited resistance to higher salt concentrations, potentially due to their less robust osmotic stress tolerance mechanisms compared to species like *B. velezensis*(Kothari et al., 2013, Djelid et al., 2023). However, it is important to note that *B. safensis* have been shown to enhance plant growth under moderate salinity stress, suggesting that while AL33 might not thrive in extremely high saline conditions, it could still offer benefits in environments with lower salinity levels.

The growth responses of the two endophytic bacterial isolates, Alon18 (*B. velezensis*) and AL33 (*B. safensis*) to varying temperatures highlight their distinct thermal tolerances and preferences, which are crucial for their potential application in plant growth promotion under different environmental conditions. Alon18 exhibited robust growth at moderate temperatures (10ºC, 25ºC, and 30ºC), with peak growth at 30ºC, aligning with findings from previous studies that *B. velezensis* thrives at temperatures ranging from 25ºC to 30ºC, suggesting its preference for temperate climates (Chen et al., 2018). However, its diminished growth at 37ºC indicates that *B. velezensis* may struggle in high-temperature environments, corroborating research by Wu et al. (2020) which showed a similar decline in growth at temperatures exceeding 35ºC. Conversely, AL33, identified as *B. safensis*, demonstrated consistent growth across the temperature range, with peak growth at 30ºC, resembling the temperature tolerance observed for other *Bacillus* species, which generally show resilience to moderate temperature fluctuations (Aqel et al., 2024). Both isolates showed delayed or minimal growth at 4ºC, a finding consistent with the typical growth patterns of most bacterial species, which tend to slow down or cease activity in cold environments (He et al., 2019). These results suggest that while both isolates share a preference for moderate temperatures, AL33 may have a slight advantage in withstanding temperature fluctuations, making it a potentially more versatile endophyte for varying climatic conditions, whereas Alon18 appears better suited to warmer environments.

The comparative genomic analysis of *B. velezensis* KCN2 and *B. safensis* KCN5 further revealed distinct differences in genome size, GC content, and assembly metrics, which align with existing literature on the genomic diversity of the *Bacillus* genus. The larger genome size of *B. velezensis* KCN2 (3.83 Mb) compared to *B. safensis* KCN5 (3.69 Mb) is consistent with previous studies reporting that *B. velezensis* typically exhibits a more expansive genome architecture, enabling it to encode a greater diversity of secondary metabolites and antimicrobial compounds (Nifakos et al., 2021, Wilson et al., 2023). This genomic feature is particularly relevant given the biocontrol potential of *B. velezensis*, which has been widely recognized for its role in plant growth promotion and disease suppression (Myo et al., 2019, Torres et al., 2020) . On the other hand, the relatively smaller genome of *B. safensis* aligns with findings by Satomi et al. (2006), who identified *B. safensis* as an environmental isolate with a streamlined genome, possibly reflecting adaptation to specific ecological niches, such as extreme environments or endophytic lifestyles.

The observed differences in GC content between *B. velezensis* KCN2 (46.5%) and *B. safensis* KCN5 (41.6%) may have implications for their genomic stability, thermal tolerance, and environmental adaptability. Higher GC content has been linked to greater thermal stability and resistance to mutational pressures (Wu et al., 2012, Musto, 2023). This is further explained by the

broader temperature tolerance of *B. velezensis* KCN2 compared to *B. safensis* KCN5 as observed in this study (Table 4.3.2). The growth data indicates that *B. velezensis* (Alon18) exhibits robust growth across a wider temperature range, maintaining high growth rates (≥++) from 10°C to 37°C, with optimal growth at 30°C. In contrast, *B. safensis (*AL33) demonstrates strong growth at moderate temperatures (25–30°C) but shows reduced adaptability at lower (4°C) and higher (37°C) extremes, with a clear decline after day five at 37°C. These findings align with previous studies suggesting that *B. velezensis* is well-adapted to diverse ecological niches, particularly in fluctuating agricultural and soil environments, where temperature resilience is advantageous (Liu et al., 2023, Marco et al., 2022).

Both strains harbour plant growth-promoting (PGP) genes, reinforcing their potential role as beneficial endophytes. The presence of IPyA and ipdc in both genomes suggests their ability to produce IAA via the indole-3-pyruvate (IPyA) pathway, which is widely recognized for its role in promoting root elongation and plant development(Figueredo et al., 2023). Furthermore, genes such as PatA and fusE, associated with cellular stress response and adaptation, may contribute to increased tolerance to environmental fluctuations, enhancing plant-microbe interactions (Etesami and Glick, 2024).

The presence of the pvd gene in both strains suggests the ability to produce siderophores, which facilitate iron acquisition, a crucial trait for plant health, particularly in iron-deficient soils (Ansari et al., 2017). Similarly, the detection of nifU, involved in nitrogen metabolism, implies a potential role in nitrogen assimilation or fixation, which could contribute to soil fertility and plant nutrient acquisition (Bloch et al., 2020). Additionally, phosphate solubilisation genes (phoA, phoD, phoE, and phoR) indicate that both *B. velezensis* and *B. safensis* may enhance phosphorus bioavailability, further supporting their roles in plant growth promotion (Xue et al., 2023).

The subsystem category distribution analysis revealed both shared and distinct metabolic capabilities between *B. velezensis* KCN2 and *B. safensis* KCN5, shedding light on their functional potential and ecological adaptability. The substantial representation of protein metabolism, carbohydrate metabolism, and amino acid derivatives in both genomes underscores their roles in nutrient cycling and environmental resilience, consistent with findings on metabolic versatility of *Bacillus* species (Aqel et al., 2024). The higher count of cofactors, vitamins, and prosthetic groups in *B. velezensis* suggests a more extensive enzymatic repertoire, which may enhance its ability to synthesize essential biomolecules and support diverse biochemical functions (Richter, 2013). In contrast, the greater representation of amino acid metabolism in *B. safensis* aligns with previous studies highlighting its efficient nutrient assimilation and potential for bioremediation and stress

adaptation (Laamarti et al., 2022). These metabolic distinctions may influence their ecological roles, with *B. velezensis* potentially excelling in plant-microbe interactions and bio control, while *B. safensis* may be more adapted to nutrient-limited or extreme environments, making both strains valuable for various biotechnological applications.

The genomic analysis of *B. velezensis* and *B. safensis* revealed critical genetic elements related to antibiotic resistance, CRISPR-Cas immunity, and virulence. The identification of vancomycin resistance genes (vanY, vanT in the vanG cluster) in both strains is consistent with reports of *Bacillus* species harbouring intrinsic resistance mechanisms against glycopeptide antibiotics (Sumi et al., 2015). Additionally, the presence of fosfomycin resistance genes (FosBx1) and quaternary ammonium compound resistance genes (qacJ) in *B. velezensis* highlights its potential resilience in antimicrobial-stressed environments, as documented in multidrug-resistant *Bacillus* strains isolated from soil and clinical settings (Jin et al., 2021). The CRISPR-Cas systems, particularly cas3 Type I and Type III variants in both strains, suggest a defense mechanism against foreign genetic elements, reinforcing findings that CRISPR-Cas plays a crucial role in bacterial immunity and horizontal gene transfer regulation in *Bacillus* species (Makarova et al., 2015). The presence of the BcI gene, associated with bacteriocin production, supports the notion that *Bacillus* species employ antimicrobial peptides to outcompete other microbes in their ecological niches (Rekadwad, 2024).

The genomic features of *B. safensis* further indicates a broader spectrum of antimicrobial resistance, particularly with multiple copies of vanT and cmr efflux pump genes, which are known to facilitate resistance against various antibiotics and biocides (Jacoby, 2018, Depardieu et al., 2007, Périchon et al., 2009). The presence of qacJ-qacG in *B. safensis* suggests additional resistance to antiseptic agents, a characteristic commonly observed in *Bacillus pumilus* and other closely related strains from industrial and clinical environments (Pusenkova and Lastochkina, 2024). The CRISPR-Cas complexity in *B. safensis*, with Type I and Type IIIB systems, suggests a more evolved immune defense, consistent with research showing that CRISPR diversity in *Bacillus* species contributes to environmental adaptability and phage resistance(Westra et al., 2014). Furthermore, the genetic link to *B. pumilus* (cat86) may indicate horizontal gene transfer events, highlighting the dynamic nature of microbial genomes in acquiring beneficial traits for survival in diverse conditions (Koonin et al., 2017). These findings emphasize the ecological resilience of *B. safensis*, potentially making it a candidate for biotechnological applications requiring robust stress tolerance.

The phylogenetic analysis showed the evolutionary relationships within the *Bacillus* genus, reaffirming taxonomic classifications observed in previous genomic studies (Fan et al., 2017). The close clustering of *B. velezensis* with *B. amyloliquefaciens*, *B. siamensis*, and *B. methylotrophicus*

supports the hypothesis that these species share a common evolutionary origin and metabolic potential, particularly in plant growth promotion and biocontrol applications. The strong bootstrap values indicate high confidence in the phylogenetic placement, reinforcing the stability of these taxonomic groupings. Similarly, *B. safensis* clusters tightly with *B. pumilus* and *B. altitudinis*, supporting previous findings that these species share environmental niches, particularly in extreme or high-altitude ecosystems(Mbozo et al., 2017). The inclusion of metadata such as genome size, GC content, and protein counts further elucidates genomic variations that may drive niche specialization and functional differences. These phylogenetic insights contribute to the broader understanding of *Bacillus* taxonomy, with potential implications for strain selection in industrial, agricultural, and environmental applications.

4.5 Conclusion

This chapter investigated the potential plant growth-promoting attributes of endophytic bacteria from *A. longibracteata* and *A. lettyae*, a critically endangered species. The findings revealed that both *Aloe* species harbour beneficial endophytes, particularly from the genera *Bacillus* and *Enterobacter*, which exhibit plant growth-promoting traits such as phosphate solubilisation, siderophore production, and nitrogen fixation. Notably, *B. velezensis* and *B. safensis* demonstrated strong growth-enhancing properties, including enzyme activity and salt tolerance, making them promising candidates for improving the adaptability of *A. lettyae* to degraded or novel environments. Additionally, comparative genomic analyses confirmed the presence of key genes associated with stress tolerance and plant growth promotion, further validating the ecological relevance of these endophytes in conservation efforts.

A primary limitation of this study is that all assessments were conducted *in vitro*, without greenhouse trials, due to the slow growth of *Aloe* species. While the *in vitro* results strongly support the potential of these endophytes in plant conservation, further validation in real-world conditions is necessary. Nevertheless, this study contributes to the broader understanding of plant-endophyte interactions, highlighting the potential for cross-species endophyte application in conservation. By demonstrating that beneficial bacteria from a non-endangered species can be leveraged to enhance the resilience of an endangered relative, this research provides a foundation for developing microbiome-assisted conservation strategies, potentially aiding in the restoration and re-establishment of *A. lettyae* populations.

4.7 References

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Chapter 5

Plant-part substitution and seasonal metabolite dynamics for *Ocotea bullata* conservation: A metabolomic approach

This chapter is intended for submission to the Frontiers in plant sciences journal.

[a] Cynthia K. Marokane-Radebe, [a]Adekunle R. Raimi, [a,b] Stephen O. Amoo, [a] Rasheed A. Adeleke*

[a] Unit of Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa

[b] Agricultural Research Council - Vegetables, Industrial and Medicinal Plants, Roodeplaat, Pretoria, 0001. South Africa.

Abstract

Ocotea bullata (Burch.) Baill., an endangered medicinal tree, faces significant threats due to overharvesting of its bark, necessitating alternative, sustainable harvesting strategies. This chapter investigated the potential of *O. bullata* leaves as a substitute by analyzing seasonal variations in metabolite composition. Leaf and bark samples were subjected to 1H NMR spectroscopy and multivariate data analysis to profile their metabolite composition and assess seasonal metabolite fluctuations. The findings highlight key secondary metabolites such as chlorogenic acid and other phenolic compounds, while primary metabolites like amino acids and sugars were also identified. Summer leaves exhibited higher composition of metabolites compared to winter leaves, suggesting optimal harvest periods for medicinal applications. While 1H NMR effectively provided an overview of metabolite composition, certain secondary metabolites commonly reported in *O. bullata* could not be definitively confirmed due to spectral overlap and the limitations of 1H NMR in complex crude extracts. Future studies integrating LC-MS will provide higher resolution and more comprehensive metabolite profiling. This study underscores the importance of metabolomic approaches in sustainable harvesting and medicinal plant conservation, offering insights into the seasonal metabolite dynamics of *O. bullata* for optimized utilization and conservation strategies.

5.1 Introduction

Ocotea bullata (Burch.) *Baill,* commonly known as the black stinkwood, is an ecologically and medicinally significant tree species endemic to the forests of southern Africa (Grace et al., 2002). Renowned for its medicinal properties and ecological importance, *O. bullata* has attracted considerable scientific interest. Traditionally, the bark of *O. bullata* has been extensively utilized by indigenous communities for its therapeutic benefits, including its anti-inflammatory and antimicrobial properties, and as a remedy for headache and urinary disorders (Zschocke et al., 2000, Ogundajo et al., 2018). In addition to its medicinal value, *O. bullata* contributes to forest ecology by potentially providing food and habitat to other organisms, including insects, birds, and small mammals that depend on it for survival(Van Wyk and Prinsloo, 2017). *Ocotea bullata* is currently classified as an endangered species due to its overharvesting and habitat loss (Williams et al., 2013). Given its ecological and medicinal significance, there is a growing need to explore sustainable utilization strategies for *O. bullata*. One promising approach is to investigate the metabolite composition of its different parts, such as the leaves, which are less commonly harvested but could offer comparable valuable metabolites. By shifting the focus from bark to leaves, we can potentially reduce the pressure on this threatened species and promote its conservation. Previous study by Zschocke et al. (2000) concluded that leaves may contain significant metabolites, as demonstrated through *in vitro* cyclooxygenase -1 inhibitory assay, suggesting they may serve as a viable alternative to bark and providing a foundation for the current investigation

Metabolomics is the comprehensive analysis of metabolites in biological samples and offers a powerful approach useful for investigating the chemical ecology within *O. bullata*. Moreover, seasonal changes significantly influence plant metabolism, impacting the concentration and composition of metabolites (Soni et al., 2015, Verma and Shukla, 2015). These fluctuations are driven by environmental factors such as temperature, light, and water availability, which in turn affect physiological processes, including photosynthesis, respiration, and secondary metabolite production (Verma and Shukla, 2015). However, the effects of seasonal variations on the metabolite composition of *O. bullata* remain underexplored. Understanding these variations with seasonal changes is crucial for optimizing the use of this species medicinally and in various applications. By profiling the metabolites present in the bark and leaves, and the influence of seasons on leaf metabolite contents, we can gain insights into the plant's adaptive strategies and identify potential beneficial metabolites.

The overharvesting of the bark poses a significant threat to the sustainability of the species (Williams and Raimondo, 2008, Williams et al., 2013). Therefore, the primary goal of this study is

to determine if the leaves of *O. bullata* contain metabolites similar to those found in the bark, which is the preferred part for medicinal and other uses. If the leaves are found to contain similar or additional metabolites with known therapeutic potential, their use may be promoted over that of the bark. Additionally, we aim to compare the metabolite profiles of the leaves harvested in different seasons, particularly winter and summer, to determine which season yields metabolites with significant potential for human health management. Aligning harvesting practices with seasonal variation, we can provide more information about the best period when the leaves have the highest concentration of targeted beneficial metabolites. This approach may help conserve *O. bullata* by reducing the need to harvest large quantities of, or resort to using bark, which is more destructive to the plant.

5.2 Materials and methods

5.2.1 Sample collection

Leaf samples from *O. bullata* were collected from cultivated individual plants growing in the University of KwaZulu-Natal Botanical Gardens in Pietermaritzburg. Sampling was conducted during the winter season (leaves and bark) and again in the summer season (leaves only) to assess potential seasonal variation. As the plants were part of a managed garden collection, only small, non-destructive portions of leaf and bark were sampled to minimize any impact on plant health. All sampling procedures followed ethical guidelines approved by the institution (Ethics reference: NWU-01480-24-A9). The samples were placed in sterile zipper bags, stored in a cooler box maintained at 4 °C, and immediately transported to the laboratory. Samples were then dried at room temperature for 7 days before being ground into a fine powder.

Figure 5.2.1: An *Ocotea bullata* tree located in the research garden of the University of KwaZulu-Natal

5.2.2 Sample preparation and NMR analysis

Following the method of Kim et al. (2010) with modifications, the air-dried powdered leaf samples (50 mg) of the *O. bullata* leaves and bark (Figure 5.2.1) were extracted with 750 µL methanol-d4 (CH_3OH-d4) and 750 µL potassium dihydrogen phosphate (KH_2PO_4) buffer in deuterium water (D_2O) (pH 6.0) containing 0.01% (w/w) trimethylsilanepropionic acid (TSP). The mixture was vortexed for 1 min, ultrasonicated for 20 min, and then centrifuged for 20 min (at 10,000 rpm). Samples were then filtered through a 0.22 µm syringe filter and 500 µL of the filtrates were transferred to 5 mm Norell standard NMR tubes. All the proton NMR spectra were acquired using a 600 MHz NMR spectrometer (Varian Inc, CA, USA). Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisitions. All spectra were Fourier-transformed, and phase and baseline were corrected manually.

5.2.3 Multivariate data analysis

Data analysis and processing were performed using MestReNova software (10.0.1, Mestrelab Research, Spain), and the correction of phasing and baseline, normalisation and peak alignment was done manually on the 1H NMR spectrum. All processed data were divided into 0.04 ppm bins, representing 0 – 10 ppm and converted to Excel comma-separated values (CSV) file format for

pattern recognition multivariate data analysis. Transformed data were statistically analysed, and all the imported data were Pareto scaled in the soft independent modelling of class analogy (SIMCA) software (SIMCA, Version 14.0, Umetrics, Umeå, Sweden). Similar and non-similar samples were statistically discriminated using the Principal Component Analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA). Annotations of compounds were performed using the Chenomx software (NMR suite, version 8.3) and published NMR data.

5.3 Results

The 1H NMR spectra of *O. bullata* bark and leaves aqueous methanolic extracts displayed a complex pattern of resonances, indicating the presence of many metabolites (Figure 5.3.1). The presence of resonances across the entire spectral width (0-10 ppm) suggests the detection of varied chemical features, ranging from aliphatic to aromatic groups in the extracts. Notably, intense signals in the sugar region were predominantly observed in leaf samples, (Figure 5.3.1). A visual comparison of the spectra also indicates some differences between the metabolic profiles of *O. bullata* leaves and bark, particularly in the patterns of metabolites in the aliphatic (0.5-3 ppm) and aromatic regions (6-9 ppm). Based on the NMR profiles, the relative differences between the leaves and bark samples of *O. bullata* were visualised by plotting the scores of a principal component analysis (PCA).

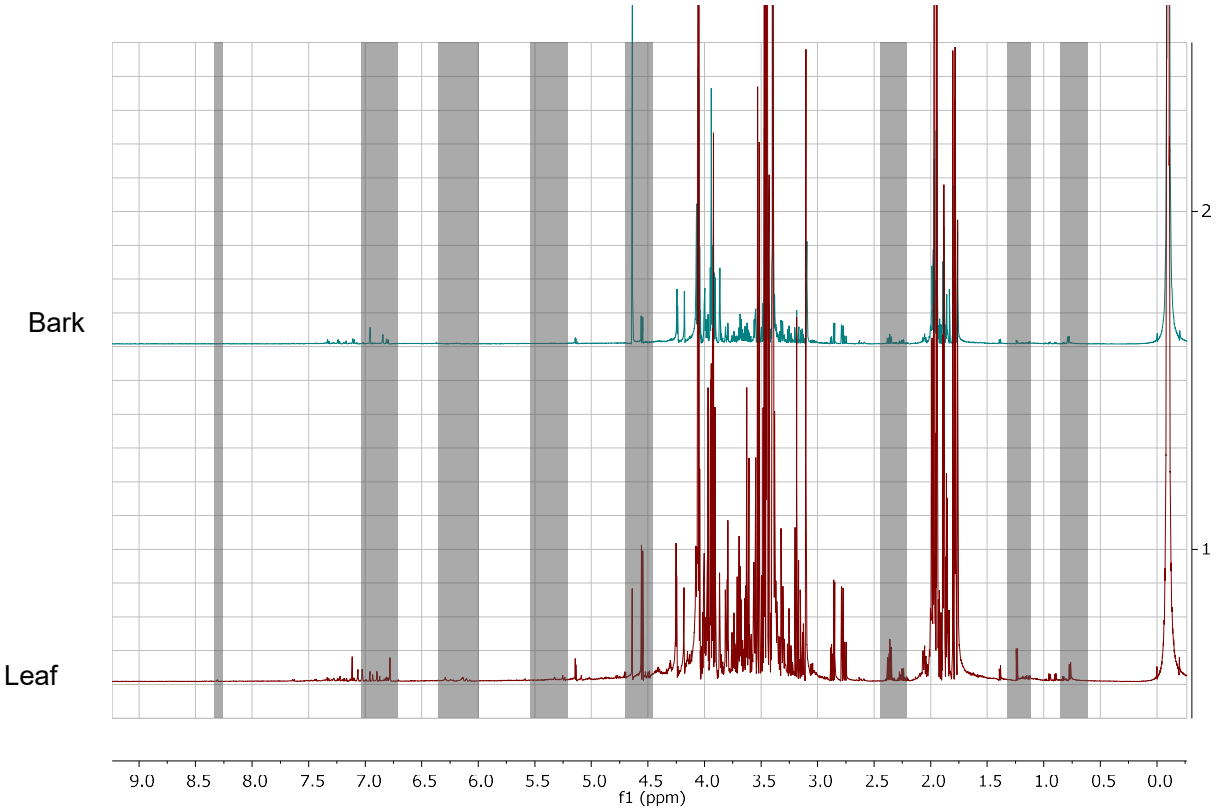

Figure 5.3.1: 1H NMR spectrum of *Ocotea bullata* leaves and bark, with shaded areas highlighting differences in metabolite occurrences between the spectra.

The unsupervised pattern recognition analysis using PCA was applied to give an overview of the dimensions of the samples (Figure 5.3.2a). OPLS-DA analysis revealed clear discrimination between the groups, indicating distinct metabolic profiles (Figure 5.3.2b). The model demonstrated strong reliability, with 95% variance explained, and high goodness-of-fit (R2X = 0.98) and predictive ability (Q2 = 0.75), supporting the robustness of the observed group separations. Observations of the PCA scores plot (Figure 5.3.2a) showed an overlap between the bark and the leaf samples which means that the principal components have captured similarities between the two sample groups, which could be due to shared features, similar metabolite profiles, or other common variables present in the data. The OPLS-DA score plot (Figure 5.3.2b) showed a clear separation among the samples, with the leaf samples clustering distinctly away from the bark, indicating differences in the metabolite profiles between these groups. The unsupervised PCA showed that the bark and leaves shared some common metabolites while the supervised OPLS-DA confirmed that despite the commonalities there are also distinct differences between the groups. The HCA dendogram constructed from the PCA and OPLS-DA (Figures 5.3.2c and d) grouped samples with similar features into different clusters.

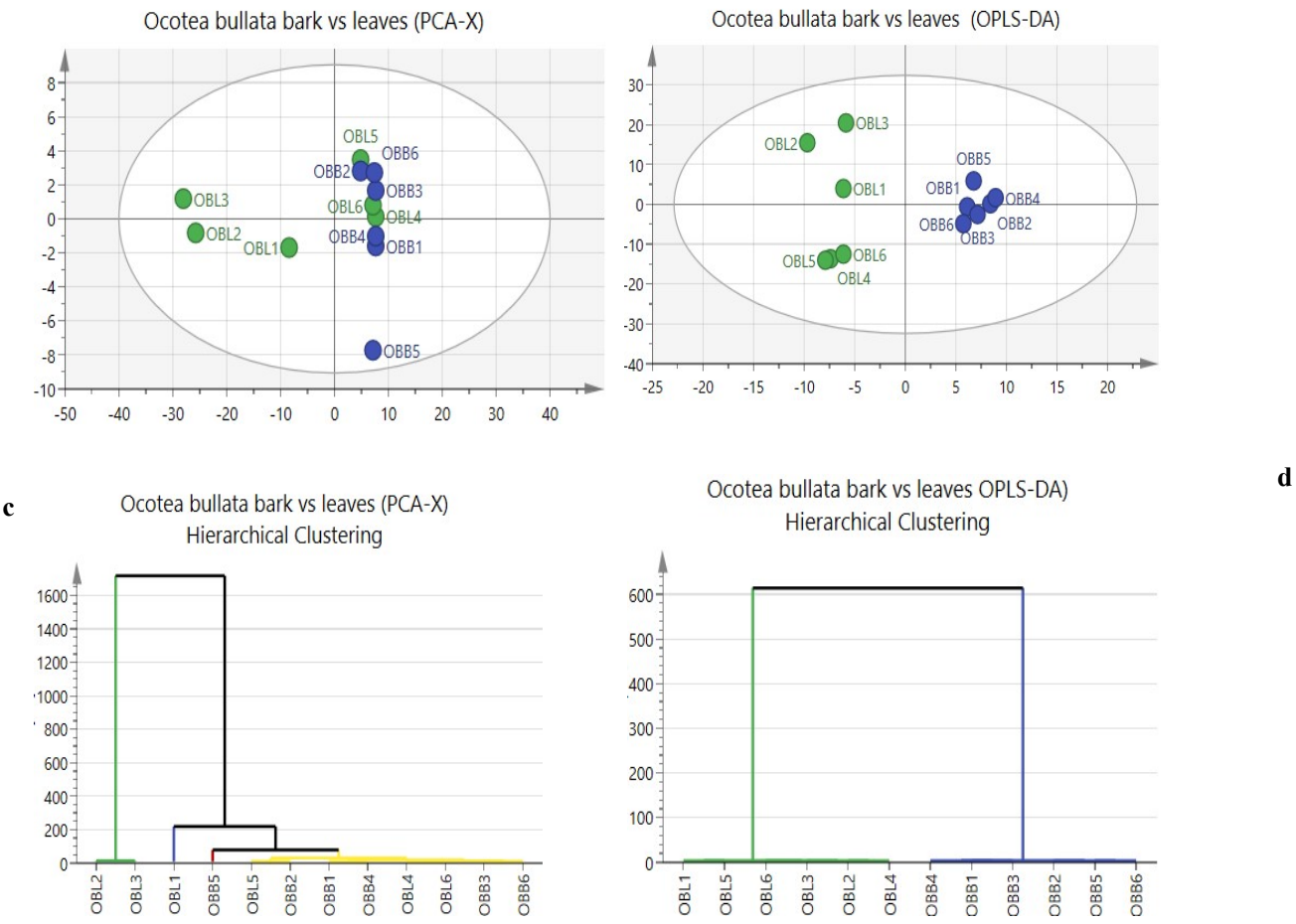

Figure 5.3.2: Principal Component Analysis (a) and Orthogonal Partial Least Squares Discriminant Analysis (b) scores of *Ocotea bullata* leaves and bark with Hierarchical Cluster Analysis (HCA) Dendrogram derived from the PCA (c) and OPLS-DA (d) showing metabolite relativity of the samples.

To validate the robustness of the OPLS-DA discriminant model, statistical interference analysis was applied, including 120 permutation tests to assess the predictor variable Y based on measured data from variable X (Figure 3.3a). The low Q^2 and R^2X values observed after permutation confirmed the model's statistical significance and indicated minimal overfitting, affirming that the patterns in the data are genuine rather than random fluctuations. Additionally, the Receiver Operating Characteristic (ROC) curve, with a high area under the curve (AUC), supported the model's strong predictive ability (Figure 5.3.3b). Cross-validated predictive residual analysis further corroborated the model's reliability (CV-ANOVA, $p < 0.05$), indicating that the model successfully captures real and meaningful differences between groups.

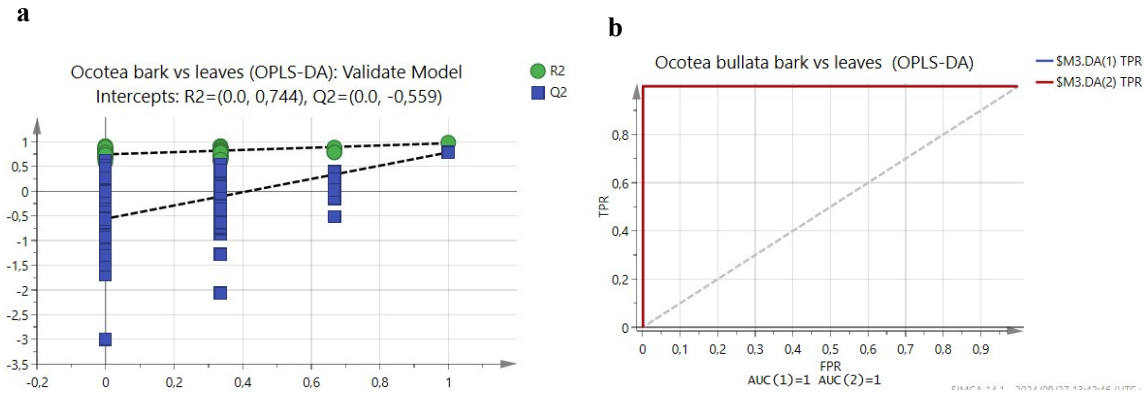

Figure 5.3.3: Statistical validation of the OPLS-DA model by permutations (a) and diagnostic performance through ROC (b).

The Variable Important in Projection (VIP) scores shown in figure 5.3.4 below are used to effectively identify the key metabolites that are most responsible for chemical diversity and uniqueness between the leaves and the bark. The VIP scores indicate the significance of each metabolite in distinguishing between the samples based on their chemical composition. Higher VIP scores signify a greater influence of a specific metabolite in differentiating the samples from the leaves and the bark. Chlorogenic acid has the highest VIP score (Figure 5.3.4), indicating that the abundance of the metabolite varies significantly between the samples from the leaves and bark and is a major discriminating factor in their metabolite compositions. Metabolites like tyrosine, citrulline, xylose, acetate and leucine also have relatively high VIP scores, suggesting their levels or structural features contribute substantially to differentiating the chemical profiles of the samples from the leaves and bark. Metabolites with lower VIP scores such as trigonelline, alanine and catechin are less influential in distinguishing between *O. bullata* leaf and bark samples based on their metabolite data.

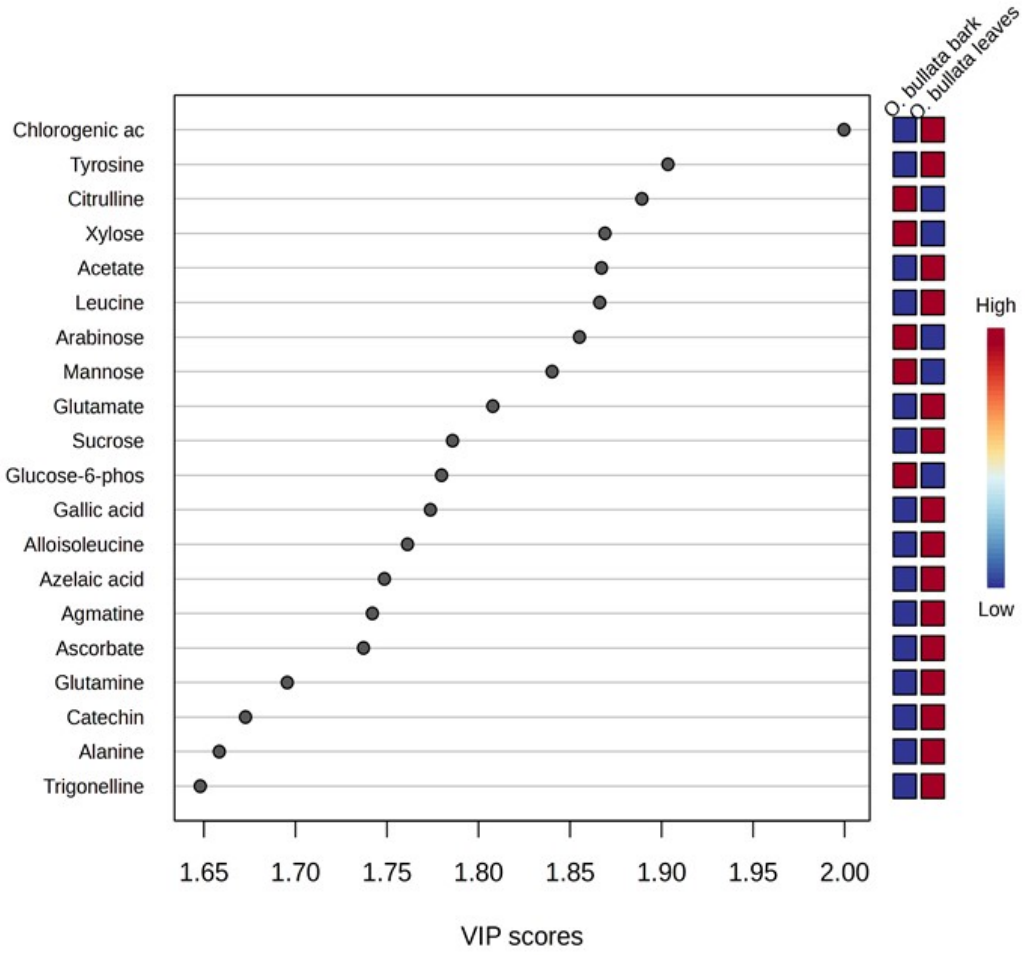

Figure 5.3.4: Variable Importance in Projection (VIP) plot highlighting key metabolites including, chlorogenic acid, tyrosine, citrulline, xylose, acetate, leucine, arabinose, mannose, glutamate, sucrose, glucose-6-phosphate,gallic acid, alloisoleucine, azelaic acid, agmatine, ascorbate, glutamine, catechin, alanine and trigonelline contributing to group separation, arranged from top to bottom based on their significance.

The metabolites annotated from the leaf and the bark samples are shown in Table 5.3.1. Among the unique metabolites observed from the leaves and bark, there were also 7 commonly present metabolites between the bark and leaves. Figure 5.3.5 below shows the chemical structures of the common metabolites between the bark and the leaves of *O. bullata*.

Table 5.3.1: Chenomx assisted annotations of differentially produced metabolites from *O. bullata* leaves and bark

Leaf		Bark	
Metabolites	Chemical shift	Metabolites	Chemical shift
Acetamide	7.40(s), 6.80(s),1.99(s)	Alanine	3.76(q), 1.46(d)
Acetate	1.91(s)	Anthranilic acid	7.29(q),6.80(t)
Ascorbate	sucro	Xylose	3.69(m)
Adenine	8.22(s)	Ascorbate	4.50(d),3.99(t), 3.71(m)
Adipate	2.16(m),	Asparagine	6.95(s), 3.98(q), 2.82(dd)
Alanine	3.76(q), 1.46(d)	Aspartate	3.87(dd), 2.78(dd), 2.64(q)
Alloisoleucine	3.73(d),2.02(m),1.38(m),1.28(m),0.92(m)	Azelaic acid	2.14(t), 1.50(m),1.24(m)
Xylose	3.69(m)	Arabinose	5.31(d),4.52(d),3.47(q)
Aspartate	3.88(q),2.78(dd),2.64(q)	Choline	4.04(m),3.51(s),3.20(s)
Betaine	3.89(s), 3.25(s)	Erythritol	3.76(dd),3.60(m)
Glycine	3.56(s)	Glyceric acid	4.07(q),3.80(dd)
Carnitine	4.52(m),3.40(m),3.21(s),2.40(m),2.39(m)	Glycine	3.56(s)
Choline	4.04(m),3.51(s),3.20(s)	Glycolate	3.94(s)
Chlorogenic acid	7.6(s), 7.2(d), 6.9(d), 4.3(q), 3.9 (q)	Acetate	1.91(s)
D-Threitol	3.67(m)	Catechin	7.06(d),6.10(dd),5.01(s)
Malonate	3.11(s)	Mannose	5.18(d),4.89(d),3.40(m)
Fumarate	6.51(s)	Glucose-6-phosphate	4.65(d),3.97(m)
Gallic acid	7.04(s)		
Gentisic acid	7.28(d),6.97(dd),6.83(d)		
Glutarate	2.16(t), 1.74(m)		
Glutamate	3.73(q),2.00(m)		
Homoserine	3.83(q),3.74(m),2.12(m),1.98(m)		
Isobutyrate	2.34(m), 1.04(d)		
Lactate	4.08(q), 1.31(d)		
Levulinate	2.75(t), 2.38(t), 2.21(s)		
Myo-Inositol	3.6 (t), 4.1(t), 3.5(dd)		
Anthranilic acid	7.29(q),6.80(t)		
Serine	3.84(q), 3.92(m), 3.99(m)		
Thymol	6.81(d),1.17(d), 3.12(m)		
Tyrosine	7.16(d),6.87(d),3.92(dd)		
Valine	3.61(d),2.22(m),1.02(d)		
Xylitol	3.78(m),3.62(m)		

Peak multiplicity (s = singlet; d = doublet; t = triplet; dd = doublet of doublet; q = quartet; m = multiplet)

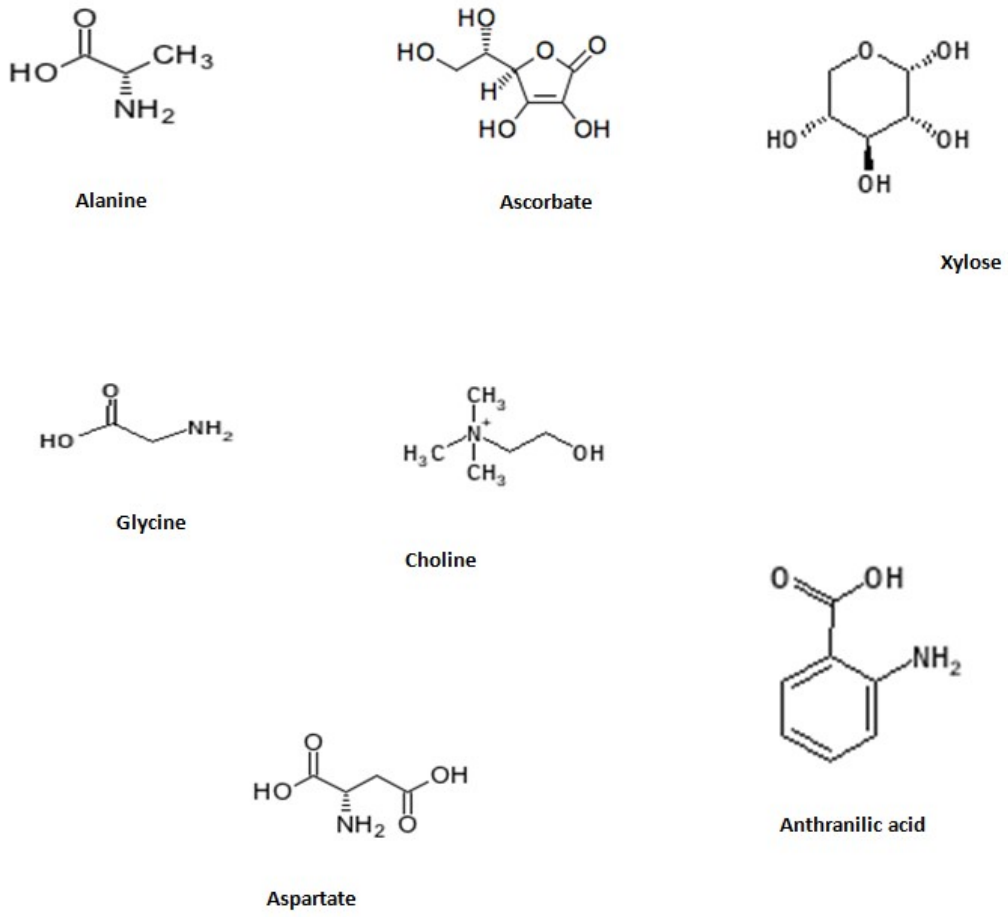

Figure 3.5: Kyoto Encyclopedia of Genes and Genomes (KEGG) drawn chemical structures of metabolites commonly found in the leaves and bark of *Ocotea bullata.*

Following the observation that, despite differences in metabolite composition between the bark and leaves, there are still common metabolites shared by both plant parts, it is evident that the leaves can also be beneficial when used traditionally. Consequently, attention shifted to investigating whether the metabolites present in the leaves are influenced by seasonal changes, as this could provide valuable insights for sustainable harvesting practices and determine the optimal timing for leaf collection to maximize their medicinal benefits. The multivariate data analysis was performed on *O. bullata* leaves collected in summer compared to those collected in winter and both the unsupervised PCA and supervised OPLS-DA confirmed distinct differences between the summer and winter samples (Figure 5.3.6a, b). A clear separation in the unsupervised model (PCA), indicates that the seasonal differences are significant enough to be captured by the natural variance in the data. Furthermore, figure 5.3.7 confirmed that the model did not over fit, and that the

separation is based on inherent patterns in the dataset rather than noise or overly complex modelling.

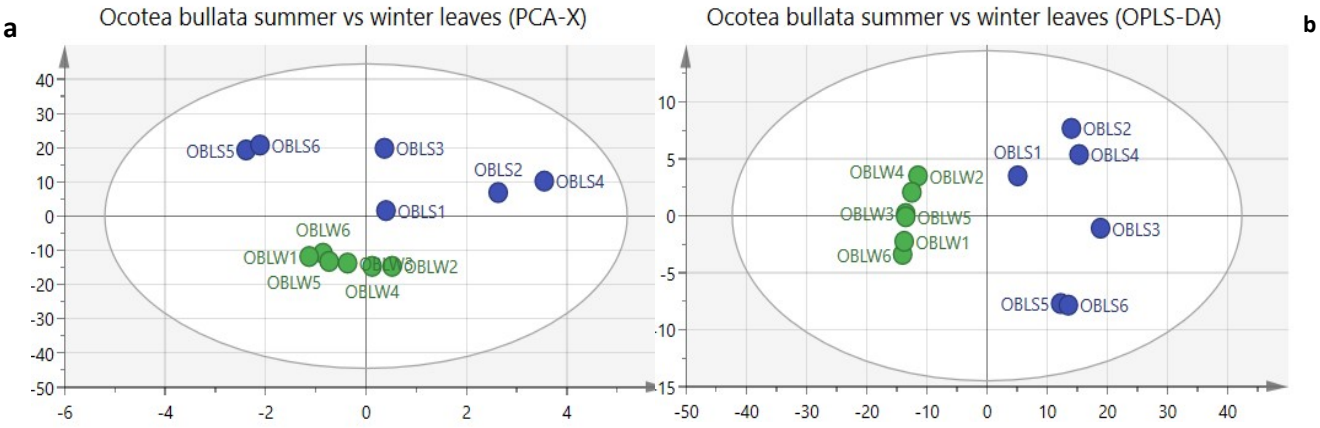

Figure 5.3.6: Principal Component Analysis (a) and Orthogonal Partial Least Squares Discriminant Analysis (b) scores of *Ocotea bullata* summer and winter leaves. The green circles indicate the winter samples and the blue circles indicate the summer samples.

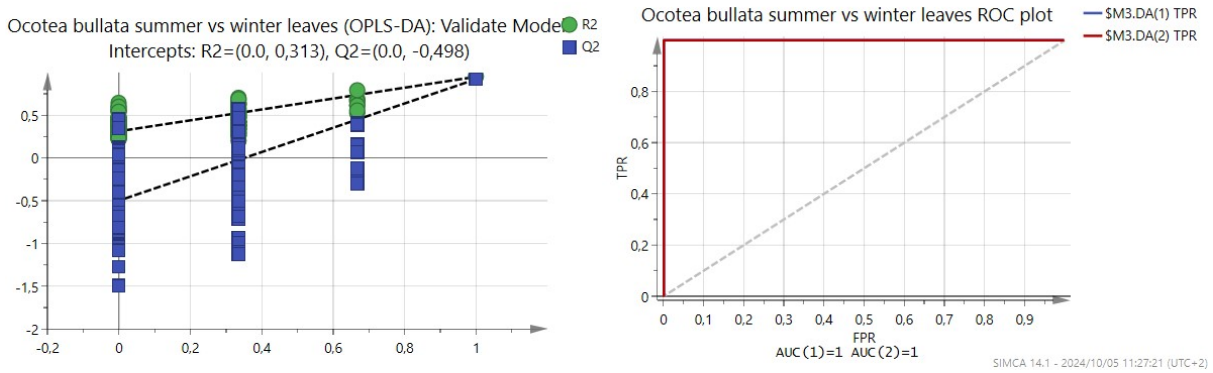

Figure 5.3.7: Statistical validation of the OPLS-DA model by permutations (a) and diagnostic performance through ROC (b).

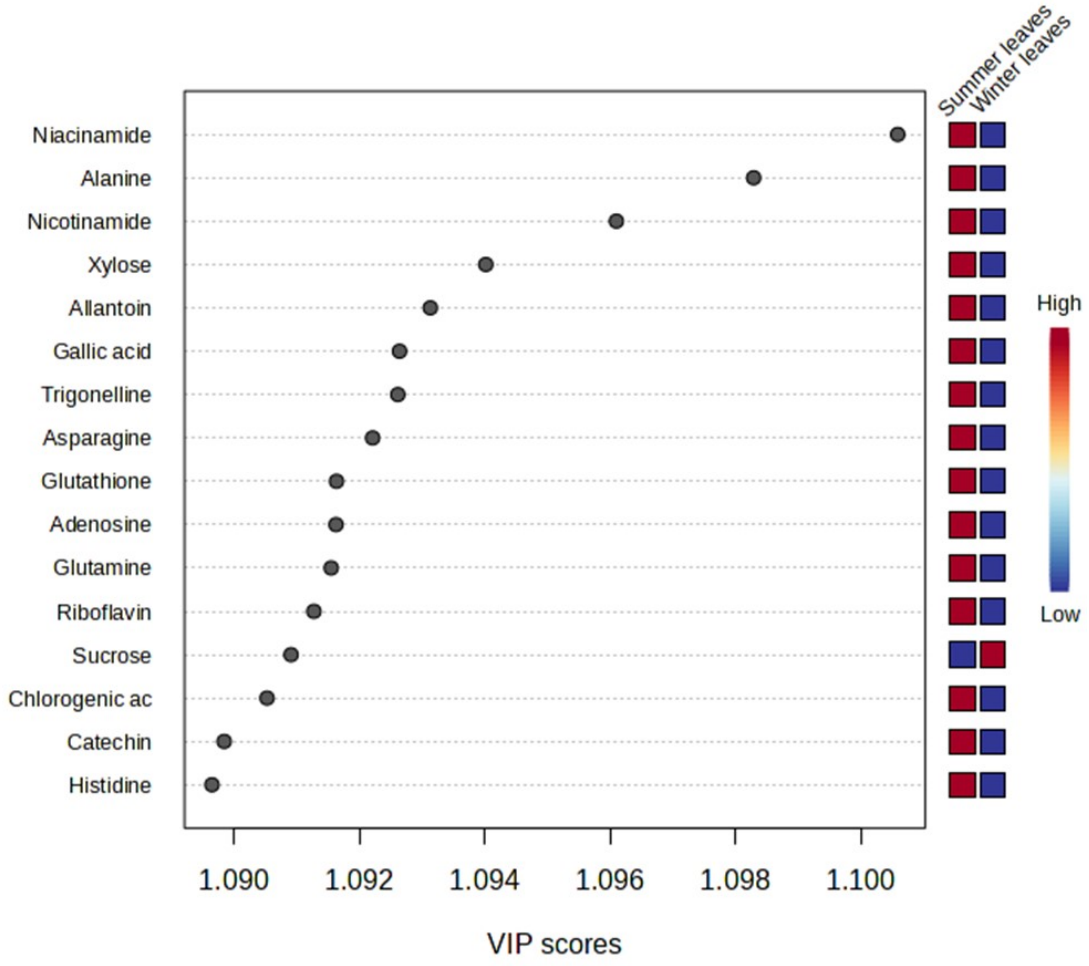

Figure 5.3.8: Variable Importance in Projection (VIP) plot highlighting key metabolites including, niacinamide, alanine, Nicotinamide, xylose, allantoin, gallic acid, trigonelline, asparagine, glutathione, Adenosine, glutamine, riboflavin, sucrose, chlorogenic acid, catechin, Histidine and furoic acid, contributing to group separation between the leaves seasonally, arranged from top to bottom based on their significance.

The metabolites annotated between the summer and winter leaves of *O. bullata* are shown in Table 3.2, with the highest composition of metabolites observed in the summer leaves. The VIP plot identified niacinamide (Vitamin B3), alanine and nicotinamide as the top three key metabolites that differentiate between the summer and winter leaves of *O. bullata*. These metabolites were more intense in the summer samples (Figure 5.3.8). In addition to these key discriminating metabolites, 10 other metabolites were commonly present in both the summer and winter leaves (Figure 5.3.9).

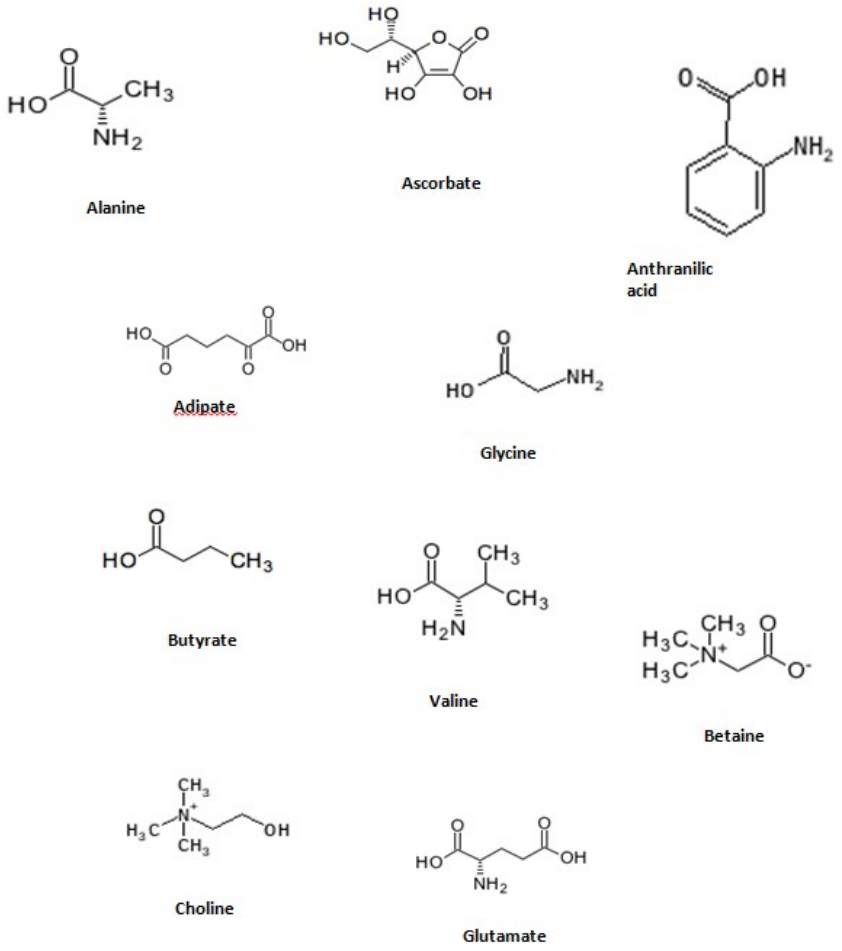

Figure 5.3.9: Kyoto Encyclopedia of Genes and Genomes (KEGG) drawn chemical structures of metabolites commonly found in the summer and winter leaves of *Ocotea bullata*

Table 5.3.2: Chenomx assisted annotations of differentially produced metabolites from *O. bullata* summer and winter leaves

Summer		Winter	
Metabolites	Chemical shift	Metabolites	Chemical shift
Acetate	1.91(s)	Aspartate	3.88(q),2.78(dd),2.64(q)
Ascorbate	4.50(d),3.99(t), 3.71(m)	Malonate	3.11(s)
Acetoacetate	3.43(s), 2026(s)	Xylitol	3.78(m),3.62(m)
Adenine	8.22(s)	Ascorbate	4.50(d),3.99(t), 3.71(m)
Adipate	2.16(m),	Valerate	2.15(t),1.48(m),0.86(t)
Alanine	3.76(q), 1.46(d)	Valine	3.60(d), 2.22(m), 1.02(d)
Alloisoleucine	3.73(d),2.02(m),1.38(m),1.28(m),0.92(m)	Valproate	2.20(m),1.38(m),1.28(m),0.85(t),1.22(m)
Allantoin	8.0(s),7.3(s),6.0(s),5.4(s)	Choline	4.04(m),3.51(s),3.20(s)
Aspartate	3.88(q),2.78(dd),2.64(q)	Alanine	3.76(q), 1.46(d)
Betaine	3.89(s), 3.25(s)	Adipate	2.16(m), 1.50(m)
Butyrate	2.13(t), 1.52(m),0.87(t)	Betaine	3.89(s), 3.25(s)
Glycine	3.56(s)	Butyrate	2.13(t), 1.52(m),0.87(t)
		Creatine	3.92(s),3.02(s)
		Glutamate	3.73(q),2.00(m)
Carnitine	4.52(m),3.40(m),3.21(s),2.40(m),2.39(m)	Anthranilic acid	7.29(q),6.80(q)
Choline	4.04(m),3.51(s),3.20(s)	Glycine	3.56(s)
Chlorogenic acid	7.6(s), 7.2(d), 6.9(d), 4.3(q), 3.9 (q)		
D-Threitol	3.67(m)		
Fumarate	6.51(s)		
Gallic acid	7.04(s)		
Gentisate			

Glutarate	7.28(d),6.97(dd),6.83(d)
Glutamate	2.16(t), 1.74(m)
Isobutyric acid	3.73(q),2.00(m)
Lactate	2.34(m), 1.04(d)
Levulinate	4.08(q), 1.31(d)
Myo-Inositol	2.75(t), 2.38(t), 2.21(s)
Anthranilic acid	3.6 (t), 4.1(t), 3.5(dd)
Niacinamide	7.29(q),6.80(t)
Serine	8.92(d), 8.69(dd),7.57(q)
Taurine	3.84(q), 3.92(m), 3.99(m)
Thymol	3.40(t),3.24(t)
Tyrosine	6.81(d),1.17(d), 3.12(m)
Valine	7.16(d),6.87(d),3.92(dd)
	3.61(d),2.22(m),1.02(d)

Peak multiplicity (s=singlet; d= doublet; t= triplet; dd= doublet of doublet; q= quartet; m = multiplet)

5.4 Discussion

The metabolomic analysis of *O. bullata* leaves and bark, as revealed by 1H NMR spectroscopy, provides a comprehensive view of its biochemical diversity. The presence of a broad range of metabolites, from aliphatic to aromatic groups, underscores the plant's metabolic versatility and its ability to synthesize diverse metabolites across different parts/tissues (Stochmal et al., 2022).

The leaves of *O. bullata* exhibit a more diverse array of metabolites compared to the bark (Table 5.3.1). Other studies involving other plant species also found that leaves generally produce more metabolites than bark (Wu et al., 2022, Jin et al., 2020) . The metabolites such as ascorbate, and gallic acid observed in *O. bullata* leaves have multiple functions including energy production, defence against oxidative stress, and protection against herbivores (Singh et al., 2021b, Saleem et al., 2022). Additionally, the need to support rapid growth and adapt to environmental stressors likely drives the synthesis of various amino acids, organic acids, and antioxidants, further contributing to the rich metabolic profile observed in the leaves (Toscano et al., 2019).

The bark showed fewer metabolites compared to the leaves (Table 5.3.1), which can be linked to its structural role in the plant. The bark primarily serves as a protective barrier, reducing the need for the extensive production of diverse metabolites that are more prevalent in leaves, which are directly exposed to environmental stressors(Lamalakshmi Devi et al., 2017). The metabolite profile of *O. bullata* bark showed the presence of compounds such as alanine, anthranilic acid, xylose, and ascorbate suggesting a complex metabolic response aimed at stress management and protection(Wishart, 2019).

The distinct separation observed in the PCA and OPLS-DA plots (Figure 5.3.2a, b) further emphasizes the robustness of the metabolic differences between the bark and leaves of *O. bullata.* Such statistical analyses not only confirm the presence of unique metabolite profiles between the leaves and bark but also highlight metabolites like chlorogenic acid, tyrosine, and citrulline as top 3 key discriminators (Figure 5.3.4). Each of these metabolites serves a different purpose in plant physiology and potentially offering various medicinal values. Chlorogenic acid, a phenolic metabolite commonly found in the leaves of many medicinal plants including *Ocotea diospyrifolia*, is known to play a role in plant defense against pathogens, pests, and environmental stresses (Al-Khayri et al., 2023, Hammerschmidt, 2014, Silva et al., 2021). It acts as an antioxidant, reducing oxidative damage in plant tissues(Mei et al., 2020). The leaves are exposed to environmental factors and chlorogenic acid helps protects them from stress and damage. The medicinal value of Chlorogenic acid is linked to its antioxidant potential, anti-inflammatory and weight management activities (Naveed et al., 2018, Kumar et al., 2020, La Rosa et al., 2023). This may suggest that the

leaves of *O. bullata* have potential for medicinal use; however, further bioassays are required to confirm their efficacy.

Moreover, based on the roles of tyrosine in plants, the observed abundance of this metabolite in *O. bullata* leaves (Figure 5.3.4) aligns with its known functions in protein synthesis, defense mechanisms, and hormone production (Cashmore, 1976, Tcherkez et al., 2020). As plant leaves are active sites for these processes, tyrosine as a precursor for proteins, secondary metabolites, and hormones (Moon et al., 2018, Feduraev et al., 2020, Kelly, 2000), would naturally be expected to be present in notable quantities in the leaf tissues. The medicinal use of tyrosine is usually with the skin health, where it is involved in melanin production (Wagstaff et al., 2022). Moreover tyrosine supplements (capsule or powdered L-tyrosine) are sometimes used to improve alertness, focus and attention particularly under stress and fatigue (Attipoe et al., 2015).

Citrulline, another key discriminating metabolite between the bark and leaf samples was prominently detected in the bark of *O. bullata* (Figure 5.3.4). This prevalence of citrulline in the bark is likely related to its role in resource storage and long-term metabolic processes (Ahmadi et al., 2020). The bark, as a protective outer layer, serves as a reservoir for vital compounds, including those involved in nitrogen metabolism (Shukla, 2009). Citrulline helps manage nitrogen efficiently, supporting the metabolic needs of the bark over time (Cañas et al., 2016). Furthermore, the noticeable presence of citrulline in the bark may contribute to its traditional use in treating urinary tract infections, owing to citrulline's antioxidant properties and potential benefits for erectile dysfunction (Cormio et al., 2011, Muncey et al., 2021).

Moreover, the presence of shared metabolites like alanine, xylose, ascorbate, aspartate, butyrate, choline, glycine, and anthranilic acid (Figure 5.3.5) between the bark and leaves of *O. bullata* highlights key metabolic processes crucial for the plant's survival and overall function. These compounds play significant roles in both plant growth and human health. Alanine and glycine are essential amino acids involved in protein synthesis and metabolic regulation, supporting plant growth and resilience (Planchet and Limami, 2015, Ganie, 2021, Khan et al., 2020). Ascorbate, a powerful antioxidant, helps protect plants from oxidative damage and enhances their stress tolerance, while also contributing to human health by neutralizing free radicals (Devasagayam et al., 2004, Hasanuzzaman et al., 2019).

Butyrate influence energy metabolism and osmotic regulation, which are vital for maintaining cellular functions in plants (Liu et al., 2019). Choline supports membrane integrity and neurotransmission, benefiting both plants and humans(Korsmo et al., 2019, Penry and Manore, 2008). The presence of anthranilic acid may indicate its role in aroma production, which aligns with

the strong herbal smell of *O. bullata* leaves and bark. However, it is primarily the bark that is currently sniffed to relieve headaches(Zschocke et al., 2000). The analysis of *O. bullata* bark and leaves observed in this study confirms that the leaves (a sustainable plant part) also have as much potential as the bark to be used traditionally in the treatment of various ailments.

Therefore, since seasonal variations have been shown to impact the production of metabolites in plants (Soni et al., 2015, Chaves et al., 2013), this study also analysed the leaves of *O. bullata* collected in both winter and summer to determine which season yields metabolites with significant potential for human health and plant growth promotion. Identifying optimal seasonal conditions for beneficial metabolite production could support conservation efforts and sustainable utilization of this endangered species.

The multivariate data analysis of *O. bullata* summer and winter leaves showed a clear separation (Figure 5.3.6a, b), indicating that seasonal variations significantly influence the composition of plant metabolites. This also suggests that there are distinct biochemical profiles associated with each season. Similar to findings in studies of other plants(Sahoo et al., 2011, Tripathi et al., 2018), the summer leaves of *O. bullata* exhibited a richer composition of metabolites compared to those collected in winter (Table 5.3.2). In summer, increased sunlight and warmer temperatures boost photosynthesis and other biosynthetic pathways, leading to the production of a broader range of primary and secondary metabolites (Yeshi et al., 2022).

The winter leaves of *O. bullata* showed a reduced and more specialized metabolite profile compared to the summer leaves, reflecting the plant's adaptation to colder, less favourable conditions. Generally, metabolic processes slow down as the plant conserves energy and resources during winter (Fürtauer et al., 2019). The lower temperatures and reduced sunlight availability limit photosynthesis and the biosynthesis of many metabolites, leading to a decrease in the overall metabolic diversity (Pant et al., 2021). The metabolites that remain abundant in winter leaves are likely those that help the plant cope with cold stress, prevent cellular damage, and maintain basic physiological functions (Janská et al., 2010, Ritonga and Chen, 2020). During winter, the presence of metabolites like ascorbate in *O. bullata* leaves may suggests a focus on maintaining cellular integrity and combating oxidative stress, which can be heightened in cold environments. Ascorbate, known for its antioxidant properties, plays a critical role in protecting the plant tissues from damage due to cold-induced reactive oxygen species (Akbasova and Samat, 2021, Roychoudhury and Basu, 2012).

The VIP plot identified niacinamide (Vitamin B3), nicotinamide, and alanine as the top three key metabolites that differentiate between the summer and winter leaves of *O. bullata*. These

metabolites were annotated as the most important in distinguishing summer leaf samples of *O. bullata* samples (Figure 5.3.8), and are important for various biochemical processes, from energy production to amino acid synthesis, in both plants and other organisms (Gakière et al., 2018, Noctor et al., 2011). Niacinamide supports essential metabolic processes in plants by contributing to NAD^+/$NADP^+$ biosynthesis, which is critical for cellular energy balance, redox reactions, and stress responses. Through these functions, it indirectly aids in plant development and defense regulation (Kirkland and Meyer-Ficca, 2018). In terms of medicinal importance, niacinamide is widely recognized for its anti-inflammatory and skin-repair properties in humans, contributing to its use in dermatological treatments (Tempark et al., 2024, Glass, 2020).

The metabolites commonly found in both the winter and summer leaves of *O. bullata* including alanine, anthranilic acid, ascorbate, choline, adipate, caffeine, betaine, valine, glutamate, sucrose, butyrate, and glycine (Figure 5.3.9) further highlight the medicinal potential of the leaves. These compounds are known for their diverse bioactive properties, which support various health benefits. Ascorbate (Vitamin C) acts as a potent antioxidant, while glutamate plays a role in cellular detoxification and stress response (Padayatty et al., 2003, Matés et al., 2002). Betaine is involved in liver protection and anti-inflammatory activities (Socała et al., 2020, Tellone et al., 2019, Veskovic et al., 2019). Anthranilic acid contributes to the synthesis of aromatic compounds, and valine, an essential amino acid, aids in tissue repair and muscle metabolism(Fuchs, 2008, Soeters et al., 2004). The presence of these metabolites suggests that *O. bullata* leaves could serve as a sustainable alternative to bark harvesting for medicinal purposes, offering a rich profile of bioactive compounds that promote human health.

In the light of this metabolite profiling results, which revealed both seasonal and plant-part differences in *O. bullata*, it is essential to consider other options such as investigating how endophytes within this species might similarly vary and contribute to broader conservation goals. Knowing that endophytes and plants are partners in producing metabolites, the presence of health-promoting metabolites in both bark and leaves not only underscores the medicinal potential of the leaves but also highlights the role that endophytes adapted to these tissues and conditions could play in promoting plant health and resilience. Understanding seasonal dynamics and the distribution of these endophytes can enhance conservation strategies, support sustainable metabolite production, and contribute to ecosystem protection by strengthening the plant's natural defense. The next phase of this study, therefore, examines the potential of culturable bacterial endophytes from leaves and bark considering seasonal variation, for their antimicrobial properties and ability to support plant growth, providing an integrative approach to both plant and environmental health.

5.5 Conclusion

The comparative metabolite analysis of *O. bullata* barks and leaves revealed differences and also highlighted some similarities of the metabolites contributing to both plant growth promotion and human health. Seasonal variation further influenced metabolite composition, with summer yielding a generally higher abundance of metabolites than winter. This seasonal variability allows for strategic harvesting, where leaves collected in summer may be prioritized for medicinal and agricultural applications, while winter-harvested leaves still retain value depending on specific needs. Notably, the findings underscore the potential of leaves as a sustainable alternative to bark harvesting, reducing pressure on the species and promoting conservation efforts. However, because only NMR was used, certain low-abundance metabolites may have remained undetected or unresolved due to spectral overlap. Despite this limitation, the study provides a comprehensive overview of metabolite distribution in *O. bullata*, offering valuable insights into its chemical diversity. To fully harness the potential of leaf-derived metabolites, future studies should employ additional analytical techniques such as LC-MS to detect a broader spectrum of metabolites, and also conduct targeted bioassays to assess biological efficacy. These advancements will strengthen the foundation for sustainable utilization and conservation of *O. bullata*.

5.6 References

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Chapter 6

Endophytic bacteria from *Ocotea bullata*: dual potential in plant growth promotion and antimicrobial activity

This chapter is intended for submission to the Journal of Applied Microbiology.

[a] Cynthia K. Marokane-Radebe, [a]Adekunle R. Raimi, [a,b] Stephen O. Amoo, [a] Rasheed A. Adeleke*

[a] Unit of Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa

[b] Agricultural Research Council - Vegetables, Industrial and Medicinal Plants, Roodeplaat, Pretoria, 0001. South Africa.

Abstract

The endangered *Ocotea bullata (O. bullata)*, commonly known as black stinkwood, is a highly valued timber species native to South Africa, with its stem bark historically utilized for medicinal purposes, highlighting the importance of exploring its plant growth promotion and microbial interactions. This study isolated endophytic bacterial strains from the leaves (collected across two seasons) and bark of *O. bullata* and thoroughly screened them for their *in vitro* plant growth-promoting traits, including phosphate solubilisation, nitrogen fixation, the production of hydrogen cyanide, siderophore, and IAA as well as hydrolytic enzymes activity. Additionally, the antimicrobial activities of these bacterial endophytes were evaluated against human pathogenic bacteria and yeast. The 16S rRNA were isolated from the leaves and bark of *O. bullata*, gene sequencing and the subsequent bioinformatics analysis of the fifteen endophytic bacterial strains revealed *Bacillus* species being predominant across different seasons and plant parts. The bacterial endophytes exhibited significant plant growth-promoting traits, and enzyme production, with Streptomyces sp, isolate B8 displaying the highest amylase activity and *Bacillus toyonensis* (SL7) showing the most robust lipase activity. Among the isolated endophytes, *Enterobacter asburiae* (WL2), *Bacillus toyonensis* (SL7), and *Enterobacter sp*. (B1) demonstrated potent antimicrobial activity against multiple human pathogens and significantly enhanced the germination rate of tomato seeds, highlighting their potential as bio primers and potential sources of novel antimicrobial compounds. The findings underscore the potential of these endophytes in sustainable agriculture, particularly as biofertilizers and biocontrol agents, warranting further research into their practical applications.

6.1 Introduction

Sustainable agriculture requires the recruitment of diverse bacteria endophytes to manage the on-going emergence of plant diseases. Bacterial endophytes colonize and inhabit internal plant tissues without causing any apparent damage (Morales-Cedeño et al., 2021). Within the plant, these bacteria exert plant growth by the action of Phytohormone or the production of metabolites (Morales-Cedeño et al., 2021). Moreover, bacterial endophytes also protect their plant host through biocontrol pathogens or by inducing plant innate immune system (Chaudhary et al., 2022). Although endophytes assist plants in coping with abiotic stresses, they also become constrained by environmental factors such as intense UV radiation, droughts and extreme cold leading to limited vitality of some endophytic strains (Liu et al., 2024). Therefore, environmental conditions not only affect plant species and growth status but also define microbial community structure (Dastogeer et al., 2020). A study by Zhu et al. (2021) investigating the endophytic diversity in *Kalidium schrenkianum*, reported a higher diversity of endophytic community in summer and autumn, with the peaked endophytic diversity during spring. In another study, the diversity of endophytes of *Huperzia serrate* varied significantly across different seasons, with lower diversity observed during summer compared to other seasons (Shen et al., 2022).

Additionally, plants of the *Lauraceae* family have been investigated and found that they host a large number of bacterial endophytes (Elmagzob et al., 2019, Frizzo et al., 2000). Endophytes isolated from *Cinnamomum camphora* have shown that they are able to inhibit pathogens and are effective in the utilization of cellulose (Strobel et al., 2004, Elmagzob et al., 2019). Furthermore, endophytic fungi, *chaetomium globosum*, and *penicillum minioluteum* sourced from *Litsea cubeba*, showed efficacy in suppressing the growth of six plant pathogenic fungi, including *Colletotrichum gloeosporioides*, *Phytophthora capsici*, *Fusarium andiyazi*, *Alternaria alternata*, *Phomopsis sp*, and *Rhizoctonia solani* (Urooj et al., 2018, Wu et al., 2019).

Moreover, in a study by Quach et al. (2021), an endophytic actinomycete strain associated with *Litsea cubeba* demonstrated significant inhibition against seven bacterial pathogens and three human tumor cell lines. This strain was identified as *Streptomyces* variabilis and exhibited phenotypical resistance to fosfomycin, trimethoprim-sulfamethoxazole, dalacin, cefoxitin, rifampicin, and fusidic acid (Quach et al., 2021). Additionally, it carried two antibiotic biosynthetic genes, PKS-II and NRPS (Quach et al., 2021). Despite the extensive research conducted on various members of the *Lauraceae* family, a significant gap exists in our understanding of the diversity, plant growth promotion capabilities, and antimicrobial potential of endophytes specifically

associated with *O. bullata*. This gap is particularly concerning given the imminent threat of extinction facing *O. bullata*.

The endangered *O. bullata* of the *Lauraceae* family, commonly known as the black stinkwood, is a highly valued timber species native to South Africa (Zschocke et al., 2000). Throughout history, the stem bark of *O. bullata* have been utilised for medicinal purposes, targeting a spectrum of human aliments such as diabetes, inflammation, envious disorders, and headaches (Ogundajo et al., 2018, Zschocke et al., 2000). The utilization of *O. bullata* in treating various conditions underscores the presence of potentially valuable metabolites warranting further investigation especially given the endangered status of this species. Furthermore, its efficacy in treating urinary bladder infections (Zschocke et al., 2000) suggests the presence of beneficial microorganisms associated with this species.

Given the critical importance of *O. bullata* in ecological systems ((Zschocke et al., 2000) and the potential benefits that may arise from studying its endophytic microbiome, urgent attention and research efforts are warranted to fill this knowledge gap and inform conservation strategies aimed at preserving this endangered species. It is therefore hypothesized that that there will be variation in the culturable bacteria with plant growth promotion capabilities isolated from leaves of *O. bullata* collected during the summer and winter seasons. Additionally, it was anticipated that the culturable microbial communities from the leaves for both seasons will exhibit similarities with those from the bark, making leaf collection a viable alternative to bark collection for further investigations. This approach is particularly important as bark collection may pose harm to the plant.

Bioprospecting endophytes for sustainable agriculture remain a relevant venture, primarily driven by the imperative for reliable bio inoculants. However, the urgency intensifies with the realization that certain species housing these endophytes are facing endangerment. Hence, there is an imminent need to embark on bioprospecting ventures before these species vanish from their habitats. Additionally, recognizing the variability of endophyte communities across seasons underscores the necessity to conduct seasonal investigations into culturable endophytes. This approach not only enhances our understanding of seasonal dynamics but also aids in pinpointing optimal periods for isolation and cultivation.

The aim of this chapter is to assess the seasonal variation in culturable bacteria with plant growth promotion attributes isolated from leaves of *O. bullata*, comparing samples collected during the summer and winter seasons, and to determine if the microbial communities inhabiting the leaves are comparable to those found in the bark, with the ultimate goal of providing a non-invasive method for further investigations that minimizes harm to the plant.

6.2 Material and methods

6.2.1 Sample collection

Leaf samples from healthy *O. bullata* plants were collected from the research garden of the University of KwaZulu Natal in Pietermaritzburg. Sampling of *O. bullata* leaves was done in summer and again in winter. To avoid putting pressure on the plants, the bark was only sampled in winter. The samples were placed in a sterile zipper bag, immediately transported to the laboratory in a portable 4ºC cooler bag and placed in a 4ºC refrigerator until use.

6.2.2 Isolation of bacterial endophytes

Ocotea bullata leaves were excised and washed under running tap water. The methodology employed in surface sterilization of *O. bullata* samples closely follows the approach outlined by Sahu et al. (2022), with slight modifications. Sterilization of leaf surfaces was done by soaking the tissues in a series of baths: sterile distilled water for 1 min, 70% ethanol for 30 s, 2.5% sodium hypochlorite for 4 min, and a final series of rinsing thrice in sterile distilled water in three different containers. The concentration of ethanol and sodium hypochlorite was reduced by half for the sterilization of the bark. Additionally, the washing time with these reagents was shortened to 20s and 1 minute respectively. A 0.1 mL aliquot of the final rinse water was plated onto nutrient agar plates to confirm the success of surface sterilization. The bark that was already opened up was washed under running tap followed by a series of five washes with distilled water in different containers.

The sterilized plant materials were then cut into approximately 5 mm segments, and twenty leaf/bark segments per individual plant were placed in four petri dishes (9 cm; five segments/plate) containing nutrient agar and incubated in the dark at 35± 2 ºC. The cultures were regularly observed for bacterial growth, for a period of forty-eight hours. Bacteria growing from the previous steps were streaked on fresh nutrient agar plates to obtain single colonies and stored at 4 ºC until further analysis.

6. 2.3. Pathogenicity screening of endophytes using haemolysis assay

All pure culture isolates of the bacterial endophytes from the aloes were evaluated for potential pathogenicity through a haemolysis assay, as described by Amaria et al. (2023). The isolates were spot inoculated onto sheep blood agar plates and incubated at 30 °C for 48 hours. Hemolytic reactions were determined based on the presence or absence of discoloration or clear zones around the colonies.

Alpha (α) haemolysis was indicated by a greenish or dark halo surrounding the colonies, reflecting partial lysis of red blood cells. Beta (β) haemolysis was confirmed by the formation of clear zones, indicating complete lysis. In contrast, gamma (γ) haemolysis, signifying non-hemolytic activity, showed no visible changes in the agar surrounding the colonies. Only isolates exhibiting gamma haemolysis were considered non-pathogenic and were subsequently selected for molecular identification and evaluation of plant growth-promoting (PGP) traits, including nitrogen fixation, phosphate solubilisation, IAA and siderophore production, as well as hydrolytic enzyme activity

6.2.4 Molecular identification of culturable endophytes

Genomic DNA of the isolated organisms was extracted using the ZymoBIOMICS™ DNA Microprep Kit (Zymo Research) according to the manufacturer's protocol. In the final step for DNA elution, the volume of the buffer was adjusted to 50 µL from the manufacturer's protocol to increase the DNA concentration and the same elution process was repeated. Each 2µL DNA sample was used as a template in 25 µL PCR. A partial 16S rDNA fragment was PCR amplified using the bacterial universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction contained: 12µL master mix, 7µL PCR buffer, 1µL of each primer (QIAGEN) and 2µL of bacterial genomic DNA. The PCR cycling conditions were 94 ºC for 3 min, followed by 30 cycles of 94 ºC for 0.5 min, 55 ºC for 0.5 min, and 72 ºC for 1 min, followed by a final extension performed at 72 ºC for 10 min. The PCR products were sent to inqaba biotech for sequencing. The 16S rRNA sequences generated in this study were deposited in Genbank and compared against the Genbank database using NCBI BLAST nucleotide search. A multiple sequence alignment was constructed on approximately 1200 bp of the 16S rRNA gene fragments using the ClustalX (2.0) software package (http://www.clustal.org/clustal2) and a phylogenetic tree was constructed using the neighbour-joining method in the MEGA v11.0.13 software (www.megasoftware.net), with confidence tested by bootstrap analysis (1000 repeats).

6.2.5 Screening for *in vitro* plant growth-promoting (PGP) traits

6.2.5.1 Phosphate solubilisation

The bacterial endophytic isolates were screened for Phosphate solubilisation as follows. Pikovskaya medium (glucose 10 g/L; $Ca_3(PO_4)$ 2.5 g/L; $(NH_4)_2SO_4$ 0.5 g/L; NaCl 0.2 g/L; $MgSO_4.7H_2O$ 0.1 g/L; KCl 0.2 g/L; $FeSO_4.7H_2O$ 0.002 g/L; yeast extract 0.5 g/L; $MnSO_4.2H_2O$ 0.002 g/L; agar 15 g/L; and 1 L dis. H_2O) was prepared and bromophenol blue was added as an indicator. The medium was inoculated with endophytic isolates and incubated for 48 h at 30ºC. The Pikovskaya medium without bacterial inoculation was used as a control. The formation of clear zones around the colony,

due to the utilization of tricalcium phosphate, was measured to assess the ability of endophytes to solubilise phosphate (Jasim et al., 2013). The phosphate solubilizing efficiency and solubilizing index were then calculated using the following formulas:

$$PSE(\%) - (Z - C)/C \times 100$$

Where,

Z=Solubilisation zone (mm)

C=Colony diameter (mm)

SI=Solubilisation index

$$SI = Z + C/C$$

6.2.5.2 Nitrogen fixation

The nitrogenase activity of the isolates was determined by the growth on nitrogen-free medium, the Burks Agar (HiMedia, India) according to Burk and Burris (1941). Pure bacterial colonies were streaked on the Burks Agar and incubated for 5–7 days at 28 ± 2°C. The appearance of the bacterial colonies indicated a positive test.

6.2.5.3. Qualitative hydrogen cyanide (HCN) production assay

Hydrogen cyanide production of bacterial endophytes was tested qualitatively following method by (Reetha et al., 2014).The bacterial endophytes were streaked on nutrient agar media supplemented with 4.4g/l glycine. A sterile filter paper saturated with picric acid solution (2.5g of picric acid;12.5g Na_2CO_3,100ml of distilled water) was placed in the upper lid of the petri plate. The plates were sealed with parafilm and incubated at 28ºC for 48h. A change of color of the filter paper from yellow to light brown, brown or reddish brown was recorded as weak(+), moderate(++) or strong(+++) reaction respectively.

6.2.5.3 Siderophore production

Siderophore production was evaluated on universal Chrome Azurol S (CAS) agar (Schwyn and Neilands, 1987). Each bacterial strain was inoculated at the centre of the plate. The appearance of an orange halo around the colony, after incubation at 28 °C for 3 days, indicated siderophore synthesis.

6.2.5.4 Indole-3-acetic acid (IAA) production

Indole acetic acid production was quantitatively measured according to Gordon and Weber (1951). Bacterial cultures were grown in test tubes, each containing 5 mL nutrient broth (Merck Millipore, UK) amended with 0.1%, 0.3% and 0.5% (w/v) tryptophan, the bacterial cultures were also allowed to grow in a tryptophan-free nutrient broth, then incubated at 28 ± 2°C for 48 h. Then, the culture medium was centrifuged at 10,000 rpm for 10 min. The total of 1 mL supernatant was mixed with 2 mL of Salkowski reagent. Tubes were incubated in dark at room temperature for 25 min. The development of pink colour indicates high production of IAA and the intensity of pink colour was read at 530 nm wavelength. The concentration of IAA produced was then extrapolated from the standard curve (Gordon & Weber 1951).

6.2.6 Enzyme activity analysis

The ability of endophytes isolated from *O. bullata* leaves (2 seasons) and bark to produce enzymes that hydrolyse starch, cellulose, casein and lipase were analysed qualitatively

6.2.6.1 Amylase analysis

For qualitative analysis of amylase activity, starch agar medium with 0.2% soluble starch and 1.5% agar was prepared, and the bacterial endophytes were point-inoculated and incubated at 25ºC for 7 days. Later, the plates were flooded with Gram's iodine solution to observe the zone of inhibition.

6.2.6.2 Protease analysis

Qualitative analyses of protease activity were carried out with casein agar medium containing 10% skim milk powder and 1.5% agar that had been autoclaved separately and mixed before plating. Then, these plates were point-inoculated with bacterial endophytes and incubated at 25ºC for or 7 days. Activity was identified by the zone of clearance around the point of inoculation

6.2.6.3 Cellulase analysis

Qualitative cellulase analysis was performed by inoculating bacterial endophytes into nutrient agar with 1% cellulose powder and incubated at 25ºC for 7 days. Later, the plates were flooded with Gram's iodine solution to observe the zone of inhibition.

6.2.6.4 Lipase analysis

Qualitative Lipase analysis was performed by inoculating bacterial endophytes into nutrient agar supplemented with 1% filtered tween20 after autoclaving. The plates were incubated at 25ºC. The plates were regularly checked for the presence of zone of inhibition over the period of 7 days.

6.2.7 Antimicrobial activity

The isolated endophytes were further analysed for their potential antimicrobial activity against the human pathogenic bacteria *Serratia marcescens*, *Enterococcus faecalis ,Escherichia coli, , Klebsiella pneumoniae,* and *Micrococcus luteus* and the yeast, *Candida krusei*. The pathogens were obtained from a microbiology laboratory technician at North West University in Potchefstroom.

6.2.7.1 Antibacterial activity

Bacterial endophytes were grown on Mueller Hinton broth for 24 h, and agar plug of the bacterial endophyte colony was placed at the centre of a Mueller Hinton agar plate that had already been spread with human pathogenic bacteria. After overnight incubation, the antibacterial activity was analysed by evaluation of the inhibition zone that had formed.

6.2.7.2 Crude extract preparation

Crude extract was prepared by modifying the method of Song et al. (2017). Bacterial endophyte B1, WL2 and SL7 that possess significant antibacterial activity from preliminary test were selected and cultured in 1L Luria broth (LB) and incubated at 30ºC on shaking incubator at 150 rpm until the stationary phase was reached (OD 1.5-1.7). After incubation the broth was centrifuged at 4000 r/m for 10 min and supernatant was collected then equal volume of ethyl acetate was added and shaken for 30 min in an extracting flask to extract secondary metabolites. Later, the organic phase of the liquid collected and evaporated using rotating evaporator. Then, the dry sample of crude extract with secondary metabolites was collected and stored in 4ºC until further analysis.

6.2.7.3 Antimicrobial assay of crude extract of endophytes

The antimicrobial assay of crude extract was evaluated by a modified broth dilution method (Charria-Girón et al. 2021). A 10 mg/mL stock solution of secondary metabolite crude extract was prepared and subjected to serial dilution to the concentrations 125, 250, 500,750, 1000, and 2500µg/ml using Mueller–Hinton broth and dimethyl sulfoxide (DMSO) 1:1 ratio. As positive controls Streptomycin (antibiotic) with 1.25, 2.5, 10, 30, 50 and 100 µg/ml concentrations was used and 1:1 ratio of DMSO and Mueller–Hinton broth was used as negative control. Antimicrobial assay was performed in 96-well micro titter plate, where, 24 h cultured broth of human pathogenic bacteria 100 µl with 1.5×10^8 CFU/ml and 100 µl of diluted crude extract was added, followed by blank (Mueller–Hinton broth), positive and negative controls were added and incubated at 37◦C for 24 h. After incubation the growth of the human pathogenic bacteria were analysed at 600 nm

6.2.8 NMR analysis of antimicrobial secondary metabolites

The crude secondary metabolite extract prepared by the above method (50 mg) was dissolved in 1500 µL potassium dihydrogen phosphate (KH_2PO_4) buffer in deuterium water (D_2O) (pH 6.0) containing 0.01% (w/w) trimethylsilanepropionic acid (TSP). The mixture was vortexed for 1 minute, ultrasonicated for 20 minutes, and then centrifuged for 20 minutes (at 10,000 rpm. Samples were then filtered through a 0.22 µm syringe filter and 500 µL of the filtrates were transferred to 5 mm Norell standard NMR tubes. All the proton NMR spectra were acquired using a 600 MHz NMR spectrometer (Varian Inc, CA, USA). Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisitions. All spectra were Fourier-transformed, and phase and baseline were corrected manually.

6.2.8.1 Spectra pre-processing and metabolites annotation

Data analysis and processing were performed using MestReNova software (10.0.1, Mestrelab Research, Spain), and the correction of phasing and baseline, normalisation and peak alignment was done manually on the 1H NMR spectrum. Annotations of compounds were performed using the Chenomx software (NMR suite, version 8.3) and published NMR data.

6.2.9 Seed priming with endophytes

Tomato and cucumber seed obtained from a local grocery store (Shoprite-Potchefstroom) were surface sterilized with a 1% sodium hypochlorite solution for 5 minutes and rinsed thoroughly with distilled water. Thereafter, they were soaked in bacterial endophytes B1, WL2 and SL7 at varying dilutions (B1 1:1,1:3,1:5; WL2 1:1,1:3,1:5 and SL7 1:1,1:3,1:5 v/v), while distilled water was included as the control. For each treatment, 20 seeds were placed in 90 mm petri plate lined with 2 layers of sterile Whatman No1 filter paper. The seeds were incubated at 25ºC with 12/12 light and dark regime for 10 days. The petri plates were laid out in a completely randomised design in triplicates. Seed germination was monitored and recorded daily, and four germination parameters; Final germination percentage (FGP), Seedling vigour index (SVI), Mean germination time (MGT) and Germination velocity (GV) were calculated according to Kader (2005) as follows.

$$\text{FGT}(\%) = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ planted} \times 100$$

Where:

- Number of seed germinated: total number of seeds that have successfully germinated by the end of the observation period.
- Total number of seeds planted: the total number of seeds initially planted in the experiment.

Mean Germination Time (MGT)

$$\text{MGT} = \frac{\sum(n \times d)}{\sum n}$$

Where:

- n is the number of seeds germinated on day d
- d is the day of observation

Seedling Vigor Index (SVI)

$$SVI = Germination\ percentage \times Mean\ seedling\ length$$

Germination velocity (GV)

$$GV = \frac{1}{MGT}$$

6.2.1 Data analysis

Data on the growth parameters of the endophytic bacterial strains were subjected to analysis of variance (ANOVA). Significance at 5% level was tested by Tukey's range tests at $p<0.05$ using Graphpad prism version 8.0.2.

6.3 Results

6.3.1 Morphological and genetic identification of the endophytes

A total of 39 endophytic bacterial isolates were obtained from *O. bullata*, comprising 18 from summer leaves (SL), 10 from winter leaves (WL), and 11 from bark samples. Following haemolysis testing to assess pathogenicity, only the non-pathogenic isolates were selected for further analysis. The 16S rRNA sequence analysis and phylogenetic reconstruction revealed notable taxonomic diversity among these endophytes across different plant parts and seasons. Summer leaf isolates included *Bacillus licheniformis* (92%), *Bacillus toyonensis* (100%), *Enterobacter hormaechei* (100%), and *Bacillus sp*. (99%), while winter leaves yielded *Bacillus licheniformis* (100%), *Pantoea brenneri* (96%), *Enterobacter asburiae* (100%), *Bacillus velezensis* (99%), Pantoea sp. (98%), and *Pantoea vagans* (99%) (Table 6.3.1). Bark isolates were identified as *Enterobacter sp.* (99%), *Bacillus velezensis* (94%), *Streptomyces sp*. (96%), *Bacillus tequilensis* (97%), and *Bacillus sp.* (97%). Phylogenetic analysis (Figure 6.3.1) confirmed the affiliations of these isolates with related taxa, forming well-supported clades within the genera *Bacillus*, *Enterobacter*, *Pantoea*, and *Streptomyces*. These results reflect a rich and seasonally influenced community of bacterial endophytes with potential functional significance for *O. bullata*.

Table 6.3.1: The 16S rRNA sequence identification of endophytic bacterial strains from *Ocotea bullata* leaves and bark

Plant species	Plant part	Bacterial strain code	Homologue sequence (sequence identity %)	NCBI accession numbers
Ocotea bullata	**Leaf summer**	**SL2**	***Bacillus licheniformis* 92%**	PQ637393
		SL7	***Bacillus toyonensis* 100%**	PQ637394
		SL12	***Enterobacter hormaechei* 100%**	PQ637611
		SL15	***Bacillus sp.*99%**	PQ637612
	Leaf winter	**WL1**	***Bacillus licheniformis* 100%**	PQ637613
		WL4	***Pantoea brenneri* 96%**	PQ637395
		WL2	***Enterobacter asburiae* 100%**	PQ637396
		WL5	***Bacillus velezensis* 99%**	PQ637397
		WL9	***Pantoea sp.* 98%**	PQ637398
		WL10	***Pantoea vagans* 99%**	PQ637399
	bark	**B1**	***Enterobacter sp.*99%**	PQ637390
		B5	***Bacillus velezensis* 94%**	PQ637391
		B8	***Streptomyces sp.* 96%**	PQ637571
		B9	***Bacillus tequilensis* 97%**	PQ637392
		B10	***Bacillus sp.* 97%**	PQ637610

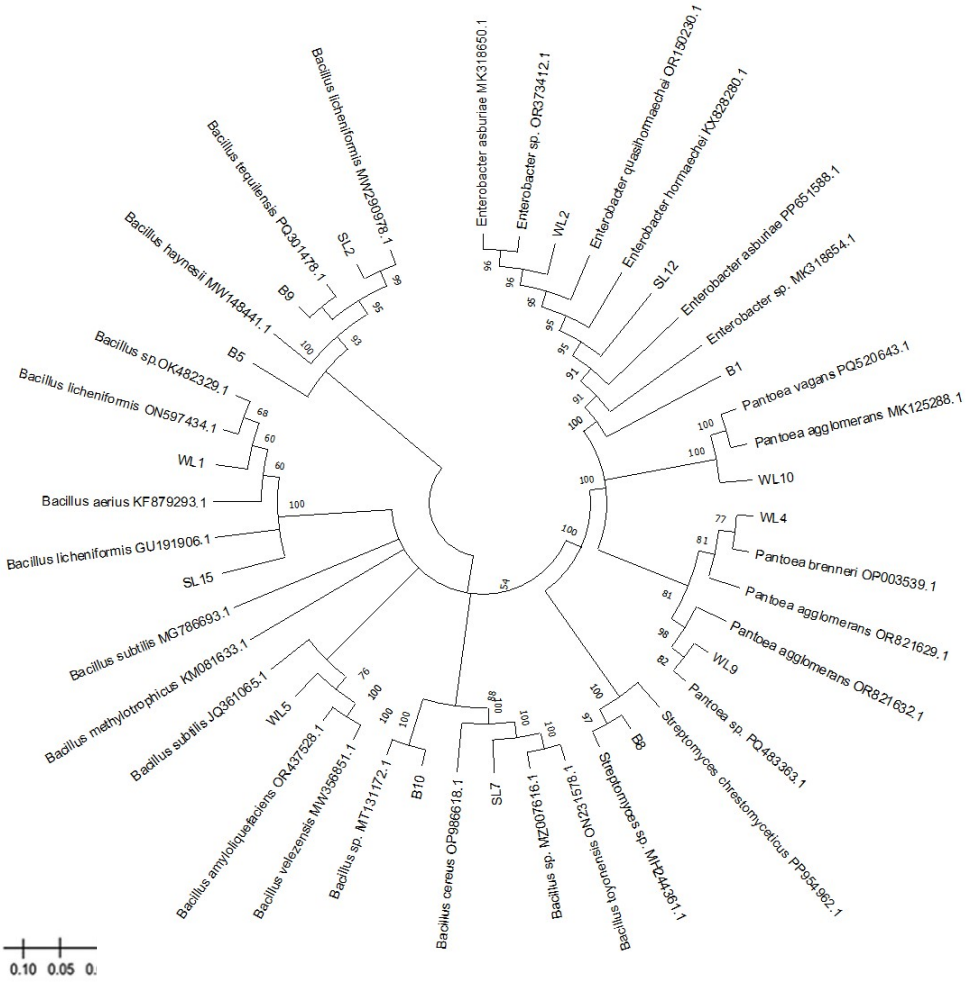

Figure: 6.3.1: Phylogeneticc analysis of 16S rRNA sequences of bacterial strains with reference sequences from NCBI. Alon refers to bacteria isolated from *Aloe longibracteata* plants whereas AL is the sequences from *Aloe lettyae* isolates. The identity of the bacterial isolates is available in table 2. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Evolutionary distances were computed using the p-distance method, and the phylogenetic tree was constructed using the Neighbour-Joining method in MEGA 11 (Tamura et al., 2021).

6.3.2 Plant growth promotion and Enzymes activity

All the bacterial endophytes isolated from *O. bullata* showed the potential to fix atmospheric nitrogen, with the highest activity observed from the isolates of winter leaf samples (WL9, WL2, WL5) and the isolates from the bark (B9, B8, B5). Moreover, 9 endophytes (SL15, SL12, SL7, B10, B8, B5, B1, WL2 and WL5) displayed significant ability to solubilise inorganic phosphate with clear zone on the Pikovskaya medium (Figure 6.3.2).

All bacterial endophytes isolated from *O. bullata* leaves in summer showed hydrogen cyanide activity (Figure 6.3.2) and only 6 bacterial endophytes (SL7, SL2, B8, B5, WL9, WL2 and WL5) were able to produce siderophore (Figure 6.3.2). The ability to produce IAA with and without the precursor tryptophan was observed across all isolates, however increasing tryptophan concentration

from 0.1 to 0.5% resulted in increased bacterial ability to produce IAA from 10 to 80µg/ml (Figure 6.3.3). Eighty per cent of the *O. bullata* bacterial endophytes were able to synthesize two or more of the following enzymes: lipase, protease, amylase, and cellulose (Figure 6.3.4). The isolate from the bark (B8) produced all these enzymes, with amylase showing the highest activity. Additionally, the isolate from the summer collection of leaves (SL7) tested positive for all enzyme activities, with the highest activity observed for protease. Although the isolate B1 tested positive for three out of the four enzymes, it had the highest protease activity among all isolates. The isolate from the winter collection of leaves (WL2) exhibited the maximum activity with cellulose (Figure 6.3.4).

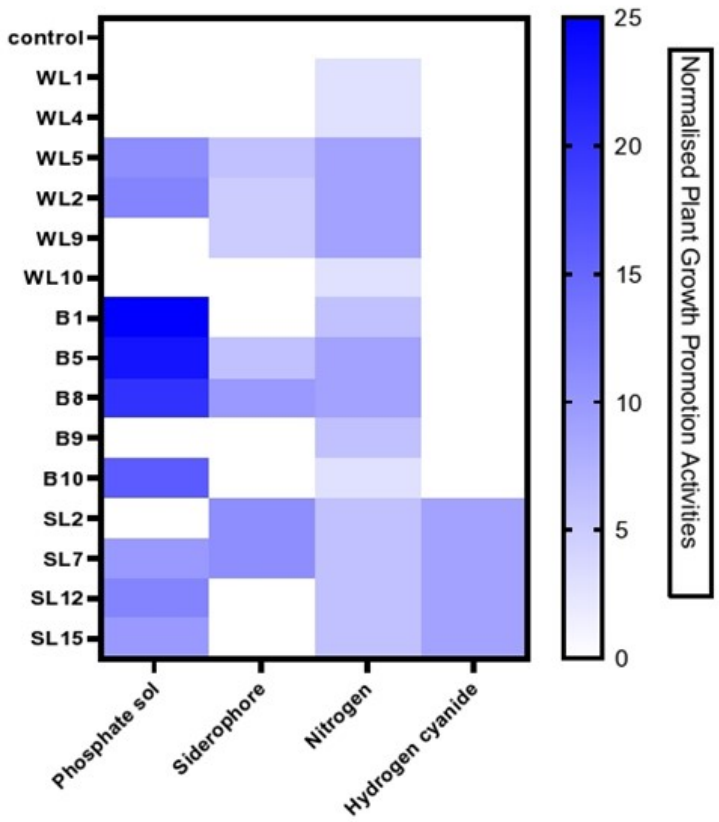

Figure 6.3.2: Phosphate solubilisation, siderophore production, Hydrogen cyanide production and nitrogen fixation activity of *Ocotea bullata* bark and seasonal leaf endophytic bacterial strains.

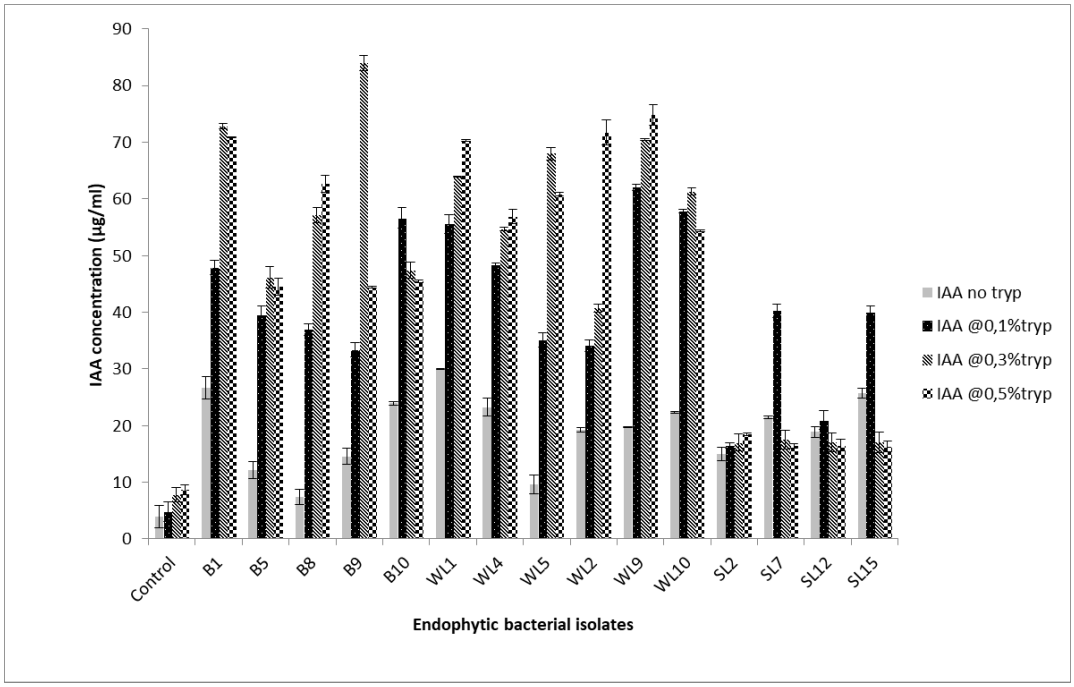

Figure 6.3.3: Quantitative production of IAA by endophytic bacterial strains with and without tryptophan. The control is without bacterial inoculation. Identity of the bacterial isolates is available in Table 6.3.1. The IAA concentration was measured at 530 nm. The data represents the mean values ± standard deviation (SD) of triplicates. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p < 0.05$), indicating significant differences in IAA production between conditions.

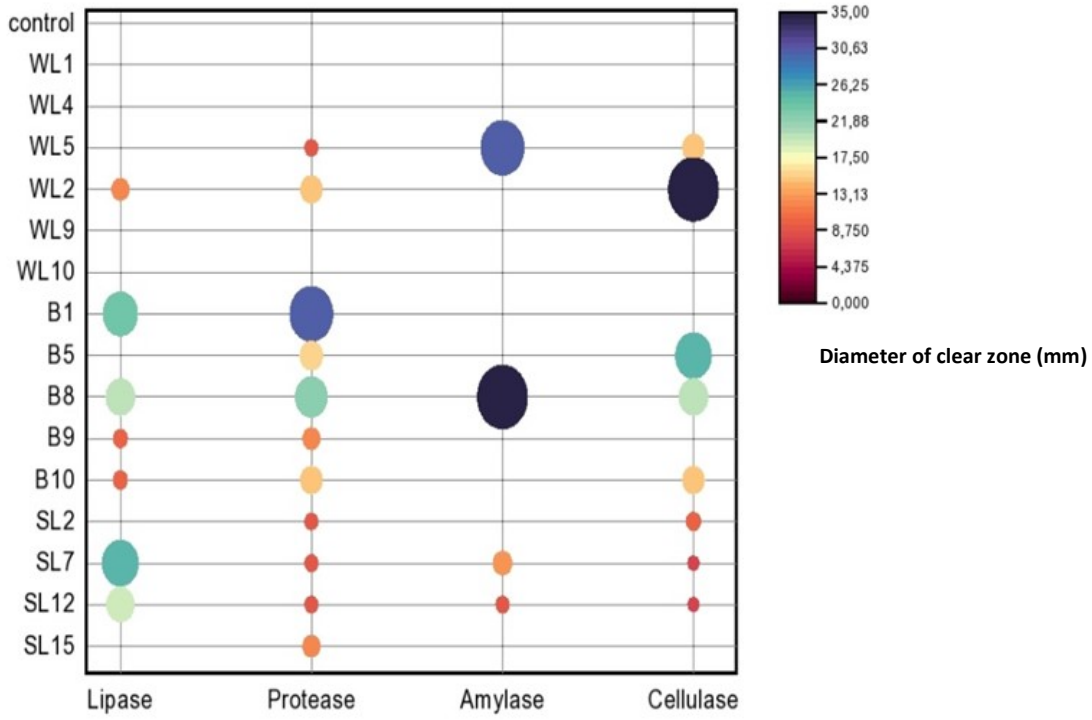

Figure 6.3.4: Extracellular enzymatic activities of bacterial endophytes.

Table 6.3.2: Antimicrobial activity representing the endophytes that possess antimicrobial activity against human pathogens, +=mild, ++=moderate. +++= very significant, and - = no activity

Sample number	Given name	*S. marcescens*	*E. faecalis*	*E. coli*	*C. krusei*	*K. pneumoniae*	*M. luteus*
1	SL2	+	+	+	-	+	+
2	SL7	+++	+++	+	+	++	+++
3	SL12	-	+	-	-	-	-
4	SL15	+	++	+	-	-	-
5	WL1	-	-	-	-	-	-
6	WL2	++	+	+++	+++	++	++
7	WL4	+	-	-	+	-	-
8	WL5	-	-	+	-	-	-
9	WL9	++	-	+	+	-	-
10	WL10	-	-	-	-	-	-
11	B1	++	+++	++	+++	+++	++
12	B5	+	-	-	-	-	-
13	B8	+	-	-	-	-	+

14	**B9**	++	-	-	-	-	-
15	**B10**	+	-	-	-	+	-

6.3.3 Antimicrobial activity

The endophytes isolated from *O. bullata* were subjected to preliminary antimicrobial screening against five human pathogens and yeast, as detailed in Table 6.3.2. Out of the 15 bacterial endophytes isolated, 12 displayed antimicrobial activities; however, only three endophytes were capable of inhibiting the growth of all six tested pathogenic strains. These three promising bacterial endophytes were selected for further analysis. The secondary metabolites crude extracts from these three endophytes, which demonstrated significant antimicrobial activity, were then extracted and analysed at different concentrations to determine their Minimum Inhibitory Concentration (MIC). The crude extract of endophyte WL2 showed MIC values of 500 µg/mL for *S. marcescens*, 750 µg/mL for *E. faecalis*, 500 µg/mL for *E. coli*, 750 µg/mL for *C. krusei*, 250 µg/mL for *K. pneumoniae*, and 750 µg/mL for *M. luteus* (Figure 6.3.5). Similarly, the crude extract of endophyte SL7 exhibited MIC values of 250 µg/mL for *S. marcescens*, 500 µg/mL for *E. faecalis*, 500 µg/mL for *E. coli*, 1000 µg/mL for *C. krusei*, 750 µg/mL for *K. pneumoniae*, and 500 µg/mL for *M.* luteus (Figure 6.3.6). Lastly, the crude extract of endophyte B1 demonstrated MIC values of 750 µg/mL for *S. marcescens*, 500 µg/mL for *E. faecalis*, 500 µg/mL for *E. coli*, 250 µg/mL for *C. krusei*, 250 µg/mL for *K. pneumoniae*, and 250 µg/mL for *M. luteus* (Figure 6.3.7). The significant MIC for a positive control, *streptomycin* was exhibited at 2.5µg/ml, 10µg/ml and 2.5µg/ml for *S. marcescens, E. faecalis, E. coli, C. krusei, K. pneumoniae*, and *M. luteus* respectively. These results underscore the potent antimicrobial activities of the crude extracts from WL2, SL7, and B1, highlighting their potential as sources of novel antimicrobial compounds.

6.3.4 Seed germination

The most effective antimicrobial agents, WL2, SL7, and B1, which also demonstrated satisfactory plant growth promotion potential during screenings, were selected to investigate their impact on the germination of tomato seeds when used as bio-primers at different dilutions. The study revealed that seed priming with these bacterial endophytes from *O. bullata* (WL2, SL7, and B1) significantly enhanced the germination of tomato seeds, with germination completing on or before 10 days (Table 6.3.3). Specifically, biopriming with WL2 at a 1:3 dilution-initiated germination with two seeds sprouting within a day, followed by SL7 at a 1:1 dilution, which resulted in the germination of one seed within a day. Other treatments began germination on the second day of the experiment, except for SL7 at a 1:5 dilution, which started on the fifth day but resulted in five seeds germinating

simultaneously (Table 6.3.3). Additionally, different dilutions of the bacterial endophytes influenced the initiation of germination, with 1:1 and 1:3 dilutions showing results comparable t o the control across all three endophytic bacterial isolates. The results indicated that biopriming treatments significantly impacted all tested germination parameters (Table 6.3.4), with the highest seed germination velocities observed predominantly at higher dilutions (1:3 and 1:5) for all investigated bacterial endophytes.

6.3.5 NMR metabolite profiling of the endophytic bacterial strains (WL2, SL7 and B1) from *O. bullata*.

The metabolites from 3 (SL7, WL2 and B1) of the selected Plant growth promoters, antimicrobial agents and potential stimulants for seed germinated were profiled for the type of metabolites they produce using 1H NMR. The 1 HNMR spectra of the 3 bacterial endophytes from *O. bullata* showed similar occurrences of metabolites with the highest intensities observed in the SL7 isolate (Figure 6.3.8). The bacterial endophytes WL2 and B1 confirmed the presence of the Osmolytes; glycine, betaine and proline but the bacterial endophytes SL7 was missing betaine (Table 6.3.5). Despite the commonality in osmolytes production, the three bacterial endophytes showed completely different patterns of metabolites (Table 6.3.5). The metabolites, tyrosine, Gluconate, lactate and glutamate with potential use in drug developments were found in bacterial endophyte WL2. The bacterial endophyte SL7 showed the presence of metabolites; allantoin, malonate, and tyrosine. And lastly the endophytic bacteria B1 showed the presences of the metabolites, betaine, acetate and indole-3-acetate.

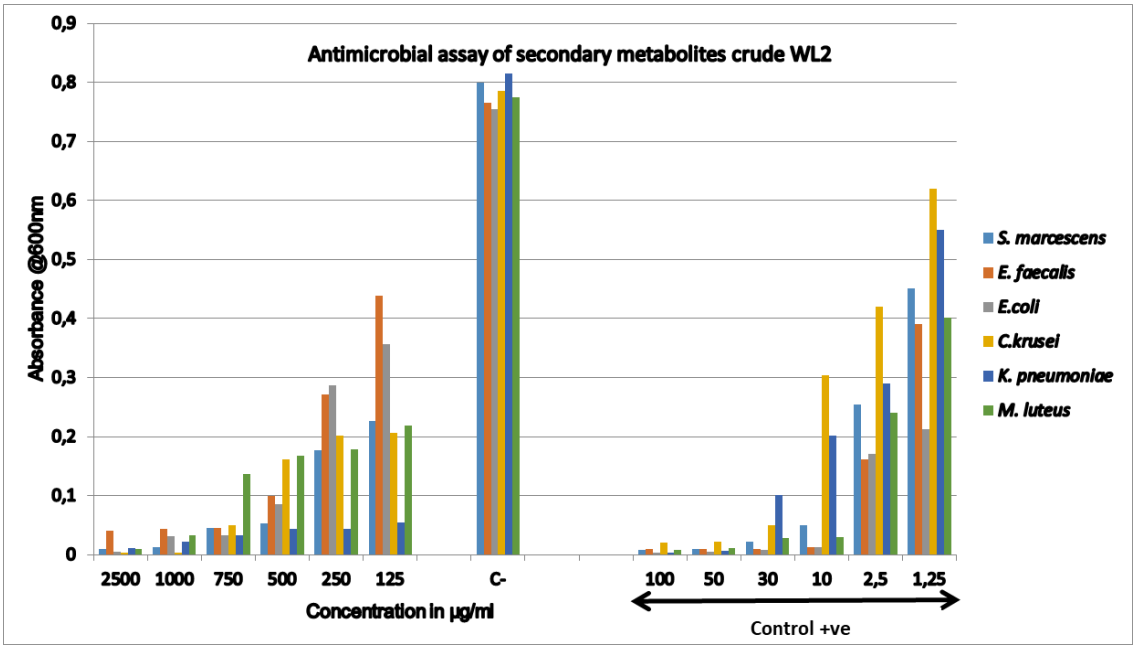

Figure 6.3.5: Antimicrobial analysis of WL2 crude secondary metabolites extract. Antimicrobial activity of endophyte WL2 was analysed against human pathogens. Here, negative control (C-) Muller-Hinton broth and DMSO at 1:1 ratio inoculated with human pathogen with 1.5x10^8 Colony Forming Units (CFU/ml), positive control (C+ve) Muller-Hinton broth and Dimethyl sulfoxide (DMSO) at 1:1 ratio inoculated with human pathogen with 1.5x10^8 CFU/ml and antibiotic streptomycin-1.25,2.5,10,30,50 and 100µg/ml.

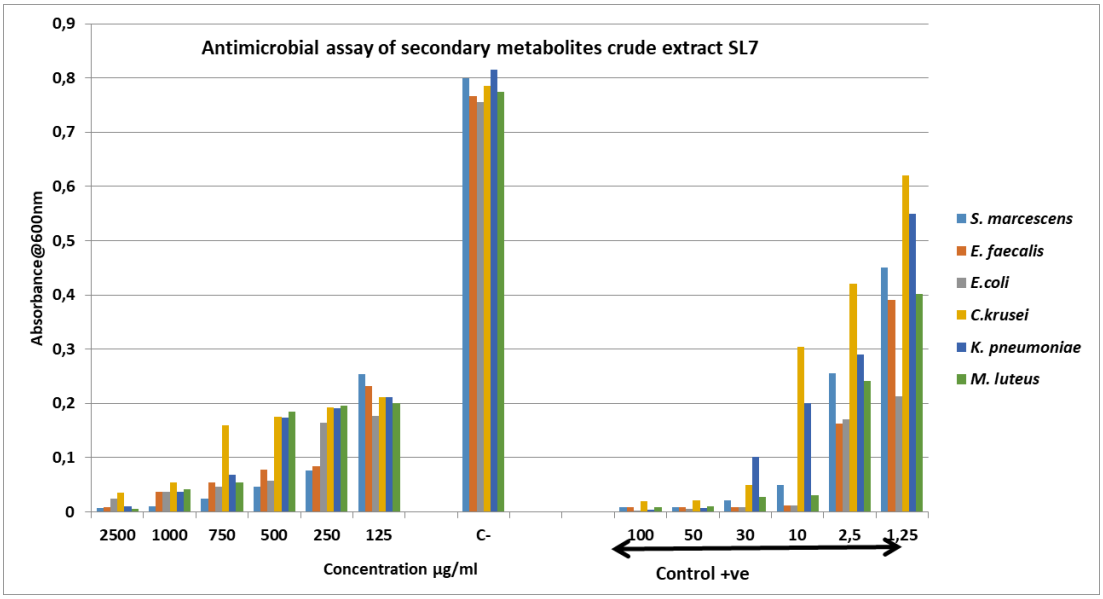

Figure 6.3.6: Antimicrobial analysis of SL7 crude secondary metabolites extract. Antimicrobial activity of endophyte WL2 was analysed against human pathogens. Here, negative control (C-) Muller-Hinton broth and DMSO at 1:1 ratio inoculated with human pathogen with 1.5x108 Colony Forming Units (CFU/ml), positive control (C+ve) Muller-Hinton broth and Dimethyl sulfoxide (DMSO) at 1:1 ratio inoculated with human pathogen with 1.5x108 CFU/ml and antibiotic streptomycin-1.25,2.5,10,30,50 and 100µg/ml.

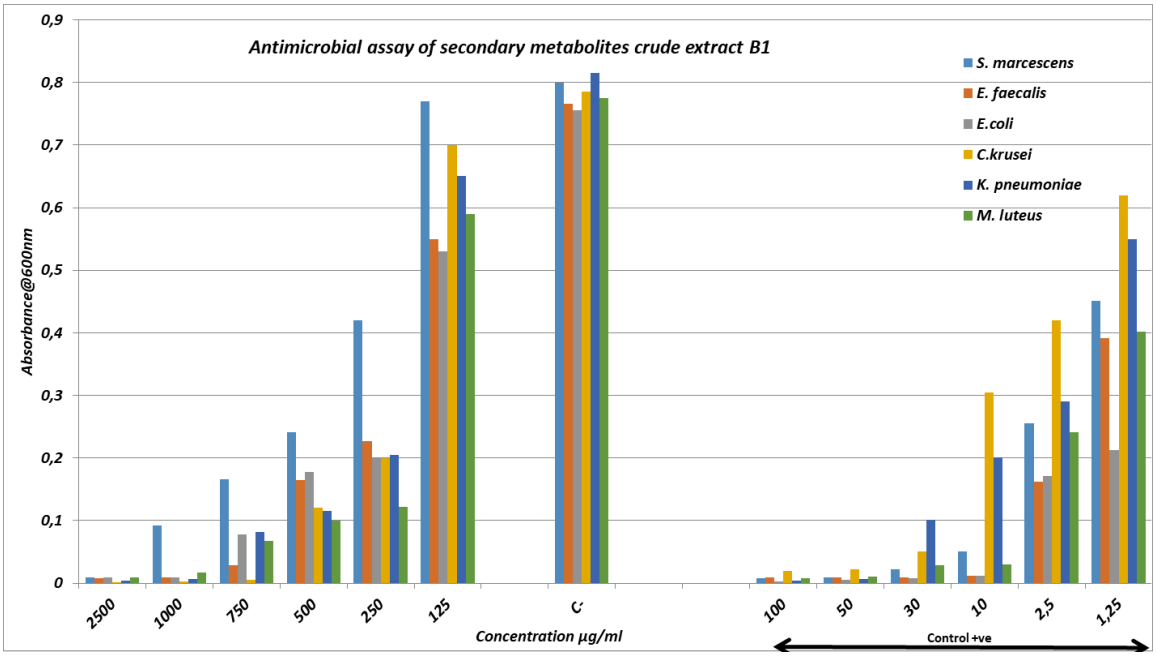

Figure 6.3.7: Antimicrobial analysis of B1 crude secondary metabolites extract. Antimicrobial activity of endophyte WL2 was analysed against human pathogens. Here, negative control (C-) Muller-Hinton broth and DMSO at 1:1 ratio inoculated with human pathogen with 1.5x108 Colony Forming Units (CFU/ml), positive control (C+ve) Muller-Hinton broth and Dimethyl sulfoxide (DMSO) at 1:1 ratio inoculated with human pathogen with 1.5x108 CFU/ml and antibiotic streptomycin-1.25,2.5,10,30,50 and 100µg/ml.

Table 6.3.3: Effect of endophyte bio priming on the germination of tomato seeds at 25ºC per day

Experimental crop	Isolate	Treatment	Seeds per lot	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Solanum lycopersicum (Tomato)	Control	-	20	0	1	1	1	1	3	6	1	1	1
	WL2	1:1	20	0	0	0	1	1	4	6	4	2	2
		1:3		2	2	2	2	4	2	3	2	1	0
		1:5		2	2	2	2	4	2	3	2	1	0
	SL7	1:1	20	1	1	2	0	4	2	3	2	3	2
		1:3		0	0	2	2	2	3	4	2	2	3
		1:5		0	0	0	0	4	6	6	2	1	1
	B1	1:1	20	0	1	1	1	1	1	1	5	5	4
		1:3		0	1	5	4	4	3	2	1	0	0
		1:5		2	4	4	4	4	1	1	0	0	0

Table 6.3.4: Effect of endophyte bio priming on tomato seed germination incubated at 25ºC. FGP, final germination percentage, SVI, seedling vigour index, MGT, mean germination time, GV, germination velocity.

Experimental crop	Isolate	Treatment	FGP (%)	SVI	MGT	GV
Solanum lycopersicum (Tomato)	**Control**		80	480	1.26	0.79
	WL2	1:1	100	550	1.39	0.72
		1:3	100	700	0.94	1.06
		1:5	100	500	0.94	1.06
	SL7	1:1	100	750	1.22	0.82
		1:3	100	720	1.36	0.74
		1:5	100	400	1.32	0.76
	B1	1:1	100	200	1.44	0.69
		1:3	100	450	0.89	1.12
		1:5	100	500	0.71	1.41

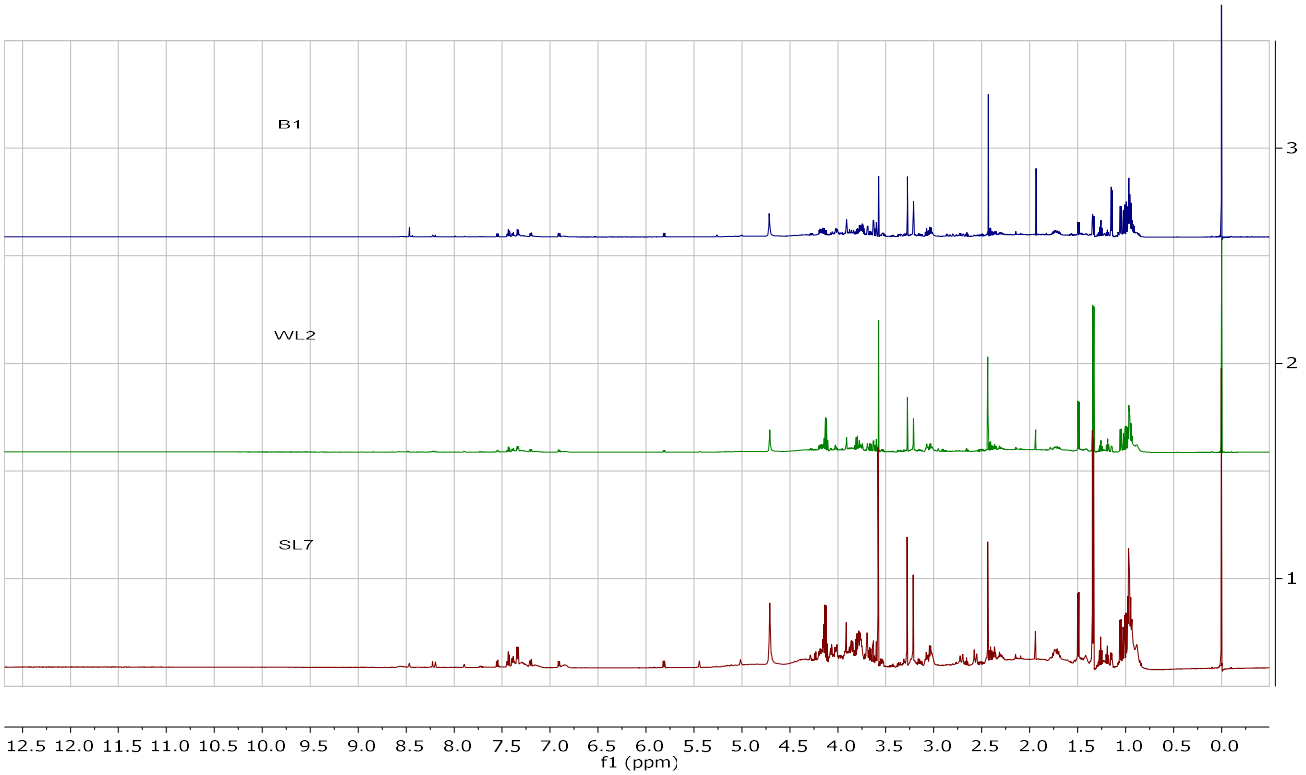

Figure 6.3.8: 1H NMR spectra of *O. bullata* endophytic bacteria

Table 6.3.: Chenomx assisted annotations of differentially produced metbaolites of WL2, SL7 and B bacterial endophytes isolated from *O. bullata*

Endophytic bacteria	Metabolite	Chemical shift (ppm)
WL2	Proline	4.10(dd), 3.40(m), 2.30(m), 2.02(m)
(*Enterobacter asburiae*)	β Alanine	3.20(t), 2.60(t)
	Gluconate	4.10(d), 4.02(t)
	Glutamate	3.73(dd), 2.32(m)
	Glycine	3.56(s)
	Lactate	1.31(d), 4.09 (dd)
	Leucine	3.71 (dd), 1.64(m), 0.93(t)
	Valine	3.61(d), 2.22(m), 1.02(d)
	Tyrosine	7.16(d), 6.87(d), 3.92(dd)
	Succinate	2.39(s)
	3Hydroxyisovalerate	2.35(s), 1.25(s)
	Betaine	3.89(s), 3.25(s)
SL7	Allantoin	1.91(s)
(*Bacillus toyonensis*)	Alloisoleucine	4.10(dd), 3.40(m), 2.30(m), 2.02(m)
	Aspartate	3.20(t), 2.60(t)
	Proline	3.89(s), 3.25(s)
	Choline	4.04(m), 3.49(t), 3.19(s)
	Glycerate	8.44(s)
	Glycine	3.56(s)
	Indole-3-acetate	7.60(d), 7.50(d), 7.20(m), 3.60(d)
	Malonate	1.31(d); 4.09 (dd)
	Myo-inositol	3.71 (dd); 1.64(m); 0.93(t)
	Pyruvate	2.36(s)
B1	Acetate	1.91(s)

(***Enterobacter sp***)	Proline	4.10(dd), 3.40(m), 2.30(m), 2.02(m)
	β Alanine	3.20(t), 2.60(t)
	Betaine	3.89(s), 3.25(s)
	Choline	4.04(m), 3.49(t), 3.19(s)
	Formate	8.44(s)
	Glycine	3.56(s)
	Indole-3-acetate	7.60(d), 7.50(d), 7.20(m), 3.60(d)
	Lactate	1.31(d); 4.09 (dd)
	Leucine	3.71 (dd); 1.64(m); 0.93(t)

6.4 Discussion

The potential to uncover unique and beneficial microbial communities from medicinal plants that vary with environmental conditions can be leveraged in sustainable agriculture by developing biofertilizers and biocontrol agents from these endophytes (Tripathi et al., 2022). This approach reduces reliance on chemical inputs, enhancing crop productivity and health naturally (Tripathi et al., 2022). *Ocotea bullata* possesses several valuable medicinal properties and has been used traditionally to treat various human ailments (Zschocke et al., 2000, Kowalski and Staden, 2001, Ogundajo et al., 2018). Although no microbial studies have been conducted on *O. bullata,* medicinal plants of *Lauraceae* family have been reported to harbour diverse endophytic bacteria that can significantly influence their growth, health, and therapeutic properties (Ho et al., 2012). Based on a detailed literature search of research articles, this is the first report on the Plant growth promotion, antimicrobial and metabolite profiling of endophytes isolated from *O. bullata*. Thus, this study analysed the microbial communities of *O. bullata* leaves across two distinct seasons (summer and winter) and from the bark, screening their potential for promoting plant growth also profiling their metabolites.

In this study, 15 putative bacterial endophytic strains were isolated from the leaves and bark of *O. bullata* growing in Pietermaritzburg, KwaZulu-Natal province. The isolation of these strains during different seasons revealed a diverse range of bacterial species, including *Bacillus, Enterobacter, Pantoea*, and *Streptomyces*. Such diversity aligns with previous studies that have demonstrated the presence of various endophytic bacteria in different plant tissues and environmental conditions, emphasizing the influence of seasonal variations on microbial communities (Santoyo et al., 2016; Hardoim et al., 2015). The presence of *Pantoea* in winter isolates suggest a possible adaptation to seasonal environmental conditions, as this genus include species that can thrive in cooler temperatures and offer bio control against phytopathogens (Walterson and Stavrinides, 2015). The identification of *Streptomyces* among the bark isolates highlights the unique adaptability and ecological significance of this genus as an endophyte. *Streptomyces* species are well-known for their ability to produce a wide range of bioactive compounds, including phytohormones like IAA, as well as antimicrobial metabolites that may contribute to the host plant's defense and stress tolerance (Vurukonda et al., 2018). Their presence in *O. bullata* bark suggests a potential role in mediating plant-microbe interactions and promoting host resilience in challenging environments.

The plant growth promotion (PGP) activities of these bacterial strains were characterised including extracellular enzyme production, antimicrobial action, Indole-3-acetate (IAA), Hydrogen cyanide

production, siderophore production, phosphate solubilisation and nitrogen fixation (Figure 6.3.2;6.3.3 and 6.3.4). Phosphorus and nitrogen are two of the essential elements necessary for plant development and growth with their deficiency mostly limiting the growth of crops (Bilal et al., 2021, Dokwal et al., 2021, Vats et al., 2021). This study demonstrated that all the isolated bacterial endophytes from *O. bullata* leaves and bark have the potential to fix nitrogen making it usable by plants. However, only 9 of the 15 endophytic bacteria were able to solubilise phosphate. These 9 endophytes were identified as the members of the *Bacillus* and *Enterobacter* bacteria, aligning with Agboola et al. (2023) who reported enhanced germination and vigour in maize and cowpea seeds primed with *Enterobacter*. Additionally, Mohamed et al. (2018) observed a high phosphate solubilisation index of 2.8 to 3.2 in *Bacillus* endophytes isolated from tomatoes.

Notably, only isolates from leaves collected in summer produced hydrogen cyanide (Figure 6.3.2). This observation is particularly important because hydrogen cyanide play a significant role in biocontrol by inhibiting root pathogens, thereby promoting plant health and growth naturally. In this study the significant production of Hydrogen cyanide by the isolated collected in summer is in agreement with the findings by Emad- Fadoul et al. (2023) which confirmed that the production of hydrogen cyanide in plants tend to be higher in warmer temperatures.

The production of extracellular enzymes is common among most reported plant growth promoters (Ismail et al., 2021, Ferrari et al., 1993), and in this study, 13 of the 15 isolated bacterial endophytes produced 1 to 4 of the tested enzymes. Remarkably, among all isolated bacterial endophytes from *O. bullata*, the isolate from the bark (B8) and two isolates from summer-collected leaves (SL7 and SL12) produced all four tested extracellular enzymes important for plant growth promotion.

Although this study presents the very first records regarding PGP screening on an endangered member of the *Lauraceae* family, *O. bullata*, Tung et al. (2022) reported the antibacterial potential of endophytic bacteria isolated from the medicinal plant *Lisea cubeba* of the *Lauraceae* family. In this study, 3 (WL2, B1 and SL7) of the thirteen bacterial endophytes of *O. bullata* that showed activity against more than 1 of the tested human pathogens were investigated further for their Minimal Inhibition Concentration (MIC).

The antimicrobial activities observed in the crude extracts from endophytic bacteria WL2, SL7, and B1 (Figure 6.3.5, 6.3.6 and 6.3.7) align with previously published research on the potential of endophytes as sources of novel antimicrobial compounds. The MIC values of WL2, ranging from 250 µg/mL to 750 µg/mL, demonstrate significant inhibitory effects against pathogens such as *S. marcescens*, *E. faecalis*, *E. coli*, *C. krusei*, *K. pneumoniae*, and *M. luteus*. These findings are

consistent with studies by Yadav et al. (2018) and Jasim et al. (2016), which highlight the broad-spectrum antimicrobial properties of endophytic bacteria isolated from various plant species.

Similarly, the SL7 extract exhibited MIC values between 250 µg/mL and 1000 µg/mL, with notable efficacy against *S. marcescens* and *M. luteus*. This is in agreement with research by Tanvir et al. (2019), who reported that endophytic bacteria from medicinal plants show significant antimicrobial activity due to their ability to produce diverse bioactive metabolites. Furthermore, the strong activity of B1, with MIC values ranging from 250 µg/mL to 750 µg/mL, particularly against *K. pneumoniae*, *C. krusei*, and *M. luteus*, supports findings by Semenzato et al. (2024) that endophytic bacteria from tree bark possess potent antimicrobial properties. Comparative analysis with the positive control, streptomycin, underscores the significant, though relatively lower, efficacy of the crude extracts, which suggests the potential for these endophytes to serve as alternative antimicrobial agents. This is corroborated by studies such as those by Ramesh et al. (2018) and Mousa and Raizada (2013), who have documented the antimicrobial potential of endophytes as promising candidates for antibiotic development.

The observed enhancement in germination of tomato seeds upon priming with the endophytic bacterial from *O. bullata*(WL2, SL7 and B1) aligns with previous research on the positive effects of endophytic bacteria on seed germination and plant growth where studies by Cardarelli et al. (2022) and Bhattacharyya and Jha (2012) have shown that bacterial endophytes can improve seed germination rates, root development, and overall plant vigour through mechanisms such as nitrogen fixation, phosphate solubilisation, and production of phytohormones like IAA.

The specific finding that biopriming with WL2 at a 1:3 dilution-initiated germination within a day, followed by SL7 at a 1:1 dilution, supports the notion that certain bacterial concentrations can optimize germination processes. This is consistent with the work of Makhanye et al. (2021), who reported that optimal dilutions of bio stimulant can significantly enhance seed germination and early seedling growth. The delayed but robust germination observed with SL7 at a 1:5 dilution, where five seeds germinated simultaneously on the fifth day, further underscores the importance of appropriate bacterial concentration in achieving desirable germination outcomes.

This study's findings that biopriming treatments significantly affected all tested germination parameters (Table 6.3.4), with higher seed germination velocities predominantly at higher dilutions (1:3 and 1:5), are in agreement with reports by Verma et al. (2019) and Ghosh et al. (2022). These researchers highlighted that endophytic bacteria can enhance seed germination and seedling vigour through mechanisms such as the production of siderophores, which enhance iron availability, and the synthesis of extracellular enzymes that facilitate nutrient acquisition. Overall, this result

underscores the potential of using bacterial endophytes from *O. bullata* as bio-primers to enhance seed germination and seedling development in agricultural practices. Moreover, these results contribute to a growing body of literature emphasizing the role of endophytes in sustainable agriculture, where they can serve as natural biostimulants, reducing the need for chemical inputs and promoting healthier crop growth (Compant et al., 2010; Hardoim et al., 2015).

The 1H NMR profiling of three bacterial endophytes (SL7, WL2, and B1) isolated from *O. bullata* revealed distinct metabolite fingerprints, despite overlapping production of key osmolytes. Notably, glycine, betaine, and proline, well-documented osmoprotectants that enhance stress tolerance and plant growth promotion Mishra et.al (2022) were detected in WL2 and B1. While SL7 lacked betaine, the isolate displayed the highest signal intensities, and it was uniquely associated with allantoin, malonate, and tyrosine. Allantoin, a purine catabolite, is known for its wound-healing and anti-inflammatory properties (Sánchez-Murcia et al., 2019), while malonate is involved in microbial energy metabolism and may act as an antimicrobial compound (Booth et al., 2011). WL2 distinguished itself by producing gluconate, lactate, glutamate, and tyrosine, all of which have medicinal potential. Glutamate and tyrosine serve as precursors for neurotransmitters and have implications in neuropharmacology (Fernstrom & Fernstrom, 2007), while gluconate has applications in wound healing and mineral supplementation (Gouider et al., 2021). The endophyte B1 produced betaine, acetate, and indole-3-acetate (IAA), the latter being a widely recognized phytohormone that promotes seed germination and root elongation (Spaepen et al., 2007). These findings suggest that although the endophytes share functional traits in osmolyte production, their unique metabolite profiles highlight potential niche-specific roles and pharmacological relevance, underscoring their biotechnological potential in agriculture and drug discovery.

6.5 Conclusion

This chapter successfully isolated and characterized 15 bacterial endophytes from the leaves and bark of *O. bullata*, revealing a diverse range of species including *Bacillus*, *Enterobacter, Pantoea,* and *Streptomyces*. The diversity and abundance of isolates differed between tissues, with leaves yielding 10 distinct isolates compared to 5 from bark. Seasonal variation was also evident, with summer samples producing 12 isolates, of which 8 displayed multiple plant growth-promoting traits, compared to winter samples that produced only 7 isolates with lower overall activity. Notably, the isolates WL2, SL7, and B1 were particularly effective, demonstrating both antimicrobial activity and the ability to enhance tomato seed germination. Metabolite profiling using 1H NMR identified key osmolytes and secondary metabolites, highlighting the potential applications of these endophytes in agriculture and medicine. These patterns suggest that both tissue type and season strongly influence the composition and functional potential of *O. bullata* endophytes, with summer leaf isolates showing the highest plant growth-promoting potential. Future research should focus on elucidating the mechanisms underlying these seasonal and tissue-specific variations, as well as exploring the practical applications of these endophytes as biofertilizers and biocontrol agents to promote sustainable agriculture. In parallel, comprehensive metabolite profiling of both *O. bullata* and its associated bacterial endophytes is essential to assess whether these microbes can act as alternative sources of key bioactive compounds. This strategy is particularly crucial given the endangered status of *O. bullata*, as it presents a sustainable alternative to direct plant harvesting, thereby supporting both conservation and biotechnological innovation.

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Chapter 7

General conclusion and recommendations

7.1 Summary of key findings

The literature review in this study highlighted the considerable potential of endophytes in producing a vast array of bioactive compounds with potential applications in medicine, agriculture, and biotechnology. Endophytes are known to synthesize compounds with ant diabetic, antioxidant, anticancer, and antimicrobial properties. However, research on bioactive compound production from endophytes specifically those associated with endangered plant species like *Ocotea bullata* and *Aloe lettyae* remains largely unexplored. This chapter underscored the urgency of investigating endophytic communities in endangered plants, as the ecological, medicinal, and conservation significance of these species suggests they could harbour unique microorganisms that may not be found elsewhere.

Understanding the diversity and functionality of these endophytes offers a dual benefit: uncovering novel resources for sustainable applications while contributing to the conservation of the host plants themselves. By identifying bioactive metabolites and microbial allies within these species, we can promote sustainable resource use that minimizes harvesting pressures on endangered plants. Such approaches not only support the preservation of these plants in their native ecosystems but also open new avenues for cultivating these endangered species in controlled environments, fostering their reintroduction and helping to secure their populations for the future. This conservation-focused research reinforces the importance of protecting these unique plants and their microbial ecosystems, ultimately contributing to broader biodiversity conservation efforts.

This foundation encourages further research into diverse endangered plant species to understand the potential of endophytes species. By exploring endophyte diversity across plant types and environmental contexts, future studies can establish an interdisciplinary approach involving microbiology, ecology, and conservation biology. This direction could reveal how endophytes contribute to ecosystem resilience and sustainability, as well as their biotechnological potential.

The first research chapter in this study investigated a comparative analysis of the metabolite profiles and endophytic communities in *A. lettyae* (endangered) and *A. longibracteata*. The chapter demonstrated the richness of metabolites with plant growth-promoting and bioactive properties. These metabolites, along with the phylogenitically diverse endophytes, suggest that both *Aloe* species could contribute to sustainable agriculture, and drug development post safety evaluations.

Additionally, the similarities between the species in shared metabolites and microbial communities point to the possibility of using *A. longibracteata* as a model or surrogate for conservation-oriented applications aimed at *A. lettyae*.

Future research should further investigate the specific roles of individual metabolites and endophytes in agricultural and medicinal applications, with a focus on evaluating their safety and efficacy. Conducting thorough safety evaluations is essential to ensure that any medicinal applications derived from these endangered plants are both effective and safe. This step is crucial for responsible development, as it prevents premature claims of medicinal value that could lead to overharvesting and put additional pressure on these already vulnerable species. By carefully evaluating safety and efficacy before promoting medicinal uses, we can expand the potential for these endangered plants to contribute to medicine in a controlled, sustainable way. This means developing methods to harness the beneficial compounds of the plants without directly impacting their populations in the wild. For instance, if useful endophytes or metabolites can be isolated and produced in laboratory settings, we could harness their medicinal properties without relying on wild harvesting. This controlled approach allows us to benefit from the unique resources of the plants while protecting them in their natural habitats, supporting long-term conservation efforts. Additionally, studies could explore how *A. longibracteata* might support *A. lettyae* conservation through endophyte transfer or metabolite-based biotechnological uses, enabling sustainable utilization of these compounds without impacting endangered populations

The second research chapter highlighted the core microbiomes of *A. lettyae* and *A. longibracteata*, dominated by *Bacillus* and *Enterobacter* species. These endophytes exhibit promising plant growth-promoting activities, including phosphate solubilisation, nitrogen fixation, and production of growth-promoting phytohormones like IAA, positioning them as beneficial agents in conservation efforts. By enhancing nutrient availability and promoting healthy growth, these endophytes can support the survival and resilience of endangered plants. Their presence enables plants to thrive under challenging conditions, which can be crucial for re-establishing endangered populations in the wild or in restoration sites. In this way, these endophytes not only promote plant health but may also contribute directly to conservation by enhancing the adaptability and survival rates of endangered species. The resilience of certain Bacillus strains to environmental stressors like salinity suggests that these endophytes could support the re-establishment of *A. lettyae* in diverse habitats, enhancing adaptability and survival. In future, confirmation of the conservation potential of these endophytes by greenhouse and field trials are necessary, through inoculation experiments. These studies could evaluate the efficacy of *A. longibracteata* endophytes in improving *A. lettyae* growth and stress tolerance in degraded or alternative habitats, aiding its reintroduction and expanding its

distribution. Additionally, further research could explore whether this approach could be applied to other endangered plants, making endophyte-based treatments a scalable strategy for conservation.

The comparative analysis in the third research chapter of this study revealed significant differences in metabolite composition between *O. bullata* bark and leaves. The leaves, with varied beneficial metabolites, emerged as a sustainable alternative to the bark for harvesting metabolites with human health importance. Seasonal analyses also showed that leaves collected in summer had higher metabolite composition than those collected in winter, offering strategic guidance for optimal and sustainable harvesting practices.

Future research should prioritize refining extraction techniques from leaves to maximize yields of desired metabolites. Additionally, bioassays assessing the efficacy of specific metabolites across applications in medicine and agriculture would further inform sustainable use practices. Studies could also evaluate the metabolic responses of *O. bullata* in different environmental conditions to inform conservation strategies and resilience under environmental change.

The final experimental chapter documented the isolation and functional profiling of diverse endophytic bacteria from *O. bullata*. The isolated strains exhibited plant growth-promoting properties such as nitrogen fixation and phosphate solubilisation, along with extracellular enzyme production, making them strong candidates for sustainable agriculture. Notably, isolates like WL2, SL7, and B1 that demonstrated antimicrobial activity and supported tomato seed germination, highlighting their biocontrol and biofertilizer potential.

Given these findings, further research should investigate the seasonal dynamics of microbial communities in *O. bullata* and assess the potential of the endophytes as biofertilizers or biocontrol agents on a larger scale. Developing bioformulations from selected endophytes could create eco-friendly agricultural solutions that reduce reliance on chemical fertilizers and pesticides. Additionally, exploring the genomic basis for these beneficial traits of these endophytes could enable bioengineering for optimized agricultural applications.

The insights garnered from each chapter collectively underscore the multifaceted role that endophytes play in enhancing plant resilience, health, and adaptability. The study of endophytic bacteria and metabolites in *A. lettyae* and *O. bullata* reveals how these microorganisms can be pivotal in promoting conservation and sustainable resource utilization. By leveraging the unique traits of endophytes, this research demonstrates a potential for reducing pressures on endangered species while fostering biodiversity through strategic conservation and sustainable agricultural practices. These findings lay a foundation for further studies aimed at expanding this knowledge

and applying it in practical contexts, such as developing biofertilizers, biocontrol agents, and novel bioactive compounds.

7.2 Limitations of the current study

While this study successfully advanced understanding of the endophytic communities and metabolite profiles of *A. lettyae* and *O. bullata*, certain limitations were encountered. For *A. lettyae*, greenhouse validation experiments to test whether endophytes from *A. longibracteata* could support its growth in alternative environments were not possible. This was primarily due to the unavailability of seeds from suppliers and the species' inherently slow growth. Although collection permits were obtained, harvesting from *A. lettyae* populations was necessarily limited to ensure minimal disturbance. This restricted the availability of seeds and sufficient plant material for greenhouse propagation experiments, as overharvesting would conflict with conservation priorities for this endangered species. The comparative approach with *A. longibracteata* therefore provided the most practical and conservation-aligned strategy. In the case of *O. bullata*, greenhouse experiments were similarly not feasible because the species grows slowly, its seeds are highly perishable, and no commercial sources exist. Finally, although the study confirmed that *O. bullata* leaves contain metabolites with reported medicinal attributes, the work did not extend to experimental validation of these compounds as functional substitutes for bark-derived metabolites. These limitations highlight the challenges of conducting research on endangered species, while also underscoring the value of the present findings as a foundation for future applied studies.

7.3 Recommendations for future research

1. Functional validation in field conditions: A significant future direction involves testing the effectiveness of beneficial endophytes in field conditions, especially for *A. lettyae*. This could validate the potential for re-establishment in alternative habitats, which is critical for conservation efforts.
2. Mechanistic studies of metabolites and enzyme activities: Future studies should explore the biochemical pathways responsible for beneficial traits, such as IAA production or phosphate solubilisation. Understanding these pathways will deepen insight into plant-microbe interactions and support the development of optimized applications for sustainable agriculture.
3. Exploring seasonal variability and sustainable harvesting: Research on seasonal effects on endophyte communities and metabolite profiles could yield more sustainable harvesting practices, particularly for endangered medicinal plants. Such research can contribute to guidelines for optimal harvest timing and reduce overharvesting pressures on end

4. angered species.
5. Development of endophyte-based biofertilizers and biopesticides: Based on promising endophyte traits identified in this study, future research could focus on formulating biofertilizers and biopesticides. These eco-friendly alternatives could reduce chemical inputs in agriculture and contribute to sustainable crop management.

In conclusion, this research has demonstrated the promise of endophytic bacteria and plant metabolites in promoting sustainable practices across agriculture, conservation, and biotechnology. The findings affirm the potential of endophytic communities to support endangered plant species and to offer novel resources for practical applications. Continued research in this area could build on the foundation established here, leading to new conservation strategies and innovative applications of plant-associated microorganisms. Together, these efforts may help safeguard biodiversity and promote the sustainable use of plant resources.

Appendix

Supplementary Table

Supplementary Table1: PERMANOVA and PERMDISP analysis between Aloe species

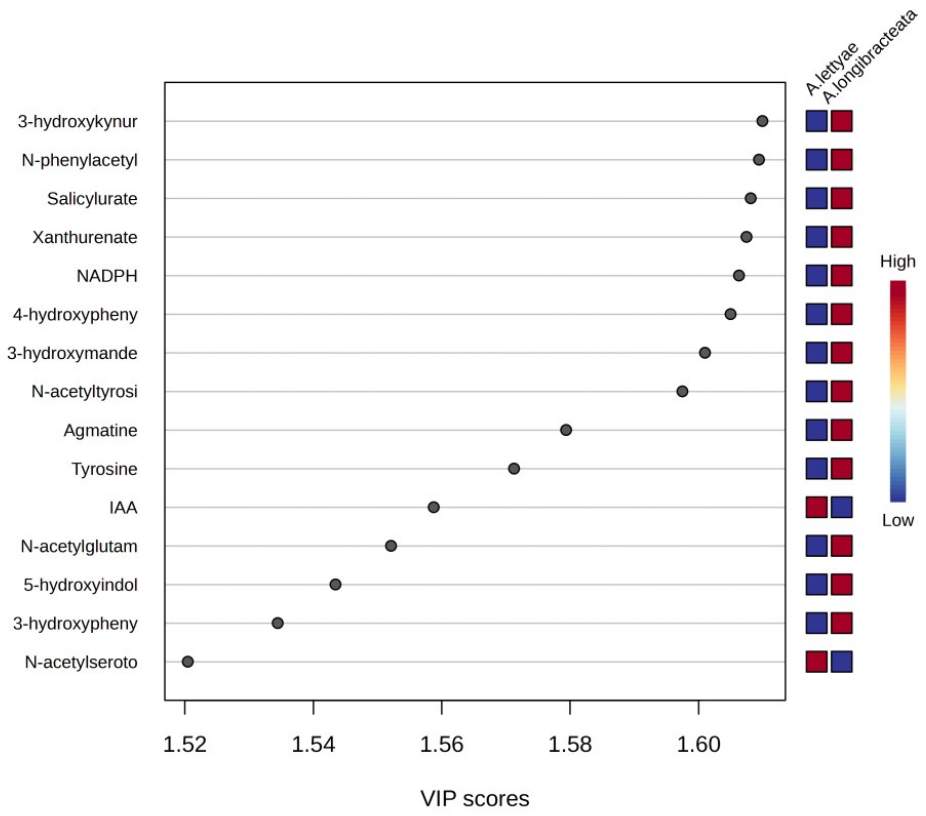
Factor	*PERMANOVA*			*PERMDISP*
	DF	*R2 (%)*	*P*	*P*
Species type	*1*	*19.947*	*0.001*	*0.058*
Residue	*17*	*80.053*		

Statistical value is based on P < 0.05, DF, degree of freedom.

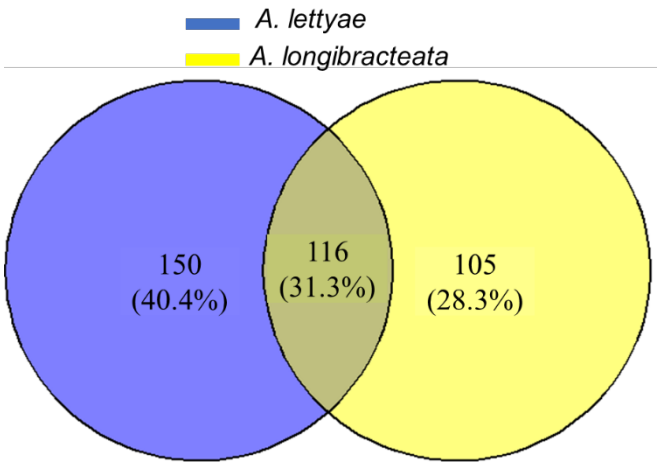
Table 2: Predicted metabolic functions of *A. lettyae* and *A. longibracteata*

Subclass 1	Frequency	Percentage
Cofactor, -Carrier, -and-Vitamin-Biosynthesis	61	15.44
Amino-Acid-Biosynthesis	36	9.11
Aromatic-Compound-Degradation	35	8.89
Nucleoside-and-Nucleotide-Biosynthesis	30	7.59
Carbohydrate-Biosynthesis	21	5.32
Carbohydrate-Degradation	20	5.06
Fatty-Acid-and-Lipid-Biosynthesis	17	4.30
Cell-Structure-Biosynthesis	16	4.05
Amino-Acid-Degradation	15	3.80
Carboxylate-Degradation	14	3.54
Fermentation	11	2.78
Secondary-Metabolite-Biosynthesis	11	2.78
Amine-and-Polyamine-Degradation	10	2.53
C1-Compound-Utilization-and-Assimilation	10	2.53
Super pathways	10	2.53
Inorganic-Nutrient-Metabolism	9	2.28
Nucleoside and Nucleotide Degradation	9	2.28
Secondary-Metabolite-Degradation	6	1.52
TCA-cycle	6	1.52
Tetrapyrrole-Biosynthesis	6	1.52
Amine-and-Polyamine-Biosynthesis	5	1.27
Generation-of-Precursor-Metabolites-and-Energy	5	1.27
Alcohol-Degradation	4	1.01
Fatty-Acid-and-Lipid-Degradation	4	1.01
Glycolysis	3	0.76
Other-Biosynthesis	3	0.76
Polyprenyl-Biosynthesis	3	0.76
Aromatic-Compound-Biosynthesis	2	0.51
Degradation/Utilization/Assimilation---Other	2	0.51
Nucleic-Acid-Processing	2	0.51
Pentose-Phosphate-Pathways	2	0.51
Aldehyde-Degradation	1	0.25
Aminoacyl-tRNA-Charging	1	0.25
Electron-Transfer-Chains	1	0.25
Energy-metabolism	1	0.25
Interconversion	1	0.25
Metabolic-Regulator-Biosynthesis	1	0.25
Respiration	1	0.25

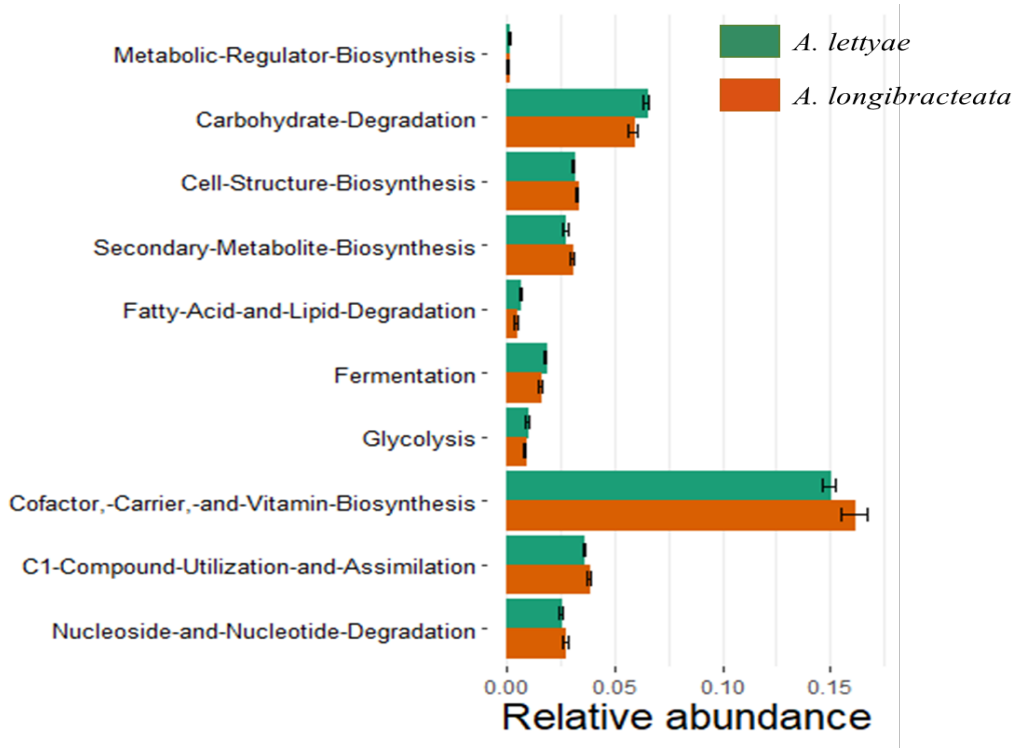
Supplementary Figure



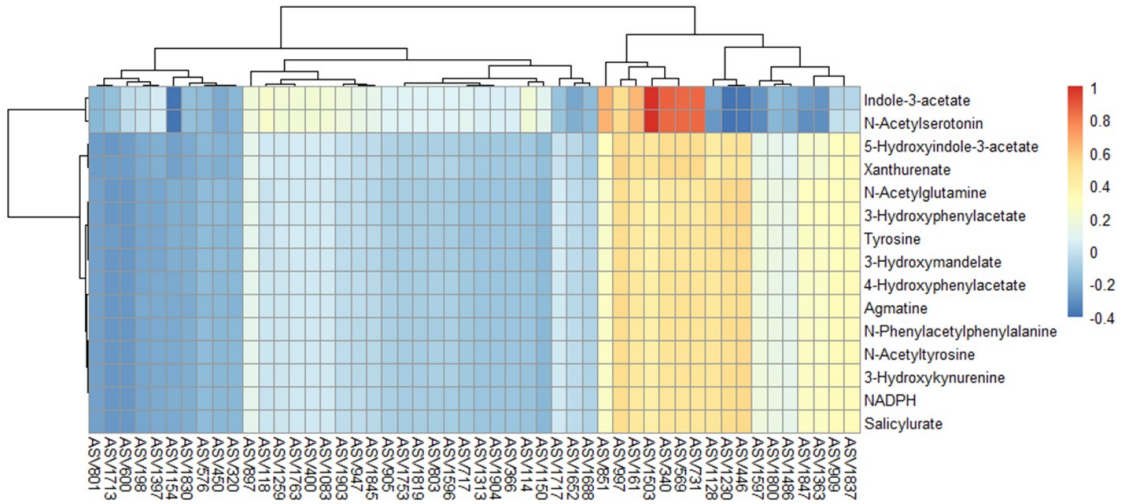
Supplementary Fig. S1. Variables Important in Projection (VIP) scores representing metabolites between the two Aloes. The metabolites are listed from top to bottom as; 3-Hydroxykynurenine, N-Phenylacetylphenylalanine, Salicylurate, Xanthurenate, NADPH, 4-Hydroxyphenylacetate, 3-Hydroxymandelate, N-Acetyltyrosine, Agmatine, Tyrosine, Indole-3-Acetate (IAA), N-acetylglutamine, 5-Hydroxyindole-3-acetate, 3-Hydroxyphenylacetate and N-Acetylserotonin. The selected metabolites were those with VIP > 1. The heatmap on the right displays red and blue boxes, indicating, respectively high and low abundance ratios of the corresponding metabolites in *A. lettyae* and *A. longibracteata.*



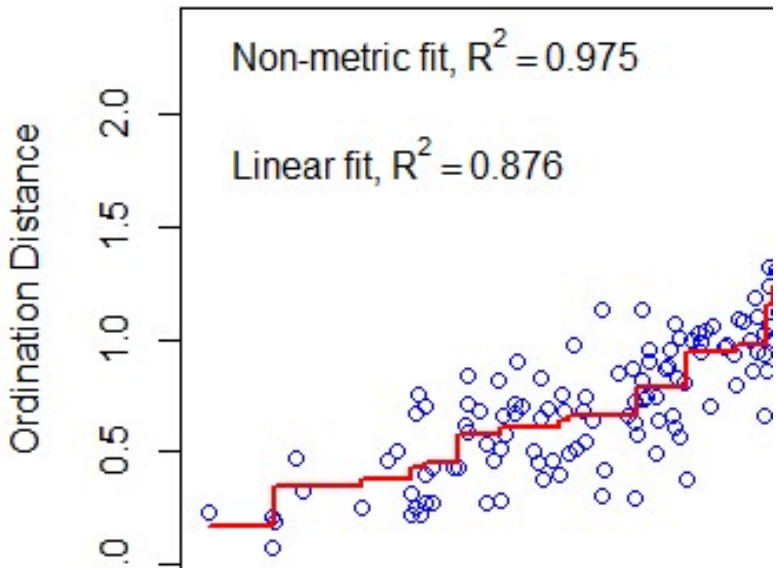
Supplementary Fig. S2. Shared and unique bacterial communities between the *Aloe* plant species



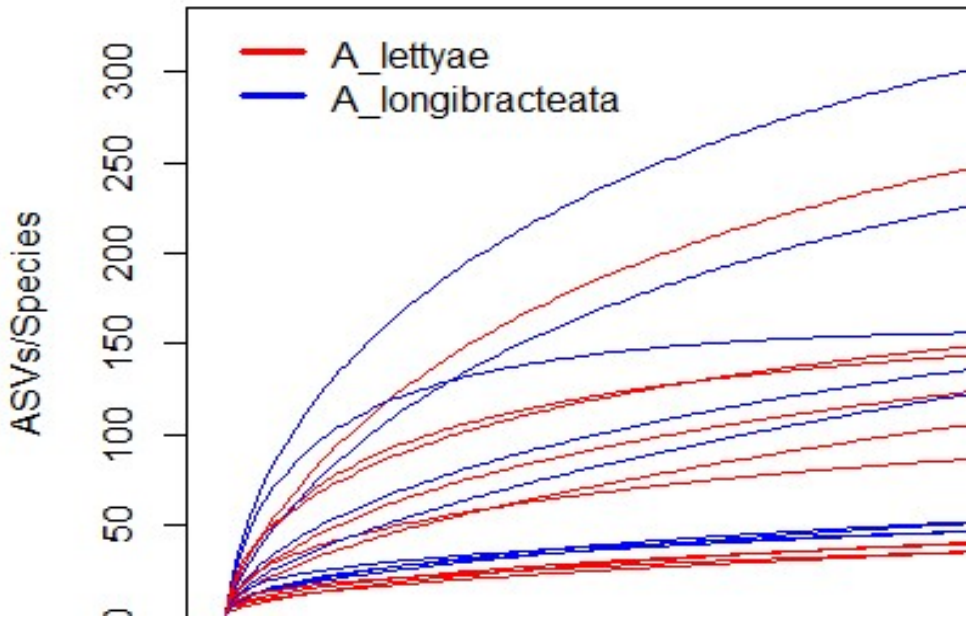
Supplementary Fig. S3. Relative abundance of the endophytic bacterial functions in the Aloe species



Supplementary Fig. S4. Correlation between differential metabolites and top 50 dominant endophytic bacteria of *Aloe* plants. The red and blue colours indicate positive and negative correlations, respectively. The colour depth indicates strong (deep colour) and weak (light colour) and correlation $P < 0.05$.



Supplementary Fig. S5. Stress plot for the nonmetric multidimensional scaling (NMDS) plot. The stress run was at 0.1941.



Supplementary Fig. S6. Rarefaction curve for sequencing reads from *Aloe* species.

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Marokane-Radebe *et al. AMB Express* (2024) 14:122
https://doi.org/10.1186/s13568-024-01784-3

AMB Express

ORIGINAL ARTICLE Open Access

Metabolomic profiling and 16 S rRNA metabarcoding of endophytes of two *Aloe* species revealed diverse metabolites

Cynthia Marokane-Radebe[1], Adekunle Raimi[1], Stephen Amoo[1,2] and Rasheed Adeleke[1*]

Abstract

Aloe species are often used interchangeably for medicinal and cosmeceutical applications, presenting a challenge to the biological efficacy consistency of some herbal preparations. Sustainable production of high-quality commonly used medicinal plants remains a limitation for commercialisation. Thus, this study investigated the potential for plant substitution by examining bacterial endophytes capable of producing similar host plant secondary metabolites. The metabolite profiles and endophytic bacterial communities of endangered *Aloe lettyae* were compared with those of *Aloe longibracteata* using nuclear magnetic resonance spectroscopy and 16 S rRNA gene sequencing. Only 15 metabolites were significantly different between *A. lettyae* and *A. longibracteata* based on metabolite concentrations. However, both plants' functionality and potential application remain comparable. Phytohormones, including indole-3-acetate and 5-hydroxyindole-3-acetate, were more concentrated in *A. lettyae* than *A. longibracteata*. Metabolites such as tyrosine, allantoin, and myo-inositol, with human health benefits, were annotated in both species. *Aloe lettyae* harboured a phylogenetically diverse bacteria community compared to *A. longibracteata*, with a higher richness of bacterial species, indicating a likelihood of diverse metabolic capabilities among the bacteria. Dominant endophytes, including *Bacillus*, *Comamonas*, and *Pseudomonas*, possess enzymes contributing to various metabolic pathways. The enzymes have the potential to impact the synthesis, or breakdown of plant metabolites, consequently influencing the overall metabolic composition of the host plant. Therefore, this study supports the interchangeability of *A. lettyae* and *A. longibracteata* due to their ability to produce similar metabolites, and although the *Aloe* species exhibit phylogenetically diverse endophytic communities, the feasibility of utilizing their endophytes as producers of secondary metabolites remains viable.

Introduction

In medicine, biotechnology and pharmacology, there is a controversy about the nature and type of bioprospecting approach that can be adopted for medicinal plants to guide drug discovery (Saslis-Lagoudakis et al. 2012). Efficacy and sustainable production of traditionally used medicinal plants are two of the most important concepts driving research interests in the bioprospecting of medicinal plants. For instance, perceived efficacy is strongly influenced by cultural biases linked to ethnobotanical data, especially when similar plants are used for the same purpose in different regions (Saslis-Lagoudakis et al. 2011). However, over-reliance on ethnobotanical data may not present complete information about the potential benefits and bioactivity of certain medicinal plants (Saslis-Lagoudakis et al. 2011). This means ethnobotanical data comparisons of medicinal plants could only be used as a guide

*Correspondence:
Rasheed Adeleke
rasheed.adeleke@nwu.ac.za

Full list of author information is available at the end of the article