Molecular Insights Into Bacterial Community Structure of Sunflower Endosphere

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DECLARATION

I, the undersigned, hereby declare that the thesis titled *Molecular Insights into Bacterial Community Structure of Sunflower Endosphere* submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Natural and Agricultural Sciences, School of Biological Sciences is my work; that, it has not been submitted in part or entirely for the award of any degree or examination in any University, and that all the sources I have used or quoted have been indicated and acknowledged by complete referencing.

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DEDICATION

This work is dedicated to the God Almighty, the giver, and source of wisdom. To my parents, lovely son, and the repose of the soul of my brother, Late Mathias Gbadebo Adeleke.

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GENERAL ABSTRACT

Environmental threats posed by chemical fertilizer application on farmlands and their persistence in the food-value chain are worrisome to stable healthy living; hence, the need for satefy precautions. Promisingly, agricultural biotechnology approaches in ensuring food security sustainably through bioinoculants exploration from copious plant microbes as an alternative to agrochemicals are helping to meet increasing world food demand. Therefore, this study aims to give insights into diverse endophytic bacteria, their isolation, and inoculation to enhance sunflower yield. In this study, a next-generation sequencing approach was employed in determining the bacterial community structure and function in the sunflower roots and stem endosphere. Also, culture-dependent methods were employed to isolate endophytic bacteria from sunflower plants, and their interdependence in enhancing sunflower yield was evaluated under greenhouse experimental conditions. Isolation, identification and characterization of the bacterial isolates were achieved by cultural and molecular techniques. Out of the bacteria isolated, 70% were Gram-positive and 30% Gram-negative with dominant genera, *Bacillus, Stenotrophomonas,* and *Pseudomonas*. The bacterial strains exhibited a positive response to almost all the plant growth-promoting tests, such as exopolysaccharide, indole-3-acetic acid (IAA), phosphate solubilization, siderophore, and enzyme and ammonia production. However, 3 bacterial isolates, namely, *B. cereus* T4S, *S. indicatrix* BOVIS40, and *S. maltophilia* JVB5 with high plant growth-promoting characteristics were selected for the greenhouse experiments. Each bacterial inoculum was standardized to 0.5 at OD_{600}. The singularly, co-and-combined bacterial inoculation yielded a significant difference in sunflower yield parameters above and below ground compared to uninoculated. Bacterial inoculation enhances root development, shoot weight, head weight, and early flowering before seed production at maturation. The inoculated pot experiment with singularly, dually, and combined inoculation (T4S+JVB5+BOVIS40) significantly affected the sunflower taproot length, root length, lateral root number, and root weight (dry and fresh), shoot weight (dry and fresh), head weight (dry

and fresh), and plant wet weight compared to uninoculated plants. The genomic assessment of strain T4S, BOVIS40, and JVB5 revealed notable genes involved in siderophore synthesis, chemotaxis and attachment, nitrogen fixation, IAA production, phosphate solubilization, and transport. Secondary metabolite gene clusters, nonribosomal peptides siderophore (NRPS) were detected in the bacteria genome. The 16S rRNA gene amplicon sequencing analysis of the DNA extracted from the growing and flowering sunflower roots and stem revealed the bacterial community structure using Nephele microbial bioinformatics platform. The bacterial distribution in the samples across sites based on the Bray-Curtis distance matrix for Principal Coordinate Analysis (PCoA) plot was performed on CANOCO software, while bacterial relative abundance at phylum, class, and genus level was generated from Shinyheatmap. Alpha (α) diversity (i.e., diversity within the sites) was analyzed using PAST software with selective indices of Pileou evenness, Simpson, Shannon, and observed species abundance were estimated to reveal the diversity and richness of the endophytic bacterial community in each sample using Kruskal-Wallis test. The rarefaction curve was obtained based on these metrics. The β-diversity was represented using PCoA based on the Bray-Curtis distance matrix, while the variance or significant difference in the bacterial diversity of individual samples tested was achieved by analysis of variance similarities. The correlations of environmental variables to the distribution of endophytic bacteria across the plant organs from different sites were performed using canonical correspondence analysis. The predictive functions of the bacterial endophytes were performed on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, and the resulting output was obtained at levels 1, 2, and 3. The relative abundance of the predicted bacterial functions was generated on Shinyheatmap at level 1. The diversity indices revealed a significant difference at the phylum level in the flowering sample across the sites, while in growing sunflower, the bacterial diversity was significantly different at phylum and class levels. The predictive functional analysis revealed that, at level 3 selection, bacteria exhibit tryptophan, nitrogen, and siderophore biosynthesis. The results obtained

showed that endosphere bacterial community structure varied in sunflowers at different growth stages and their functions across the sites suggest their impact on sunflowers in modulating endosphere competence. In conclusion, this study provides salient information on the combination of culture-dependent and culture-independent techniques in the identification of agriculturally important endophytic bacteria associated with sunflower sourced from Itsoseng and Lichtenburg, South Africa. More importantly, the detection of novel genes involved in phytohormone synthesis in the genome of the bacteria strains, using singularly or combined inoculation, suggest their ability in enhancing sunflower growth under greenhouse experiment. Furthermore, these bacteria strains can be explored in the formulation of bio-inoculant, and their incorporation in agriculture can be promising in plant cultivation under nutrient-limiting soil.

KEYWORDS: Bio-inoculants, endosphere, *Helianthus annuus*, metagenome, microbial community

1.0 CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction to this chapter

Agricultural biotechnology for food production needs to be intensified through research and innovation, to avert food insecurity, hunger, and malnutrition (Aloo et al., 2019, Sammauria et al., 2020). Therefore, designing long-term and acceptable techniques for maximum actualization to meet food demand for the world population, which is envisaged to be doubled by 2050, become important (Berners-Lee et al., 2018, Khare et al., 218, Lobo et al., 2019). As a matter of urgency, care must be taken to resolve underlining factors such as climate change, drought, pests, and disease attack, erratic rainfall, poor soil fertility, inaccessibility to pest resistance hybrid cultivars, economic instability, poor storage facilities, government policies, and restrained land use, affecting food production and supply (Mbodj et al., 2018).

Over time, agrochemical applications to improve agricultural productivity has been employed in crop production but poses threats to ecosystems, which include soil degradation and acidification, changes in soil profiling, reduction in crop yields, anoxygenic conditions in water bodies, and loss in biodiversity (Kopittke et al., 2019, Martínez-Hidalgo et al., 2019). Promisingly, the use of environmental microbes with novel functional attributes is of major interest as a substitute to agrochemicals (chemical fertilizer and pesticides) in developing ecologically friendly agriculture (Kour et al., 2020). Bioinoculant applications on farm crops can persist longer in the food chain without any toxic effects. Therefore, microbial biotechnology and applications can help achieve sustainable development goals (SDG) through research-driven concepts on some economic crops such as maize, sorghum, wheat, cowpea, soybean, etc. as well as sunflower plants which are the focus of this study.

Sunflower (*Helianthus annuus* L.) belongs to the family Asteraceae, a native of North America, now cultivated in many countries of the world, such as South Africa (Marvey, 2009). Sunflower is one of the fastest-growing edible oil-producing seed crops in the world and ranked third after soybean and rapeseed (Zehra et al., 2020). The plant exhibits high tolerance to drought and can survive drought better compared to other crops, such as wheat, sorghum, maize, and soybean that require high moisture for survival. Sunflower can strive well in nutrient-rich soil, warm weather based on direct access to sunlight, and soil pH level and optimum temperature (Ebrahimian et al., 2019). At the early stage of sunflower germination, irrigation, and fertilizer application to boost crop productivity are usually employed in sunflower cultivation in South Africa. During plant development, the well-developed tap root system of sunflower enhances nutrient uptake from soil to alleviate water stress at the early growing and flowering stage for optimum yield. In South Africa, the use of chemical fertilizer in subsistence and commercial farming to enhance sunflower productivity is high and some farmers have adopted crop rotation systems by interchanging planting of crops such as maize, sorghum, drum beans, and soybeans with sunflower to prevent soil nutrient loss (Jordaan et al., 2019). Sunflower cultivation varies from one country to another due to weather and soil conditions that support their propagation. In South Africa, because of weather conditions, land preparation and planting of sunflowers usually commence around October/November to February during summer.

In 2020, sunflower production in South Africa was estimated to be 13% higher (765,000 tonnes) compared to 681,000 tonnes production in 2019. Free State and North West Provinces are regarded as prominent sunflower-producing areas in South Africa. The nutritional profiling and medicinal values of sunflower, composites, and derivable products have been reported in review documentation (Ebrahimian et al., 2019). In terms of sunflower production in Africa,

South Africa is ranked first with more than 99% of her human population relying on sunflower oil as edible vegetable oil for domestic and industrial use, thus suggesting that boosting sunflower production in Africa can help avert food insecurity. Also, the provision of adequate agricultural inputs to farmers and implementation of farmer-oriented programmes can help alleviate poverty levels, famine, and food shortage with lots of opportunities in advancing agro-engineering and Africa's economy. Despite the current demand for sunflower and its products in homes and industries, the research to boost their yield using bioinoculants is yet to be fully explored.

The endosphere of sunflower and other plants is known to harbor diverse microbial communities with multifunctional activity in enhancing their growth and immunity for higher productivity (Fouda et al., 2021). The word endosphere denotes specific regions in plants where endophytic microbes live and complete their life cycle without eliciting pathological effects on the host plants (Khalil et al., 2021, Wang et al., 2020b). Endosphere is said to be a subdivision of the rhizosphere owing to high chemoattractants signal deposition mediating microbial infiltration from the root-soil environment into the plant roots and becoming endophyte (Watanabe et al., 2020). Bacterial interactions with the endosphere of medicinal plants have contributed to plant growth and immunity in diverse ways (Eljounaidi et al., 2016, Khalil et al., 2021), but to date, little information is available on the exploration of bacterial endophytes for improved sunflower productivity in Southern Africa. Hence, this research aims to create insights into sunflower endophytic bacterial community structure using culture-independent and culture-dependent methods to predict the novel genes underlining their importance in agriculture.

The advancement in next-generation sequencing (NGS) has enabled researchers in the field of microbiology, ecology, agronomy, and environmental sciences to explore the diverse microbes in the plant endosphere in the agricultural bio-economy (Uzoh and Babalola, 2018). The conventional microbial isolation techniques, complemented with culture-independent methods, can best validate the ability of the identifiable microbes' in *in vivo* and *in vitro* experiments. The culture-independent approach allows the discovery of genes underlining plant-microbe interactions, thus resolving the theory of endosphere ecology and consequent revelation of novel functional attributes of unculturable endophytic bacteria in an ecosystem (Almeida and De-Martinis, 2019). The culture-independent technique has widened the research scope in determining microbial ecology and coevolution with plants with specific functions. The genomic sequencing of agriculturally important microbes can help predict novel genes involved in phytohormone synthesis, biocatalysis, bacterial attachment, and biocontrol activity (Wu et al., 2018). Despite the genomic elucidation of endophytic microbes, expertise in handling and managing huge sequence datasets, ineffective screening techniques, design complexity, time-cost in data analysis, high cost of sequencing, and presence of singleton reads form major challenges. The use of NGS in determining bacterial community structure (BCS) and functions have been reported in maize, wheat, sorghum, and soybean (Wasai and Minamisawa, 2018, Shen et al., 2019b, Fadiji et al., 2020b, Kuramae et al., 2020), nevertheless, no information is available on the bacteria community structure in sunflower from Southern Africa. Consequently, the combination of culturing and non-culturing methods in the analysis of BCS in the sunflower endosphere, further identification, and application under greenhouse experimental conditions has proven to be efficient in improving sunflower yield, which affirms the importance of this study.

1.2 Problem statement

The conventional approach to farming using agrochemicals on farmlands to improve agricultural crop productivity threatens the ecosystem. Excess usage of agrochemicals has caused a negative shift in ecological balancing in terms of changes in biodiversity structure, soil degradation, and eutrophication, and anoxygenic conditions in water bodies, nutrient loss, soil acidification, and water pollution. In addition, the persistent use of chemical fertilizers, channeling into plant-food chain systems has caused human health issues and other related disease conditions (Sharma and Singhvi, 2017). How can this menace facing agricultural productivity be mitigated? One of the best measures is through agricultural biotechnology that involves harnessing copious agriculturally important microbes from plants, instead of agrochemicals usage to ensure a stable ecosystem and food production (Grieve et al., 2019).

A biotechnological approach in the study of sunflower endophytic microbes can be promising in exploring their potential as a core arsenal in agriculture for bioinoculant formulation. Harnessing bioincentives from endophytic microbes with multiple functions in agriculture has yielded success in terms of plant growth and improved crop yield sustainably. Therefore, this research aims to explore bioproducts from the bacteria associated with sunflower by testing their effects via inoculation on sunflower plants.

1.3 Justification of the study

South Africa is one of the oilseed sunflower-producing countries in the world. On a global scale, bridging researcher-farmer ideas into sunflower cultivation, as in other oilseed crops can boost sunflower oil production as an economical source of food, for use at home and in industries. Although, isolation of copious agriculturally important bacteria associated with medicinal plants is known, nevertheless, the molecular insights into endophytic bacteria

associated with sunflower in Southern Africa have not been documented, thus necessitating this study.

The use of culture-dependent techniques in the characterization of endophytic bacteria from the plant has revealed their potential in sustainable plant health, although with a lot of limitations, such as viability, cultural diversity, and genetic complexity. Therefore, employing the next-generation sequencing approach in determining yet-to-be cultured bacteria has proven efficient in the understanding of endosphere biology. In addition, the combination of culture-independent and culture-dependent techniques has provided information on the bacterial community structure and predicted functions using various bioinformatics tools, with insights into endophytic bacteria associated with the sunflower in South Africa. Therefore, for the first time, the combination of culture-dependent and culture-independent techniques in assessing bacterial diversity and functional-base application under greenhouse experiments for improved sunflower yield will be reported in this study.

1.4 Aims and objectives of the research study

1.4.1 Aims of the research study

This present study aims to elucidate the roots and stem endosphere bacterial community structure of sunflower plants using culture-independent techniques and culture-dependent techniques to examine the ability of endophytic *Stenotrophomonas* and *Bacillus* species in enhancing sunflower (*H. annuus* L.) yield.

1.4.2 Objectives of the research study

The specific objectives of the research study are:

(i) To isolate and identify endophytic bacteria by cultural and molecular techniques from the sunflower and determine their ability in enhancing sunflower growth in a growth chamber;

(ii) To determine the physical and chemical properties of sunflower rhizosphere soil;

(iii) To determine the diverse bacterial communities associated with the sunflower endosphere;

(iv) To investigate the functional diversity of endophytic bacterial compositions (BCCs) associated with sunflower endosphere using bioinformatics tools.

List of publications

Chapter two: The endosphere microbial communities, a great promise in agriculture. *Published in International Microbiology.* (2020) 24:1-17. https://doi.org/10.1007/s10123-020-00140-2.

Authors: Bartholomew Saanu Adeleke and Olubukola Oluranti Babalola.

Candidate's Contributions: Conceptualized the topic, managed the literature searches, and wrote the first draft of the manuscript.

Chapter three: Roles of plant endosphere microbes in agriculture - a review. *Published in Journal of Plant Growth Regulation* (2021). https://doi.org/10.1007/s00344-021-10406-2.

Authors: Bartholomew Saanu Adeleke and Olubukola Oluranti Babalola.

Candidate's Contributions: Conceptualized the topic, managed the literature searches, and wrote the first draft of the manuscript.

Chapter four: Screening of plant growth-promoting bacterial endophytes isolates from sunflower endosphere. *This chapter has been submitted in this format for publication in the Archives of Microbiology.*

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Chapter five: Optimization of process parameters for indole acetic acid production ability of bacteria associated with plants. *This chapter has been formatted for publication in Microbiological Research.*

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Chapter six: Genomic assessment of *Stenotrophomonas indicatrix* for improved sunflower plant. *Published in Current Genetics* (2021). 67:891-907. https://doi.org/10.1007/s00294-021-01199-8.

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro, and Olubukola Oluranti Babalola

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Chapter seven: Effect of endophytic bacterium, *Stenotrophomonas maltophilia* JVB5 on sunflower. *Accepted (In Press) in Plant Protection Science.*

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Chapter eight: Genomic analysis of endophytic *Bacillus cereus* T4S and its plant growth-promoting traits. *Published in Plants* (2021) 10:1776. https://doi.org/10.3390/plants10091776.

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro, and Olubukola Oluranti Babalola

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Chapter nine: Unveiling the influence of different farming practices on the structural diversity of sunflower microbiome endophytes using 16S rRNA gene amplicon sequencing. *This chapter has been submitted in this format for publication in the Antonie van Leeuwenhoek.*

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola**.**

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Chapter ten: Bacterial community structure of the sunflower (*Helianthus annuus*) endosphere. *Published in Plant Signaling & Behavior* (2021) 16:19742117. https://doi.org/10.1080/15592324.2021.1974217.

Authors: Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Appendix 6: Whole-genome sequencing of sunflower root-associated *Bacillus cereus*. *Published in Evolutionary Bioinformatics* (2021) 17:1-6. https://doi.org/10.1177/1176934321103894.

Authors: Olubukola Oluranti Babalola, Bartholomew Saanu Adeleke, and Ayansina Segun Ayangbenro

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Appendix 7: Draft genome sequencing of *Stenotrophomonas indicatrix* BOVIS40 and *Stenotrophomonas maltophilia* JVB5, two strains with identifiable genes involved in plant growth promotion. *Published in Microbiology Resource Announcements* (2021) 10:e00482-21. https://doi.org/10.1128/MRA.00482-21.

Authors: Olubukola Oluranti Babalola, Bartholomew Saanu Adeleke, and Ayansina Segun Ayangbenro

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Appendix 8: 16S rRNA gene amplicon sequence data from sunflower endosphere bacterial community. *Published in Data in Brief* (2021) 39:107636. https://doi.org/10.1016/j.dib.2021.107636.s

Authors: Olubukola Oluranti Babalola, Bartholomew Saanu Adeleke, and Ayansina Segun Ayangbenro

Candidate's Contributions: Designed the study, conceptualized the topic, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, and wrote the first draft of the manuscript.

Appendix 9: Oilseed crop sunflower (*Helianthus annuus*) as a source of food: Nutritional and health benefits. *Published in Food Sicence & Nutrition* (2020) 8:4666-4684. https://doi.org/10.1002/fsn3.1783.

Authors: Bartholomew Saanu Adeleke and Olubukola Oluranti Babalola

Candidate's Contributions: Conceptualized the topic, managed the literature searches and wrote the first draft of the manuscript.

Appendix 10: Plant growth-promoting root-colonizing bacterial endophytes. *Published in Rhizosphere* (2021) 20:100433. https://doi.org/10.1016/j.rhisph.2021.100433.

Authors: Bartholomew Saanu Adeleke, Olubukola Oluranti Babalola, and Bernard R. Glick

Candidate's Contributions: Conceptualized the topic, managed the literature searches and wrote the first draft of the manuscript.

2.0 CHAPTER TWO

The endosphere microbial communities, a great promise in agriculture

Abstract

Agricultural food production and sustainability need intensification to address the current global food supply and meet human demand. The continuous human population increase and other anthropogenic activities threaten food security. Agrochemicals input has long been used in conventional agricultural systems to boost crop productivity, but they are disadvantageous to a safe environment. Towards developing environmentally friendly agriculture, efforts are being directed in exploring biological resources from soil and plant microbes. The survival of rhizosphere and endosphere microbiota is influenced by biotic and abiotic factors. Plant microbiota lives interdependently with the host plants. Endophytes are regarded as colonizer microbes inhabiting and establishing microbial communities within the plant tissue. Their activities are varied, and this includes fixing atmospheric nitrogen, solubilizing phosphate, synthesis of siderophore, secretion of metabolite-like compounds containing active biocontrol agents in the control of phytopathogens and induced systemic resistance that stimulates plant response to withstand stress. Exploring beneficial endophyte resources in formulating bio-inoculants, such as biofertilizer as an alternative to agrochemicals (fertilizers and pesticides) in developing environmentally friendly agriculture and incorporation into crop breeding and disease control program is promising. Therefore, in this review, endosphere microbial ecology, associating environmental factors, and their roles that contribute to their effectiveness in promoting plant growth for maximum agricultural crop productivity were highlighted.

Keywords: Beneficial microbes, endophytes, environmental factors, microbial communities, sustainable agriculture

2.1 Introduction

Agriculture for food production needs to be intensified to meet the current trend in the world population. The world aims at ensuring food stability, but due to unpredictable climatic changes, there is a need to find alternative and fast approach techniques to ensure food sustainability (Aloo et al., 2019). The application of agrochemicals (chemical fertilizer and pesticides) has caused an improvement in agricultural productivity, thus assuring food security, and agriculture for food production needs intensification through adequate and proper use of agro-inputs (Wolde-meskel et al., 2018). The use of agrochemicals is characterized by high cost, environmental pollution, toxicity to soil microorganisms, and disease in humans (Campos et al., 2019, Sandargo et al., 2019). Their use is also surrounded with a lot of disadvantages and detrimental influences on the functional ecosystem where living organisms dwell, and their prolonged use reduces crop quality and quantity, soil fertility, causing eutrophication in the water bodies, increasing soil acidity, thus, affecting the microbial composition and their resistance, which make them persist in the environment (Segaran and Sathiavelu, 2019). The use of agro-allied farm inputs, such as synthetic fertilizer and pesticides by farmers is aimed at improving crop yields, boosting agricultural production, and averting hunger and famine among the growing human population in the world. Nevertheless, a lot of environmental problems are associated with their use, due to poor management and improper use, which sometimes escalate to grievous environmental problems (Sharma and Singhvi, 2017). In 1992, the World Health Organization (WHO) reported over 3 million pesticide poisoning affecting individuals with 220,000 deaths.

There are many facets of agriculture, but it is mainly to supply food to the growing population and make raw materials available to industries as a source of income and economic growth.

Nevertheless, their inefficient and inadequate practices have greatly reduced soil fertility. There is therefore the need to design the best options for boosting agricultural production using affordable and scalable farming approaches through technological advancement by exploring veritable microbial resources for maximum crop productivity sustainably (Igiehon and Babalola, 2017). In present-time agricultural practices, plant breeding and technological advancement, as well as application of agrochemicals have progressively improved crop yield, though with major concerns threatening the ecosystem and environment. The use of agrochemicals has contributed greatly to global warming, which has resulted in depleting oxygenation in the aquatic habitat (Zhang et al., 2018b).

Crop yield and agricultural produce are often influenced by environmental factors, causing global warming through changes in temperatures, rainfall, anthropogenic, and other human activities. Global warming is a phenomenon that challenges agricultural production and increases the risk of food insecurity, damage to crop plants, shortage, and scarcity of food for humans, hunger, and even death. Other anthropogenic and occupational activities, such as urbanization have increased from the emission of greenhouse gases as a result of deforestation and poor land use; changes in agriculture, forestry, and land use have contributed to about 25% of global emissions (Paul et al., 2019). The effects of global warming and greenhouse gas emission can be ameliorated by setting agricultural policies that can encourage the use of biological-based (biofertilizer or biopesticide) or non-toxic organic material (compost) in place of agrochemicals (fertilizer and pesticides); as biofertilizer and biopesticides are easily formulated from environmental microbes isolated from plant and soil environments (Bargaz et al., 2018).

Globally, the problem facing agricultural production vary per region. Poor soil fertility, erratic rainfall, and changes in climatic conditions affect the growth of plants with many effects on crop yield. The effect of environmental factors on living organisms, notably, the minute life, affects the nutrient mobilization and assimilation by plants in developing sustainable agriculture and improving crop productivity (Omomowo and Babalola, 2019). The reduction in agricultural crop output is usually due to continuous cropping or over-use of cultivated land that results in nutrient loss, land degradation, long term use, sole reliance on chemical fertilizer and pesticides, pest and rodent attacks, and urbanization involving clearing and removal of the topsoil where nutrients are deposited (Doni et al., 2019, Omomowo and Babalola, 2019).

Proper agricultural management and practices are the best way to ensure agricultural sustainability by application of farm inputs that promote soil health and retain nutrients in the soil. Human beings tend to amend the soil by applying synthetic agrochemicals and other agro-allies to the soil instead of using biologically synthesized products from soil-and-plant inhabiting organisms (Mahanty et al., 2017). This can be due to a lack of awareness and knowledge that a populous unseen microbial world exists naturally in a different environment with a range of activities in promoting plant growth, soil health, root elongation, seed germination, crop yields, drought control, and formulation of biofertilizer and biopesticide, etc. (Igiehon and Babalola, 2017). Poor land use by continuous application of chemical fertilizer and pesticides reduces soil nutrients, alters soil physicochemical characteristics, and makes plants vulnerable to pests and diseases, thus affecting different microbiota communities in the soil. Since adequate nutrient supply to soil boosts plant growth and soil health, proper soil management by conserving nutrients in such a way to enhance soil microbiota would better enhance crop yield and productivity (Odelade and Babalola, 2019). The activities of

microorganisms found in the soil, however, do not only limit pests and diseases but facilitate catalysis, breakdown of complex organic matter, and removal of toxic heavy metal pollutants, as well as improving soil fertility and soil health (Salam and Varma, 2019).

Beneficial plant-microbe interactions are usually symbiotic, as the beneficial microbes confer many benefits on the plant. The internal tissue of plants harboring a community of microorganisms known as endophytes directly affect the physiological features of plants due to their interaction, without developing any disease symptoms or damaging the host immune responses (Aloo et al., 2019). The peculiar niches occupied by endophytes in a habitat differentiating them from the rhizosphere might likely cause a change in their functions, adaptations, responses, competence, and specialization. Even in the endosphere, not every part is colonized by the same microbes; some are specialized as per the region or location they colonized. The colonizer and survival endophytes type are commonly found colonizing plant reproduction organs (ovules) while seeds endophytes colonizer are characterized to be competitors which make them adapt to the harsh environment (Sahu et al., 2019). The competence of endophytic bacterial isolates and their growth-promoting efficacy in the seed of rice has been reported (Banik et al., 2019, Zhou et al., 2020).

Despite the variance in the microbial population in ecological niches, the free-living microbes found in the soil and root interface i.e. rhizosphere, such as plant growth-promoting rhizobacteria, and endophytes community can share a similar mechanism of action, directly or indirectly in plant growth promotion and boosting crop immunity (Orozco-Mosqueda et al., 2018). The mutual interactions between beneficial microbes with the host plants are more advantageous in a suitable host plant. Microbial interactions benefit plants in many ways as they enhance or promote the growth of plants and improve crop yields, control of

phytopathogens, mobilization of soil nutrients through mineralization, and solubilization (Ojuederie and Babalola, 2017). The microbial endophytes attributed with plant growth-promoting potentials, such as siderophore production for iron chelation, phosphate solubilization, growth hormones (auxins, cytokinin, gibberellin, etc) stimulation, nitrogen fixation, pathogen suppression, tolerance to environmental stressors, secretion of secondary metabolites, and antimicrobial sources have been well documented (Patel et al., 2018, Ojuederie et al., 2019, Fadiji and Babalola, 2020a).

It is not only in the roots, stem, shoot, flowers, and leaves of plants that endophytes can be found, the seeds can as well represent an important source of endophytic microbes, most especially in rice and sunflower plants (Banik et al., 2016). Endophytes abruptly establish a microbial community with the roots and shoots of rice, also the sunflower during the growing stage, and this has shown the competence of rice and sunflower roots in terms of colonization, growth, and survival, and transmission to the next generation hosts (Jordaan et al., 2019). The proximity of microbial endophytes in the seeds to the shoot has been reported to be closer than to the roots (Alawiye and Babalola, 2019). The adaption of the microbial endophyte communities commonly influences their response to the prevailing environmental conditions of the host plant, as well as the entire community structure inhabiting the different host plants (Figure 2.1).

Different findings and reports have been reported on the activities of isolated and indigenous rhizobacteria and endophytic communities associated with the host plant (Enebe and Babalola, 2018, Omomowo and Babalola, 2019).

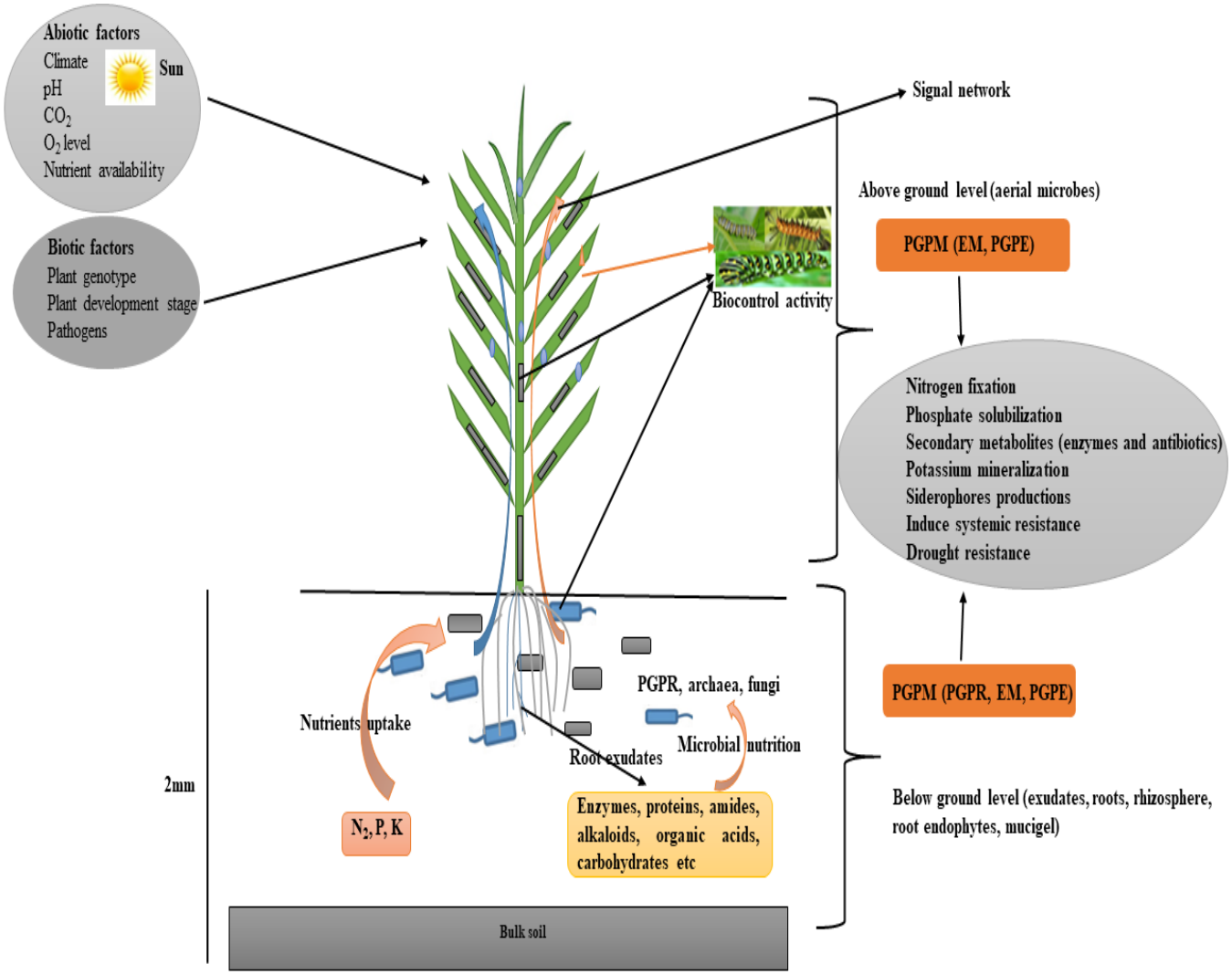


Figure 2.1: Plant growth-promoting microbes, effects of environmental factors, and plant nutrition

However, little or no information is available on the exploration and harnessing of endophytic microbes and their resources in various agricultural applications. It is, therefore, imperative to carry out an overview of the endosphere concept inhabiting the plant roots, seeds, and other parts of the plant by highlighting their significant roles in plant growth promotion for improved agricultural production and sustainability, which is the main focus of this review. The authors believe that more than 40% of microbes in the plant endosphere can possess plant growth-promoting novel genes, and harnessing them will be of great promise in agriculture. Therefore, the understanding of endophytic microbial community structures as contained in this review will reveal their possible exploitation potential and application in developing agriculturally friendly systems in formulating biological or microbial-based products as an alternative to agrochemicals in enhancing soil fertility for improved crop productivity, thus forming the principal goal of the study.

2.2 The endosphere

Microorganisms inhabiting the internal tissue of plants are referred to as endophytes. If their interactions with the host plant are beneficial, without causing disease symptoms, viz a viz, their presence does not cause harm to the plants but instead help in controlling pathogens and promoting plant growth, they can be regarded as plant growth promoters (Hong et al., 2018b, Khamwan et al., 2018a). The microbial interactions with the plant, their beneficial roles in promoting plant growth and health are still largely yet to be explored, and this has allowed researchers to find out more about endophytes novel attributes, making it an interesting area of study from complex and diverse plant species for various application in medicine, food, and agriculture (Audipudi et al., 2017, De Silva et al., 2019).

The use of culture-independent methods in the identification of endophytes using 16S rRNA gene amplicon sequencing is known. Specifically, the study performed by Bertani et al. (2016) reported the isolation of an assumed total of 318 endophytes from plant organs and the authors confirmed that the isolates displayed plant growth-promoting (PGP) ability. The microbial interactions with the host plants can be mutual, parasitic, symbiotic, or antagonistic. Microorganisms are found inhabiting various ecological niches in plants, such as the rhizosphere, endosphere, and phyllosphere, thus benefiting the host plants (Yang et al., 2018). Recent research findings focusing on the isolation of different microorganisms from various ecological niches have thrown more light on the understanding of microbes, including those inhabiting the interior part of plant tissues (Omomowo and Babalola, 2019). The internal tissues of the plant are known to be sterile without contaminants, but they contain beneficial microorganisms that stabilize the internal environment as well as contribute to the physiological functions of the plants (Soldan et al., 2019). More research is now being directed toward understanding microbial ecology in the endosphere. In the endosphere, the microorganisms inhabiting the region confer benefits to plants rather than being pathogenic. Endosphere represents an endo-compartment within the plant tissues harboring a diverse community of microorganisms living there without developing disease symptoms or negative influence on the host plant. For instance, the microbial communities which include bacteria, archaea, or fungi shall be discussed in detail in the subsection below. However, some examples of microbial domains previously isolated from the endosphere compartments of the respective economic crops are presented in Table 2.1.

Table 2.1: Microbial endophytes domain associated with commercial crops

Host plant	Microbial domains	References
Maize (*Zea mays*)	**Bacteria:** *Bacillus* species (*B. subtilis, B. amyloliquifaciens, B. velezensis, B. pumilis, B. lecheniformis*), *Pantoea* spp, *Burkholderia* spp, *Geobacillus* spp, *Paenibacillus* spp, *Cryseobacterium* spp, *Flavobacterium*, *Pedobacter* spp, *Brevibacillus* spp, *Cytophaga* spp, *Acidovorax* spp, *Brachybacterium* spp	Gond et al., (2015); Babalola et al., (2020)
	Fungi: *Alternaria alternate, Giberella moniliformis, Curcularia lunata, Cladosporium oxysporum, Auriobasidium pullulans, Cladosporium tenuissium, Fusarium graminearum, Epicoccum sorghinum, Trichoderma harzianum, Acremonium strictum, Chaetomium cochliodes, Scopulariopsis gracilis, Epicocum purpurascens, Microdochium bolleyi, Giberella zeae*	Renuka and Ramanujam, (2016); Yadav, (2018)
	Archaea: Genus, *Pyrococcus, Halomicrobium, Cenarchaeum, Nitrosopumilus, Haloferax, Methanosaeta, Aeropyrum, Desulforococcus, Thermofilum, Haladaptatus, Halalkalococcus, Methanospirillum, Methanosphaerula, Natrialba, Staphylothermus, Methanoculleus*	Fadiji et al., (2020b)
Soybean (*Glycine max*)	**Bacteria:** *Enterobacter ludwigii, Variovorax paradoxus, Norcadioides alkalitolerans, Acinetobacter oleivorans, Curtobacterium flaccumfaciens, Rhizobium pusense, Bacillus arayabhattai, Arthrobacter nicotinovorans, Bacillus subtilis, Burkholderia cepacia, Microbacterium binotii, Pantoea stewartii, Ralstonia insidiosa, Xanthomonas axonopodis, Xanthomonas arboricola, Arthrobacter niigatensis, Arthrobacter globiformis*	de Almeida Lopes et al., (2016)

	Fungi: *Phoma herbarum, Colletotrichum capsici, Colletotrichum boninense, Penicilium funiculosum, Alternaria alternata, Arthrinium phaeospermum, Aspergillus niger, Clonostachys rosea, Culvularia lunata, Fusarium graminearum, Macrophomina phaseolina, Scopulariopsis brevicaulis, Trichiderma saturnisporum* **Archaea:** rarely documented in the literature	(Russo et al., (2016); Yadav, (2018)
Sorghum (*Sorghum bicolor* L.)	**Bacteria:** *Pantoea dispersa, Acinetobacter calcoaceticus, Pseudomonas fulva, Microbacterium testaceum, Ralstonia insidiosa, Cupriavidus necator, Herbasprilum frisingense, Achromobacter xylosoxidans, Stenotrophomonas maltophilia, Rhizobium mesosinicum, Bacillus megaterium, Bacillus pumilis, Brevibacillus brevis, Paenibacillus pabuli, Kocuria varians, Curtobacterium oceanosedimentum, Lysinibacillus odysseyi*	Mareque et al., (2015); de Fretes et al., (2018)
	Fungi: *Fusarium equiseti, Leptosphaeria sacchari, Macrophomina phaseolina, Exserohilum rostrtum, Gloeocercospora sorghi, Vurvularia penniseti, Acremonium strictum, Epicoccum purpurascens, Colletotrichum sublineolum, Botrydiplodia theobromae, Melanospora zamiae, Bipolaris sorghicola, Alternaria longissimi, Rhizoctonia solani* **Archaea:** rarely documented in the literature	Zida et al., (2014)
Millet (*Pennisetum glaucum*)	**Bacteria:** *Paenibacillus dendritiformis, Bacillus axarquiensis, Pseudomonas* spp, *Enterobacter hormaechei, Enterobacter cloacae, Bacillus safensis, Bacillus subtilis, Stenotrophomonas* spp, *Macrococcus caseolyticus*	Verma and White, (2018)
	Fungi: *Diplodia seriata, Fusarium lateritium, Penicillium camemberti, Nigrospora oryzae, Epicoccum nigrum, Bionectria ochroleuca, Davidiella*	Mousa et al., (2015)

	tassiana, Aspergillus flavus, Alternaria alternate, Paraconiothyrium brasiliense, Trichoderma longibrachaitum, Torrubiella confragosa **Archaea:** rarely documented in the literature	
Cowpea (*Vigna unguiculata*)	**Bacteria:** *Bradyrhizobium kavangense, Bradyrhi. Soli, Kluyvera cryocrescens, Rhizobium leguminoserum, R. etli, R. mayense, R. multihospitium, R. pisi, R. sophoriradicis, R. endophyticum, Neorhizobium galegae, Sphingobacterium multivorum, Chryseobacterium daecheongense*	Ndungu et al., (2018)
	Fungi: *Fusarium sporotrichioides, Colletrichum lindemuthianum, F. chlamydosporum,Alternaria destruens, Alternaria* spp*, Curvularia mosaddeghi i, Penicillium oxalicum, Macrophomina phaseolina, Curvularia* spp. **Archaea:** rarely documented in the literature	Gonzaga et al., (2015)
Sugarcane (*Saccharum occifinarum*)	**Bacteria:** *Actinomadura rayongensis, Microspora amethystogenes, Streptomyces angustmycinicus, Micromonospora carbonacea, Acinetobacter nosocomialis, Kosakonia cowanii, Paenibacillus illinoisensis, Bacillus aerophillus, Pantoea cypripedii, Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae*	Kruasuwan and Thamchaipenet, (2016)
	Fungi: *Metarhizium bruneum, Diaporthe endophytica, Fusarium verticillioides, Epicoccum nigrum, Zygoascus* spp*, Dictyosporium* spp*, Alternatia* spp*, Penicillium* spp*, Resinicium* spp*, F. oxysporum, Trichoderma virens, Penicillium restrictum, Aspergillus oryzae, Epicoccum* spp*, Trichoderma* spp **Archaea:** rarely documented in the literature	Romão-Dumaresq et al., (2016); Yadav, (2018)
Sunflower (*Helinathus annuus*)	**Bacteria:** *Bacillus subtilis, B. licheniformis, B. pumilis, Achromobcater xiloxidans, Grimontella* spp*, Moraxella* spp*, Asticcacasulis* spp*, Azospirillum* spp*, Mitsuaria* spp*, Shinella* spp*, Alcaligenes* spp*, Pseudomonas* spp*, Rhizobium* spp	Leff et al., (2017)

	Fungi: *Colletotrichum truncatum, Penicillium citrinum, P. apserum, Aspergillus terreus, Alternaria destruens**,** Fusarium proliferatum, Diaporthe novem, Acremonium* spp*, Nigrospora oryzae**,** Guignardia cammillae**,*** and *Chaetomium* spp. **Archaea:** rarely documented in the literature	Thompson et al., (2018)
Cotton (*Gossypium* spp)	**Bacteria:** *Bacillus pumilus, B. brevis, B. amyloliquefaciens, Clavibacter* spp*, Xanthornonas* spp*, Erwinia* spp*, Agrobacterium* spp*, Burkholderia* spp*, Rhizobium* spp*, Variovorax* spp*, Artharobacter* spp*, Bacillus pumilis, Bulkhoderia pickettii, B. gladioli, B. Solanecearum, Agrobacterium radiobacter* **Fungi:** *Purpureocillium lilacinum, Penicillium simplicissimum, Leptosphaeria* spp*, Talaromyces flavus, Phomopsis archeri, Acremonium fusidioides, Cladosporium cladosporioides, Colletotrichum gloeosporioides, Fusarium lateritium, Glomerella cingulate, Guignardia bidwellii, Lecanicillium lecanii, Nigrospora sphaerica, Pestalotiopsis maculans, Phoma eupyrena, Rhizopus microsporus, Rhodotorula glutinis, Tritirachium oryzae* **Archaea:** rarely documented in the literature	Shaikh et al., (2017) Vieira et al., (2011); Shaikh et al., (2017)
Wheat (*Triticum* spp)	**Bacteria:** *Pantoea* spp, *Herbaspirillum* spp, *Stenotrophomonas* spp, *Peanibacillus* spp, *Flavobacterium* spp, *Janthinobacterium* spp, *Caulobacter* spp, *Pseudomonas* spp, *Rhodococcus* spp, *Dyadobacter* spp, *Pedobacter* spp, *Stenotrophomonas* spp, *Janthinobacterium* spp*, Burkholderia cepacia, Bacillus subtilis, Pseudomonas trivialis, Didymella exitialis, Alternaria infectoria,* and *Microdochium nivale* **Fungi:** *Trichoderma hamatum, Pleospora herbarum, Trichoderma atroviride, Talaromyces flavus, Phoma glomerata, Alternaria alternate, Epicoccum nigrum,*	Kuźniar et al., (2019a)

	Fusarium avenaceum, Coriolopsis halotolerans, Botrytis cinerea, Diaprothe eres, Fusarium redolens, Gaemannomyces graminis, Athelia bombacia **Archaea:** rarely documented in the literature	Ofek-Lalzar et al., (2016); Comby et al., (2016)
Rice (*Oryza sativa*)	**Bacteria:** *Bacillus altitudinis, B. velezensis, Delftia lacustris, Rhizobium oryziradicis, Flavobacterium gelidilacus, Microvirgula aerodenitrificans, Pesudomonas psudoalcaligenes, P. veronii, Sphingobium* spp*, Asticcacaulis* spp*, Halothiobacillus* spp*, Shewanella* spp*, Methylotenera mobilis, Limnobacter* spp.	Moronta-Barrios et al., (2018)
	Fungi: *Nigrospora oryzae, Exserohilum rostratum, Penicillium chrysogenum, Daldinia eschscholtzii, Fusarium oxysporum, Curvularia lunata, Chaetomium globosum, Cladosporium cladosporioides, Acrophialophora levis, Nigrospora oryzae, Pyricularia oryzae, Thielavia terricola, Trichoderma asperrellum, Aspergillus tubingensis, Ulocladium chartarum, Daldinia eschscholtzii, Penicillium simplicissimum, Aspergillus ustus*	Potshangbam et al., (2017)
	Archaea*: Halobacterium* spp.*, Halococcus hamelinensis, Haloferax alexandrinus, Haloterrigena thermotolerans, Methanobacterium bryantii, Methanosarcina, Methanospirillum* spp.*, Natrialba* spp.*, Natronoarchaeum mannanilyticum, Nitrosomonas communis, Methanospirillum* spp.*, Candidatus Methanoregula boonei*	Sun et al., (2008); Verma et al., (2017)

Consequently, the presence of microbial domains within the plant tissues enables them to elicit their biocontrol potential in the control of plant pathogens that affect crop yield (De Silva et al., 2019). Besides, they enable plants to withstand or tolerate environmental stresses and synthesize biologically active metabolites, needed for plant growth. Hence, the plants internally colonized microbes confer benefits, and secretion of metabolites needed for microbial growth in contributing to the general plant growth is referred to as the "endosphere effect". The group of microbes found in the plants that elicit their plant growth-promoting effect and synthesis of bioactive agents in the control of phytopathogens is classified as PGP endophytes (PGPE) (Soldan et al., 2019).

On the other hand, plants growing on the soil have diverse microbial communities colonizing them. However, the available data on the endosphere community is still small when compared to the success that has been recorded in the study of rhizosphere microbial counterparts. The endosphere is the internal tissue of plants colonized by microorganisms without showing any pathological or severe effects on the host plant, and the microorganisms inhabiting this region are called endophytes (Verma and White, 2018; Fadiji and Babalola, 2020a). Endophytes get into the plant via vertical means by seed inoculation and horizontally from the rhizosphere into the plant roots through signal intrusion (Santoyo et al., 2016; Omomowo and Babalola, 2019). These two measures enable microbes to establish or colonize the host plant. Endophytes stand as a promising candidate in maintaining host defense mechanisms against foreign pathogens. According to the literature on the importance of rhizosphere microbes (Enebe and Babalola, 2018, Alawiye and Babalola, 2019, Odelade and Babalola, 2019), plant endophytes have equally shared similar potential in supplying nutrients to plants by phosphate solubilization, nitrogen fixation, plant protection from foreign pathogens by secretion of essential metabolite

compounds, enhance plant tolerance to environmental stresses by induced systemic resistance (ISR), modulate physiological functions of plants, and synthesis of biopesticides in the control of crop pests (Verma and White, 2018). Research insights into the herbicidal activities of endophytes are under study.

2.2.1 Endosphere bacteria community

The large community of bacteria colonizing the inner tissue of plants predominantly defends plants against environmental stresses. They elicit strong defense mechanisms in response to various factors linked with plant growth, survival, and health (Omomowo and Babalola, 2019). The microorganisms penetrating through the soil infiltrated into the plant roots with unique biological functions in sustainable agriculture. Recruitment and exploration of plant endophytes for their ecological and agricultural importance have recently been reported (Fadiji and Babalola, 2020b). The endo-compartment is an environment suitable for only microorganisms with colonizing potential that benefits the plant. How endophytes colonize the internal tissue of plants in large numbers and elicit defense mechanisms by protecting plants is a question that has not been answered. Understanding mechanisms by which microbe-microbe and plant-microbe interactions would be promising in exploring their agricultural and biotechnological importance. In literature, it has been reported that the ability of microbes to elicit their defense responses in developing a healthy disease-free plant for sustainability depends on their communication pathways (Bamisile et al., 2018). Nevertheless, the actual mechanisms undergone by root endophytes in promoting plant growth have not been clearly defined.

The colonization of the narrow space by endophytes within the plant tissues, as well as colonization of endophyte-like pathogens make it suitable in controlling plant pathogens

through the niche exclusion and synthesis of antimicrobials (Fadiji and Babalola, 2020a). The greater the number of active microorganisms, the more effective they are in the environment. Various microbial activities are beneficial to plants through the following processes: Plant growth promotion is enhanced by microbes producing growth hormones (e.g., auxin, gibberellin, and cytokinins), suppression of biosynthetic environmental stress-related plant hormones, such as ethylene under stress, bio-fixation of atmospheric nitrogen, potassium mineralization, phosphate solubilization and a draft of systemic induced plant resistance (Igiehon and Babalola, 2017). The need for plant-microbe interactions is essential in establishing a mutual relationship in support of plant growth. In an ecosystem, plants shield and secrete exudates that supply nutrients to the microorganisms while microorganisms produce growth stimulators and biocontrol agents needed for plant growth and control of phytopathogens for improved crop productivity (Olanrewaju et al., 2019).

Endophytes bacteria are the common microbial domains found associating with plants, stimulating plant growth, and conferring stress tolerance, owing to their firm establishment with the host plant. The allelopathic actions of endophytic bacteria that improve plant growth have been reported (Polyak and Sukcharevich, 2019). Findings on bacteria endophytes characterization from different parts of plants, such as root, leaves, seed, stem, and flowers have been reported (Tyc et al., 2020, Wolfe and Ballhorn, 2020, Xie et al., 2020) (Table 2.2). Bacteria population is more pronounced in the root compartment than the aerial parts of plants above the ground level i.e., flowers, stems, and leaves. Screening of plant growth-promoting endophytic bacteria isolates from crop plants, such as maize, rice, tomato, and other essential crops, has been documented (Zhou et al., 2020, Liu et al., 2020b).

Table 2.2: Common examples of bacterial endophytes and their agricultural importance

Bacteria endophytes	**Host plant**	**Functions**	**References**
Cronobacter sakazakii, and *Kocuria rhizophila*,	Wheat (*Triticum aestivum*)	Stress tolerance	Afridi et al., (2019)
Enterobacter tabaci, Enterobacter tabaci, Stenotrophomonas maltophilia, Pantoea agglomerans, Sphingomonas sanguinis	Rice (*Oryza sativa*)	Stress tolerance to cadmium	Zhou et al., (2020)
Pseudomonas granadensis; *Rhizobium larrymoorei*	Rice (*Oryza sativa*)	Plant-growth-promotion Biocontrol and pesticide tolerance	Shen et al., (2019a)
Bacillus cereus strain, *B. sonorensis, B. subtilis, Pseudomonas chlororaphis, Ochrobactrum* sp., *Pantoea agglomerans, Ornithinibacillus* spp	Chickpea (*Cicer arietinum*)	Shoot growth	Maheshwari et al., (2019a)
Burkholderia stabilis	Ginseng (*Panax ginseng* Meyer)	Biocontrol of phytopathogens	Chowdhury and Bae, (2018)
Bacillus velezensis	Potato (*Solanum tuberosum*)	Biocontrol of phytopathogens	Cui et al., (2020)

Pantoea spp, *Pseudomonas* spp, and *Enterobacter* spp	Millet (*Eleusine coracana*)	Plant-growth-promoting	
Bacillus, *Lysinibacillus*, and *Stenotrophomonas*	Tomato (*Solanum lycopersicum*)	Biocontrol of phytopathogens	Sahu et al., (2019)
Bacillus amyloliquefaciens, *B. subtilis*, and *B. tequilensis*	Lime (*Citrus* spp)	Biocontrol of Pathogens	Daungfu et al., (2019)
Enterobacter sp., and *Burkholderia phytofirmans*	Maize (*Zea mays*)	Tolerance to drought, plant growth stimulation	de Araujo et al., (2019)
Agrobacterium radiobacter, *Enterobacter cloacae*, *Burkholderia gladioli*, *Burk. Solanacearum*, *Serratia* spp, *Rhizobium japonica*, *Variovorax paradoxus*, *Bacillus megaterium*, and *B. pumilis*	Corn, cotton (*Gossypium barbadense*)	Stimulate plant growth	Lin et al., (2018)
Bacillus amyloliquefaciens	Tobacco (*Nicotiana tabacum*)	Biocontrol of phytopathogens	Jiao et al., (2020)

The exploration of bacteria endophyte resources in formulating bio-inoculants and their application in agriculture will help sustainably in achieving the desired agriculture productivity. Endophytic bacteria genera isolated from various plants, such as *Bradyrhizobium, Enterobacter, Pseudomonas* (rice - *Oryza sativa*), *Pantoea, Pseudomonas, Enterobacter* (sugar cane - *Saccharum officinarum*), *Bacillus* (millet - *Pennisetum glaucum* and *Citrus* spp), and *Curtobacterium* (Soybean - *Glycine max*), that promote plant growth, induced plant tolerance to abiotic stress, stimulation of biological control agents against phytopathogens in controlling their infestation have been documented (De Silva et al., 2019, Sheirdil et al., 2019, Shen et al., 2019a).

In the endosphere, a large population of microorganisms interacts with each other, notably the bacteria species, and till today, little information has been documented about their isolation. In contrast, some have not been cultured at all. The culturable bacterial endophytes are suspected to be Gram-negative and Gram-positive (Kumar et al., 2016). Studies on bacteria endophytes, such as *Pantoea* sp., *Kosakonia sacchari*, *Herbaspirillum* sp., *Enterobacter ludwigii*, *Brevibacillus* spp, *Enterobacter* spp, *Bacillus megaterium, Ralstonia* spp, *Pantoea anamatis, Azoarcus* spp, *Azospirillum* spp, *B. phytofirmans*, *Erwinia* sp., *P. vagans, G. diazotrophicus, H. seropedicae*, *K. phytohabitans*, *K. pneumoniae*, *M. extorquens*, *Pseudomonas ananatis*, *P. ananatis*, *P. ananatis*, *P. fluorescens*, *P. putida*, *Staphylococcus proteamaculans*, *Stenotrophomonas maltophilia*, *Staphylococcus epidermidis*, and *V. paradoxus* from the phylum: Proteobacteria, Alphaproteobacteria, and Firmicutes; class: Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Cyanobacteria, and Bacilli; order: Rhodocyclales, Rhodocyclales, Rhodospirillales, Burkholderiales, Enterobacteriales, Pseudonocardiales, Nostocales and Bacillales from various plants have been successfully carried out by many

researchers (Liotti et al., 2018, Pinto-Carbó et al., 2018, Eke et al., 2019). The bacterial community in the endosphere grows well, due to low competition that occurs in the zone with other microbes.

2.2.2 Endosphere fungi community

The fungi form a close mutual (positive) or pathogenic (negative) association with plants. The mycorrhiza association between fungi and bacteria that makes their firm interactions with the plant contribute to the physiological functions of plants (Verzeaux et al., 2017). The mutual coexistence between fungi and host plant roots enables them to utilize nutrients secreted in the root zone as well as protect microbes from harsh environmental conditions. In turn, the microbes enhance plant growth by stimulating plant growth hormones (auxin, cytokinin, gibberellin, etc), synthesis of metabolites (enzymes and antibiotics), production of siderophores, and stimulating the plant to induce systemic resistance (Odelade and Babalola, 2019).

The beneficial fungi colonizing the root, stem, flowers, and leaves of many plants have contributed maximally to plant growth and improve crop yields. The ability of fungal endophytes to secrete antimicrobial compounds confer much resistance on plants against any form of pests or pathogens attack (Segaran and Sathiavelu, 2019). Endophytic fungi use as a biocontrol agent is achieved by breaking the intracellular metabolism of most pests at the early stage of their growth, thereby affecting their respiration, reproduction, as well as cell development that leads to their death. Also, the use of entomopathogenic fungi in controlling plant pests is known (Jaber and Ownley, 2018). Mycotoxigenic fungi secrete certain metabolites that weaken the attack of pests on plants due to toxic effects on the pests. Endophytic fungi secrete biologically active compounds that enable the host plant to withstand

environmental stresses and improve plant growth. They as well produce certain plant growth stimulators, such as growth hormones that confer growth and health benefits to the plants. Some of these hormonal compounds include indo-acetic acid, gibberellic acid, and piperine (Cheng et al., 2019).

Concerning the aforementioned relevance of endophytic fungi, their significant roles in the ecosystem cannot be overemphasized. Endophytic fungi directly or indirectly maintain interaction with the host plant by making nutrients available for plant growth, exhibiting fungicidal effects on the plant pathogens, and their systemic induced resistance enables plants to tolerate high salinity environments (Ali et al., 2019). Most endophytes produce enzymes needed in the biodegradation of environmental pollutants, although few findings have been documented in this regard. The use of endophytic fungi as a source of bio-inoculants in formulating biofertilizer is known, as this has tremendously contributed to achieving the desired sustainable agriculture through their application in improving crop yield, and they are less toxic to living organisms (Maheshwari et al., 2019a). Similarly, endophytic fungi confer natural immunity to the host plant, boosting their adaptations and survival in a stressed or disturbed environment in fighting against phytopathogens (Omomowo and Babalola, 2019). The potential application of endophytic fungi is becoming a hub of research interest by many researchers; their valuable incorporation in sustainable agricultural and industrial biotechnological applications is promising in modern agriculture. The attractive features of these microbes are that they can easily be harnessed to formulate bioinoculants and applications without negative environmental impacts.

Endophytic fungi resources, if adequately harnessed, can make a turnaround in agro-industry in replacing chemical fertilizers as they naturally synthesize essential compounds that mediate

plant growth promotion (Maheshwari et al., 2019a). The growth of fungi in an acidic environment can be a determinant in selecting their genetic traits in improving agricultural production. Endophytic fungi, such as *Aspergillus oryzae, A. awamori, A. fumigatus, Paecilomyces variotii, Penicillium purpurogenum, P. aurantiogriseum, Alternaria alternate, Piriformospora indica, Fusarium proliferatum,* and *Porostereum spadiceumm* that stimulate plant growth promotion and increase plant tolerance against environmental stress have been reported (Ali et al., 2019). The agricultural relevance of endophytic fungi is shown in Table 2.3.

2.2.3 Endosphere archaeal community

The endosphere archaeal community is the third microbial domain to be studied in this review. The endosphere archaeal community mostly populates the soil, and internal tissues of plants, as many studies have validated this (Zhang et al., 2018a, Moissl-Eichinger et al., 2018, Watanabe et al., 2020), but documentation about archaea is still lacking. The current research into various microbial domains in the internal tissues of the plant continues to receive more attention from researchers since their significant role in an ecosystem is huge.

Naturally, archaea form a close association with the soil and plant root environment as their growth is usually influenced by soil and plant types, as well as other pathogens (Moissl-Eichinger et al., 2018). The intense research into archaea, as well as their recruitment, can be similar to bacteria and fungi, although their isolation is quite challenging. In this review, we hypothesized that the large biomass of archaea community in the soil might be due to exudates released from the plant roots which encourage them to build a biofilm in the plant root region, although this is based on speculation because little or no findings have established their colonization, diversity, functions, and symbiotic interactions with the host plant.

Table 2.3: Common examples of endophytic fungi and their agricultural importance

Endophytic fungi	**Host plant**	**Function(s)**	**Reference**
Sarocladium strictum, Penicillium olsonii, Anthracocystis floculossa	Wheat (*Triticum aestivum*)	Biocontrol of phytopathogens	Rojas et al., (2020)
Streptomyces hygroscopicus	Rice (*Oryza sativa*)	Biocontrol of phytopathogens	Xu et al., (2019a)
Trichoderma hamatum, Penicillium spp, *Paecilomyces lilacinus*	Wheat (*Triticum aestivum*)	Biocontrol of phytopathogens	
Phialophora mustea, Cadophora malorum	Corns (*Crocus sativus*)	Promote plant growth	da Silva et al., (2020)
Penicillium citrinum, Aurobassium pullunts, Dothideomycetes spp	Mandarin (*Citrus reticulata*)	Induce stress tolerance	Sadeghi et al., (2020)
Yarrowia lipolytica, Aspergillus fumigatus, Fusarium proliferatum	Crown of Thorns (*Euphorbia milii* L.)	Plant-growth-promoting, salinity stress tolerance	Bilal et al., (2018)
Alternaria spp, *Colletotrichum* spp, *Diaporthe* spp.	Soybean (*Glycine max*)	Fungicidal effect	Rojas et al., (2020)
Metarhizium robertsii	Maize (*Zea mays*)	Promote plant growth	de Araujo et al., (2019)

		Biocontrol activity	
Penicillium chrysogenum and *P. crustosum*	Felty Germander **(***Teucrium polium***)**	Plant growth promotion	Bilal et al., (2018)
*Acaulospora colombiana, Claroideoglomus etunicatum, Gigaspora margarita, Rhizophagus clarus***,** *Scutellospora calospora*	Sugarcane (*Saccharum officinarum*)	Plant growth stimulation	Fors et al., (2020)
Porostereum spadiceum	Soybean (*Glycine max*)	Stimulate plant growth promotion, tolerance to salinity stress	(Omomowo and Babalola, 2019)
Aspergillus oryzae	Radisg (*Raphanus sativus*)	Plant-growth-promoting, biocontrol of phytopathogens	Sun et al., (2018)
Talaromyces spp, *Hyalodendriella* spp, *Aureobasidium pullulans, Paracamarosporium leucadendri*, *Cladosporium* spp, *C*	Bugweed (*Solanum mauritianum*)	Biocontrol activity Antimicrobial synthesis	Pelo et al., (2020)

ollectotrichum	*boninense,*	Control of plant
Fusarium spp,	*Penicillium*	pathogens
chrysogenum		

Examples of identifiable archaea include *Methanobacterium thermoautotrophicum Methanococcus jannaschii, Nitrosopumilus maritimus, Haloferax sulfurifontis, H. prahovense, Halogranum gelatinilyticum, Methanoregula boonei, M. palustris, Nitrosopumilus, Nitrososphaera,* and *Nitrosocosmicus oleophilus* (Song et al., 2019).

The archaea community in the endosphere of many plants remains abundant and yet unexplored. Archaea contribute to plant growth owing to their ability to synthesize growth-promoting stimulators, and biological control agents in the control of plant pathogens as well as enriching soil nutrients (Odelade and Babalola, 2019). Endosphere archaea can serve as a premise to rhizosphere archaea studies because their activities positively influence plant growth and physiological fitness. Archaea community in the endosphere can exert beneficial effects on the host plant by i) producing plant hormones (auxin, gibberellins, cytokinins, etc.), ii) enhancing plant nutrition by nitrogen fixation, iron chelation, phosphate solubilization, potassium mineralization, etc. iii) synthesis of antimicrobials as biocontrol agents against phytopathogens, and iv) inducing systemic resistance in the response of the plant to withstand various ecological stressors (Moissl-Eichinger et al., 2018, Odelade and Babalola, 2019).

Endophytic archaea participate in the nitrification process that converts ammonia to nitrate in the presence of ammonia monooxygenase, though this mechanism is still not well understood. Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) can be promising in the biodegradation of complex molecules due to their oxidizing potential. Archaea occupy up to 20% of the world's biomass, yet the understanding of the archaea community in the terrestrial habitat is still inadequate, thus limiting their exploration for agricultural use. From literature, little documentation is available on the biotechnological and agricultural importance of archaea. Archaea have the potential to add benefits to plants, such as halo-archaea and

methanogenic archaea groups that exhibit similar functions to plant growth-promoting rhizobacteria in producing siderophore, antibiotics, plant hormones (auxin, gibberellins, cytokinins, etc.), solubilization of phosphorus, iron chelation, mineralization of potassium and nitrogen fixation (Odelade and Babalola, 2019).

For the past few decades, the study of archaea has taken a new dimension in understanding their diversity in various ecological functions. Archaea occupy a major habitat in the biosphere, though till now, little information is available on their environmental impacts and functions. Archaea are distributed across various ecospheres within the plants as they are found inhabiting the caulosphere, laminosphere, rhizosphere and endosphere, although they are scarcely found in the phyllosphere. The colonization, distribution, and adaptation of endophytic archaea in a microenvironment are affected by various environmental factors (biotic and abiotic), such as energy stress and pathogen competition, thus causing a great shift in the microniche. Despite culturing archaea under different environmental conditions, which has been experimented (Doni et al., 2019), how they interact with plants by eliciting their functional and ecological roles still needs to be researched, because it is still unclear.

The isolation of archaea phylum, Crenarchaeota from the roots of leguminous plants has been reported (Yadav et al., 2019), though its further exploration in developing biological products and applications in agriculture is still lacking. Studies on the isolation of AOA, *Nitrosocosmicus loeophilus* from the rhizosphere soil of *Arabidopsis thaliana* as well as other ammonia-oxidizing bacteria showed that their interactions stimulate plant growth and induced systemic resistance against phytopathogens (Jung et al., 2016). The plant-growth-promoting ability and antibiosis potential of archaea, *N. loeophilus* against bacterial pathogens, *Pectobacterium carotovorum*, and *P. syringae* have been reported (Song et al., 2019). Based

on the phylogenetic comparison, archaea are classified as Crenarchaeota mostly hyperthermophilic-sulfur utilizers, Euryarchaeota (methanogens), and halophiles. The type of environmental samples collected determines the archaea's activities and gene expression. The phylogenetic trees showing the genetic correspondence of the archaea genome depict their similarity to that of Gram-positive bacteria, thus making antibiotic options easier since archaea are resistant to many antibiotics synthesized by Gram-positive bacteria (Samson et al., 2019).

The characterization of archaea belonging to phyla halo-archaea, family halobacteriaceae from the hypersaline environment, and mesophilic archaea from swamps, soils, oceans, marshlands, sewage, oceans, the intestinal tract of animals, and decomposed organic matter has been reported (Hassani et al., 2018), and their ability to colonize the plant-root region contributes to plant growth for agricultural sustainability. The contribution of archaea to plant growth can be monitored under greenhouse and field experiments. The direct isolation of archaea by culture-dependent methods from environmental samples can be tedious, time-consuming, and difficult to cultivate.

Since culturing of archaea is difficult and complex, their mechanisms of interactions with plants, and pathogenic nature are not currently known, which may be due to inefficient research, hence the need to acquire knowledge about archaea domains is important. Based on general microbiology knowledge that microorganisms are present everywhere (ubiquitous), found associated with healthy plants, we can say for this reason that, archaea interaction with the plant is species-specific with a positive effect based on their important functions in promoting plant growth, improving crop yield, adaptation to ecological stress, plant diversification due to nutrient absorption at the same time soil health. In the root endosphere, archaea closely interact with other microbes. Therefore, the use of track methods can be

employed in determining their phenotype and genotype, as well as physiological functions, which include solubilization of mineral elements, nitrogen fixation, plant hormone, and antibiotic syntheses, and production of chelating agents (Hassani et al., 2018).

Many research findings on the identification of PGPM (bacteria and fungi) by both culture-dependent and culture-independent methods have been documented (Samson et al., 2019, Fadiji and Babalola, 2020b). Recently, the use of shotgun metagenomics, high throughput sequencing techniques in analyzing plant-soil microbial community, interestingly, has made identification of total microbes easy, particularly unidentified archaea with novel genes or traits. Harnessing their biological resources in amending soil, formulation of biofertilizers will help ensure agricultural sustainability. The bioremediation of xenobiotic substances, such as non-biodegradable pollutants in the soil by soil bacteria has been documented (Ayangbenro et al., 2019). Nevertheless, few reports are available on the use of bacteria in an engineered environment, while no documentation has been reported on archaea. The synergistic actions between archaea with other microbes in the endo-rhizosphere region can contribute to plant efficiency in achieving various environmental tasks. The study of the microbial community in the endosphere is not limited to determining only the microbial type or diversity but is notably used in identifying novel traits applicable in agriculture, medicine, and industries. To date, the knowledge regarding the archaea community inhabiting endo-rhizosphere is limited; hence, this limits their full exploration for various agricultural applications. Therefore, employing next-generation sequencing approaches in studying archaeal communities from environmental samples will provide researchers with a fast approach method in detecting their novel genes or traits for specific functions in promoting plant growth for improved agricultural productivity sustainably.

2.3 Factors influencing microbial diversity in the endosphere

The colonization, diversity, and abundance of microbes in the endo-rhizosphere region are influenced by many factors (biological, chemical, and physical) (Table 2.4). The physical factors include temperature, soil pH, light intensity, geographical locations, time, water availability (rainfall), while chemical factors include exudates, mineral elements, other organic substances, and biological factors include pathogens, nematodes, viruses, protozoans, and beneficial plant growth promoter (Ojuederie et al., 2019). All these factors positively or negatively influence the survival of microorganisms in the endosphere. The adaptation strategies of microbes to strive well in an econiche depend on their ability to tolerate or resist environmental stresses (Enebe and Babalola, 2018).

The erratic rainfall, abnormal high temperatures that led to the release of toxic gases (CO_2) to the atmosphere due to human activities have resulted in the greenhouse effect, causing global warming, and this affects the ecosystem, hence, making it unfriendly for both plants and microorganisms to inhabit. As stated in the previous section 2.1, that rhizosphere microbes form a premise in establishing endosphere community in the plant roots because of the high rhizodeposits and exudation secretion containing nutrients, which provide nutrients for the microorganism to stimulate growth factors that enhance plant growth and health.

Availability of abundant organic substrates in the soil can influence the abundance and performance of diverse endophytic microbes in the root zone, unlike endophytes found in the leaves and stems that are affected by physical factors (Li et al., 2019). The key attributes of environmental factors influencing the survival of microbial endophytes are presented in Table 2.4.

Table 2.4: Factors influencing microbial diversity in the rhizosphere and endosphere

Factors	Key attributes
Physical factors	
• Soil	The soil components (humid), leaching, composting or manuring, soil amendment with chitin, biodegradation of organic matter content, soil management (crop rotation), soil particle size, soil pH, nutrients limiting, chemical fertilizers and pesticides application, presence of soil pathogens.
• Temperature	High accumulation of carbon(iv)oxide, less atmospheric oxygen.
• Water availability (rainfall)	Erratic or low rainfall, drought, poor irrigation system
• Time and seasons	Early planting season, growth stage monitoring, climate change, and autumn season, wet or dry season.
• Geographical locations	Arid and semi-arid zones, Sahara and sub-Sahara regions, low land or highland.
Chemical factors	
• Exudates (metabolites)	Enhance the microbe's infiltration, inhibition of pathogens, facilitate microbe metabolism.
• Mineral elements (N, P, K, Fe), other inorganic substances	Proliferation, inhibition of cellular biosynthesis, nutrients availability, nutrients acquisition, synthesis of biomolecules, source of energy
Biological factors	

• Pathogens, nematodes, viruses, protozoans • Beneficial microorganisms • Plants	Cause diseases in plants, reduce crop yields, compete for nutrients and space with indigenous microflora Secretion of growth hormones, induce plant immune responses, suppress plant diseases, nutrient acquisition. Plant type/species, age of the plant, plant genotype, root zone, plant developmental stages, plant cultivars.

Moreover, soil mineral composition, such as iron, phosphorus, and nitrogen can affect the microbial population found in the rhizosphere, thus causing a shift in the bacterial community both outside and in the internal root environment. The nitrogen fixer, as well as phosphate solubilizer microbes, make nutrients more abundant in the soil by enhancing nitrogen, potassium, and phosphorus uptake by the resident root microflora, in response to the improvement in crop yield (Ramakrishna et al., 2019, Maheshwari et al., 2019a). The microbial population in the endosphere often originates from the growing plant seeds or rhizoplane or bulk soil and grows under different environmental conditions. Factors, such as plant age, type, soil pH, plant developmental stages, excess or limited organic compounds, and nutrient availability are known to influence microbial competence and activities in the root zone (Shymanovich and Faeth, 2019). It has been affirmed that plant growth stage, soil physicochemical parameters, and plant species attest to be the principal factors influencing the endosphere bacterial communities (Ojuederie et al., 2019).

2.4 The roles of plant growth-promoting rhizobacteria and endophytes

The biotransformation of organic residue in our environment can be subjected to environmental treatment using microorganisms. Plant decomposition due to the activities of microorganisms influences the microbial community in the soil environment. Changes in the soil composition and characteristics can cause a difference in the microbes found in the soil. The ability of PGPR to grow under harsh environmental conditions buttresses their potential in subverting the effects of environmental stress on crop yields (Ojuederie et al., 2019, Enebe and Babalola, 2018). Under harsh conditions, plant growth and crop productivity are affected by reducing crop yield. One example of soil bacteria, *Bacillus* spp has been used in formulating biopesticides and plant growth-promoting factors. Application of biopesticide formulated from

soil microbes, *Bacillus thuriengensis*, and *B. sphaericus* have been extensively used in agriculture in controlling phytopathogens and pests, due to its antimicrobial secondary metabolites produced, as the best alternative to chemical pesticides in disease prevention programs (Jiao et al., 2020).

However, since there is a potential of unseen allies with vast importance in soil, exploring their resources can resolve various agricultural challenges through bioproducts production, such as biofertilizer and biopesticides, as an alternative to agrochemicals (Igiehon and Babalola, 2017). The growth of a plant in ecological niches can be said to be the function of plant-microbe interactions mediating vital responses allying the plant root as well as the soil environment. The beneficial microbes can enhance plant growth by the bioavailability of soil nutrients to plants through nitrogen fixation, phosphate solubilization, and potassium mineralization. All these attributes are cumulated for better crop productivity (Omomowo and Babalola, 2019).

Microbes are classified based on their responsiveness to plant growth (Table 2.5). The microbes involved in direct association with soil or plant root, for example, *Rhizobium* partake in plant nutrition by supplying essential soluble nutrients, and in-plant growth by phytohormone production (Duan et al., 2019). The use of active microorganisms in formulating bio-inoculants to replace chemical fertilizers in promoting plant growth and improving crop yields for better agricultural productivity has been reported (Igiehon and Babalola, 2017, Rostami and Azhdarpoor, 2019).

Table 2.5: Roles of plant growth-promoting rhizobacteria (PGPR) and endophytes

	PGPR	**Growth influence**
1.	*Pseudomonas aeruginosa, Serratia* spp, *Streptomyces* spp	Reduce plant toxicity, stimulate plant growth
2.	*Bacillus cereus, B. subtilis Stenotrophomonas*, *Azotobacter* spp	Induce systemic resistance, enhance plant, growth, nitrogen fixation, enhance nutrient acquisition
3.	*Pseudomonas fluorescens, Azospirillum* spp, *Rhizobium* spp, and *Pseudomonas* spp	Control of plant pathogens, induced systemic resistance, enhanced plant growth, production of siderophores, nitrogen fixation
4.	*Coniothyrium* spp, *Trichoderma* spp, *Ampelomyces* spp	Stimulate plant growth, biocontrol of phytopathogens, synthesis of biocontrol agents, siderophores, and enzymes production
	Plant endophytes	
1.	*Bacillus subtilis, Curtobacterium* spp, *Pseudomonas granadensis, Rhizobium larrymoorei*	Stimulate plant growth, plant tolerance to biotic and abiotic stresses, induce systemic resistance, secretion of plant growth hormones and other bioactive compounds
2.	*Rhizobium larrymoorei, Pantoea dispersa, Enterobacter cloacae, Pseudomonas granadensis*	Produce plant growth hormones, nitrogen fixation, acquisition of soil nutrients, synthesis of bioactive metabolites against phytopathogens
3.	*Gordonea terrae, Pseudomonas* spp, *Bacillus amyloliquefaciens Bacillus tequilensis*	Promote plant growth, secretion of metabolites, production of biocontrol agents

4.	*Porostereum spadiceum, Penicillium aurantiogriseum, Alternaria alternate, Yarrowia lipolytica*	Plant growth enhancement, stimulate plant tolerance to stresses, enhance plant tolerance to biotic and abiotic stresses
5.	*Aspergillus oryzae, Paecilomyces variotii, Penicillium purpurogenum, Piriformospora indica*	Stimulate plant growth, bioactive metabolites synthesis in the control of phytopathogens, production of plant growth stimulators

Source: Odelade and Babalola, (2019); Sadeghi et al., (2020).

Some groups of microorganisms can function neutrally but remain in the soil; as such, they can either be classified as beneficial or saprophytic. The neutral saprophytic microbes involved in the degradation of organic material and ensuring nutrient mineralization in the soil seem not to harm or benefit plants but transform soil microbial dynamics, thereby influencing plant growth, health, and crop productivity (Arora et al., 2019).

The free-living bacteria, archaea, and fungi exerting beneficial effects on the plant for improved crop production, contributing to sustainable plant health are referred to as plant growth-promoting microorganisms (PGPM).

Due to advancements in the taxonomic classification of bacteria, studies have shown that some bacteria stimulating plant growth are referred to as PGP bacteria, most especially the rhizobacteria (Sadeghi et al., 2020). In the present era, interest has been drawn towards PGPR by grouping them into different taxa according to their nomenclature and mode of action.

The use of PGPR in the bio-fertilization of farm crops has yielded successes by inoculation. Research findings have identified PGPR as classified into various genera, which include *Stenotrophomonas, Azotobacter, Azospirillium, Streptomyces, Bacillus, Pseudomonas, Rhizobium,* and *Serratia*. Similarly, some fungi genera, such as *Trichoderma, Coniothyrium,* and *Ampelomyces* have been reported to be beneficial to the host plant (Sadeghi et al., 2020).

The plant growth-promoting mechanisms of PGPR encompass multiple mechanisms in enhancing plant protection for higher productivity.

The major benefits of PGPR to plant growth can be achieved, firstly; by bio-fertilization in making soil nutrients readily available to plants; secondly; phytostimulation that involves the synthesis of phytohormones (auxin, gibberellin, IAA, and cytokinins); thirdly; biocontrol of phytopathogens through the synthesis of secondary metabolites possessing antimicrobial

properties, and lastly; induction of systemic resistance by stimulating defense responses in plants against environmental stress. Bacteria genera *Pseudomonas* and *Bacillus* have been reported as the most dominant PGPR found in the rhizosphere (Xu et al., 2019b).

2.5 Conclusion

In this review, various endophytic microbial domains were studied with more emphasis on their roles in ecosystems. Evidence has shown that bacterial and fungal endophytes colonize above (leaves, stems, flowers, and seeds) and below (root/soil) plant zones. Most rhizosphere bacteria and mycorrhiza infiltrating the root tissues can be regarded as root endophytes. Their presence is said to mediate various biological functions and physiological processes in the host plants. The roles of plant endophytes cannot be overemphasized as some of them have been identified and displayed their relevance in the agricultural, biotechnological, and pharmaceutical industries. Better still, their significance in agriculture is promising but yet to be fully explored. The coexistence of various microorganisms in the endosphere can either be beneficial or pathogenic depending on the prevailing environmental factors. Plant age, type, developmental stages, soil pH, soil nutrients, presence or absence of water/moisture, temperature, and pathogens form major factors influencing the activities of endophytes in the host plants. However, the important roles of endophytes in agricultural crop productivity by secretion of certain metabolites can facilitate their various activities in promoting plant growth through phytohormone synthesis, siderophore biosynthesis, and production of antimicrobial substances, such as antibiotics, ammonia, cyanide, and other volatile compounds that suppress activities of nematodes, protozoans, viral, bacterial and fungal pathogens on plants, thus improving crop productivity.

Many studies have been carried out on plant-root-associated microbes, but harnessing their potential use in agriculture is still limited. Findings have equally established that endophytes possessing growth-promoting traits enhance plant growth by various biological activities, thus promising in sustainable agricultural production. The application of endophytes has been employed in various environmental studies, such as in the removal of recalcitrant metals, decaying of organic materials, mineralization, and solubilization of soil nutrients, as well as the synthesis of biocontrol agents against plant pathogens. Also, endophytes exert beneficial effects on plants by enhancing their tolerance to various environmental stressors, such as drought or salinity. Under crop improvement, harnessing endophytes potential can be employed in formulating bioinoculants, on application to improve crop yield and reduce dependence on chemical fertilizer and pesticides that posed serious threats to the environment, ecosystem, and human health.

Hence, this review provides information on the roles of endophytic microorganisms living within the tissues of plants i.e. “microbial endophytes”, their biologically active properties in formulating bioinoculants, and biostimulants to improve crop production in sustainable agriculture: as such as an alternative in mitigating the menace posed by the use of agrochemicals to the ecosystem. Consequently, further study on how vital microbial metabolites can be extracted can enhance their potential application in agriculture. Therefore, the study of the endosphere community can revolutionize agriculture if adequately harnessed.

3.0 CHAPTER THREE

Roles of plant endosphere microbes in agriculture - a review

Abstract

Despite the importance of diverse plant growth-promoting endophytes in agricultural production, their biotechnological and agricultural applications are not well documented. The diversity of microbial communities interacting with the endosphere contributes to plant functions and immunity, thus leading to higher productivity. Plant-microbe interactions range from beneficial in terms of influencing plant growth to harmful, as they also cause plant diseases. Microbial survival in the internal tissues of plants depends on their colonization tendencies and their ability to compete with the indigenous plant microflora. The infiltration of microbes through the external soil-root environment into the plant endosphere significantly enhances growth-promoting attributes of plants such as antibiosis, siderophore production, induced systemic resistance, bioremediation, and growth hormone synthesis. However, the growth and diversity of endophytic microbes are influenced by the availability of soil nutrients, presence of pathogens, plant growth stages, plant genome, and other abiotic factors. Knowledge and understanding of the possible use and biotechnological relevance of endosphere communities in sustainable agriculture cannot be overemphasized. Hence, this review discusses the importance of endophytic microbes in agriculture for enhancing crop productivity.

Keywords: Agriculturally important microorganisms, crop productivity, eco-friendly agriculture, endosphere microbial communities, plant growth-promoting endophytes (PGPE)

3.1 Introduction

Agrochemical use in various agricultural practices such as fertilizer and pesticide applications for enhancing crop yield has been the conventional means of crop production for a very long time (Qaswar et al., 2020). However, the long-term use of agrochemicals deteriorates soil parameters, alters soil ecological stability, and reduces plant performance (Gouda et al., 2018). Research into plant endosphere communities is gaining attention, and the use of agriculturally important microbes to mitigate plant stressors has yielded promising results. Hence, there is a need to optimize soil conditions to favor the resident microorganisms in the biotransformation of organic matter; this will make soil nutrients available to plants and therefore improve the soil's health (Uzoh and Babalola, 2018). Maximum agricultural food production for the world's growing population can be achieved through agricultural intensification with modern technologies.

Different elemental nutrients, such as iron, carbon, phosphorus, nitrogen, and potassium are recycled by microorganisms inhabiting the root endosphere (Adegbeye et al., 2020). They are involved in the overall plant performance by contributing to the acquisition of nutrients and carbon as energy sources in a given niche. The agricultural importance of soil microorganisms in crop productivity cannot be overemphasized and this has led to research in plant endosphere colonizers (Alster et al., 2020). Generally, soil microbes form a close interaction with the host plant either by inhabiting the rhizosphere or colonizing the internal tissues, called the endosphere (Truyens et al., 2015, Alawiye and Babalola, 2019). The endosphere is the region within the tissues of plants that harbor diverse microbial communities comprising bacteria, fungi, and archaea with complex multifunctional properties (Adeleke and Babalola, 2020a). Different microbial colonizers inhabit the external surface of plants compared to intracellular

space within the plant endosphere (Potshangbam et al., 2017). The microbes that colonize the internal tissues of the host plants referred to as microbial endophytes (Gupta et al., 2020b) have the potential to establish mutual, harmful, or neutral relationships with diverse plant species (Dobereiner, 1992, Hardoim et al., 2015). Beneficial microbial endophytes symbiotically colonize the intracellular space of the plant tissues without causing harm or eliciting disease conditions in the host plants (Kandel et al., 2017).

Endophytes are regarded as a subset of rhizosphere microbes that infiltrate plant tissues by a vertical method via seed inoculation or by horizontal networking from the rhizosphere (Adeleke and Babalola, 2021a). In either of these methods, plant-microbe interactions have either mutual benefits or pathological effects on the host plant (Huang et al., 2016). Endophyte infiltration is facilitated by exudate secretion containing signal molecules in exchange for nutrients in-and-out of the soil-root environment (Glick and Bashan, 1997, Alawiye and Babalola, 2019). The complex root structure determines the number of exudates secreted. Exudates contain biomolecules such as fatty acids, oligosaccharides, sugars, phenolic, carbohydrates, enzymes, flavonoids, amino acids, volatile compounds, mucilage, nucleotides, sterol, vitamins, and structural carbohydrates, which provide carbon-energy sources for the microorganisms (Jambon et al., 2018). The endophytic community uses these multifunctional compounds as nutrient sources (Adeleke and Babalola, 2020a). The number of endophytes found in plants can influence plant physiology, thereby affecting plant growth and fitness (Verma and White, 2018).

The root endophyte community facilitates the exchange of molecules in response to interactions between microbes and chemical substances secreted from the plant roots. This exchange enhances plant health and growth (Santoyo et al., 2016). Under certain environmental

conditions, endophytic microbes are responsible for reducing the effects of drought, high temperature, the stress of nutritional deficiency, and infestation of phytopathogens on crops (Jha and Subramanian, 2018, Adeleke and Babalola, 2021a). Thus, adequate utilization of plant nutrients by plant-associated microorganisms enables them to exert diverse beneficial effects on the host plant.

Soil and endosphere microbial populations may vary in terms of diversity and relative abundance due to geographical locations (Lin et al., 2020), age of plants, weather conditions (winter or summer) (Hashem et al., 2019), and agricultural practices such as tilling and fertilizer application (Wang et al., 2018). The survival of microorganisms within the host plant depends on their colonization potential in establishing a cell biomass community. Factors including geographical location, plant type, soil quality, genotype, and tissue, presence of pathogens such as bacteria, viruses, nematodes, plant growth stages, and mechanisms of colonization pathway influence the endophytic population in plants (Zhang et al., 2020).

The use of endophytes as bioinoculants as an alternative to conventional crop improvement and plant disease prevention techniques in agriculture has gained prominence in protecting crops against phytopathogens, thereby enhancing healthy plant growth (Alawiye and Babalola, 2019). The use of bioformulants (biofertilizers/biopesticides/bioherbicides) derived from microbial endophytes are useful in crop management to control weeds (Souza et al., 2017). Since endophytic microbes are involved in the cycling of mineral elements and can boost plant immunity, their introduction through biotechnological approaches into the endosphere community may eventually reduce dependence on the use of agrochemicals by replacing them with helpful agricultural microbes.

It is important to understand the diverse potentials of the bacterial communities inhabiting the plant endosphere to ensure sustainable improvement in crop productivity through agricultural intensification (Adeleke and Babalola, 2020a). Based on the varied effects of endophytic microbe on plant growth promotion, it is necessary to explore the potential application of endophytes in agriculture and understand how they influence plant physiology. This review will discuss microbial endophyte ecology and highlight its role in agriculture.

3.2 Endosphere microbial communities associated with plants

The root, stem, leaf, and seed endosphere harbor diverse microbial communities that form close interactions with the host plant, i.e., promoting plant growth and health, as plants shelter the endophytes (Mukherjee et al., 2020). Mechanisms of endophyte colonization with the host plant are not yet fully understood. Endophytic microbes are predominantly associated with most economical plants such as maize, wheat, sorghum, wheat, and soybean. The culturable microbial endophytes occupy the discreet regions in the endosphere (Adeleke and Babalola, 2020a). The major bacteria group dominating the plant endosphere includes Gram-negative and Gram-positive bacteria like rhizobacteria while endophytic mycorrhiza fungi found in the soil also form a close interaction with the roots of the plants (Saharan and Nehra, 2011). Fungal and bacterial endophytes in the endosphere of different plant organs have been characterized (Tyc et al., 2020). For example, microbial endophytic genera such as *Bacillus, Lysinibacillus, Brevibacillus, Paenibacillus, Nocardioides, Curtobacterium, Microbacterium, Brachybacterium, Kocuria, Micrococcus, Fusarium, Xylaria, Cladosporium, Acremonium, Epicoccum, Curvularia* have been identified in seeds, leaves, and stems of common bean (*Phaseolus vulgaris*) and *Vitis vinifera* (Parsa et al., 2016). The common examples of endophytic microbial genera associated with plants are shown in Table 3.1.

Table 3.1: Some common examples of microbial endophytes associated with plants

Plant	Microbial endophytes	References
Maize (*Zea mays*)	*Acidovorax, Burkholderia, Enterobacter, Brachybacterium, Enterobacter, Rhodococcus, Rhizoctonia, Aspergillus, Penicillium, Rhizopus, Colletotrichum, Cladosporium*	Mashiane et al., (2017)
Rice (*Oryza sativa*)	*Aspergillus, Penicillium, Fusarium, Paecilomyces*, *Phoma*, *Verticillium, Paecilomyces, Acidovorax, Paenibacillus, Caulobacter, Sphingomonas, Acinetobacter*	Wang et al., (2016a)
Potatoes (*Solanum tuberosum*)	*Fusarium, Emericella, Penicillium, Bacillus, Stenotrophomonas, Xanthomonas, Rhodanobacter, Phyllobacterium, Rahnella*	Ardanov et al., (2016)
Moringa (*Moringa oleifera*)	*Fusarium, Penicillium, Glomerella, Scopulariopsis, Acremonium, Bacillus, Azotobacter, Aspergillus* spp	Abonyi et al., (2018)
Soybean (*Glycine max*)	*Penicillium, Porostereum, Cochliobolus, Cladosporium, Emericella, Aspergillus, Paecilomyce, Bacillus, Deinococcus, Clavibacter*	Hamayun et al., (2017)
Teak (*Tectona grandis*)	*Alternaria, Colletotrichum, Nigrospora, Phomopsis, Fusarium, Schizophyllum, Bacillus, Enterobacter, Pseudomonas*	Singh et al., (2017)
Neem (*Azadirachta indica*)	*Eupenicillium, Alternaria, Aspergillus, Fusarium, Penicillium, Verticillium, Bacillus, Azotobacter, Azosprillum, Acetobacter, Xanthomonas, Pseudomonas, Burkholderia*	Tiwari and Thakur, (2014)

Rubber tree (*Hevea brasiliensis*)	*Clonostachys, Colletotrichum, Trichoderma, Serratia, Pseudomonas, Azotobacter, Bacillus*	Vaz et al., (2018)

Harnessing bioincentives from microbial endophytes isolated from legumes and non-legume plants such as cotton wool (*Populous deltoids*), sweet potato (*Ipomoea batata*), soybean (*Glycine max*), and tomato (*Solanum lycopersicum*), and their application have contributed to crop yields (Mashiane et al., 2017). Similarly, the co-existence between bacteria genera *Methylobacterium, Pseudomonas, Caulobacter, Erwinia*, *Acidovorax, Chryseobacterium, Paenibacillus, Sphingomonas, Corynebacterium, Acinetobacter, Devosia,* and *Brevundimonas;* and fungi genera *Verticillium, Aspergillus, Eurotium, Fusarium, Wallemia, Septoria, Pestalotiopsis, Penicillium, Trichosporon,* and *Cryptococcus* in sprout, stems, and roots of rice (*Oryza sativa*) have supported plant growth and improvement in rice production (Wang et al., 2016a). The importance of endophytic microbes in plant growth promotion depends on their ability to fix atmospheric nitrogen, synthesize IAA, and siderophore production, antimicrobial compounds, and phytohormones (Pham et al., 2017). Examples of such endophytes include *Azoarcus* spp and *Pseudomonas stuzeri* from rice (Wang et al., 2016a, Pham et al., 2017), *Gluconacetobacter diazotrophicus* from sugarcane (James et al., 2001), *Azospirillum lipoferum* from maize (Garcia et al., 2017), *Enterobacter cloacae* from pepper (Santoyo et al., 2016), and *Burkholderia phytofirmans* from grapevine (Compant et al., 2008). The endophytic community found in the root zone easily derived all-inclusive multifunctional compounds as nutrient sources (Dubey et al., 2021). Most microbes found in the endosphere might display competent novel genes in promoting plant growth. However, only 1% of the total microbial population in the endosphere has been isolated and identified by conventional isolation techniques. Thus, further research into their isolation can make them useful tools in future agricultural production (Hiruma et al., 2018).

Microbial endophytes can directly influence plant growth by nitrogen fixation, production of phytohormones (cytokinins, gibberellins, and auxin), phosphate solubilization, synthesis of biocontrol agents in combating plant and soil pathogens, secondary metabolites, such as siderophores, antibiotics, ammonia, and hydrogen cyanide (Verma et al., 2015). Additionally, microbial endophytes play an important role in plant response to withstand harsh environmental conditions by inducing systemic resistance (Zhang et al., 2020).

The survival of bacterial endophytes in the root region or other plant organs can support plant adaptation to different environments by synthesizing metabolic chemical compounds and lytic enzymes (Bashan et al., 1995). The metabolic activities contribute to the development, growth, and immunity of plants. For instance, auxin production from endophytes can mobilize or activate plant growth by cell division, elongation, and differentiation depending on the plant response to environmental stimuli (Shi et al., 2009). 1-aminocyclopropane-1-carboxylate deaminase (ACCD) synthesis by plant endophytes can enhance plant growth under drought stress by lowering the rate of ethylene production and reducing pathogen effects on the host plants (Glick, 2014). Ethylene production in the endosphere modulates plant physiology and is an emerging strategy for microbial signaling in a host plant (Santoyo et al., 2016). The strong affinity, i.e., the chelating potential of endophytic microbes to Fe (III) by synthesizing siderophores also enables them to limit plant pathogen infestation on crops (Jiao et al., 2020). Plant endophytes function in phosphate solubilization, further aided by organic acid secretion to facilitate the conversion of insoluble phosphates into soluble phosphate derivative (monobasic or dibasic) ions, thus making it accessible and utilizable by plants (Shahid et al., 2015).

The production of specific metabolic compounds by fungal endophytes acts as biocontrol (Mane and Vedamurthy, 2018). These fungi positively influence plant growth through the secretion of biological control agents, which act against potential plant pathogens (Bashan and De-Bashan, 2010). Like bacterial endophytes, endophytic fungi have been isolated from plants. The study of fungal endophytes has attracted mycologists, soil ecologists, and environmental microbiologists because of their inherent importance in agriculture (Afzal et al., 2019, Adeleke and Babalola, 2021a).

3.3 Below-ground and above-ground microbial endophyte inhabitants

The plant types and nutrients available in a microhabitat facilitated the recruitment and establishment of different microbial communities in the endosphere (van Overbeek and Saikkonen, 2016). Diverse microbial communities inhabit different ecological niches, including below-ground and above-ground habitats that provide unique environments in an ecosystem (Oliveira et al., 2002).

These below-ground and above-ground habitats contain agriculturally important microorganisms, and their exploration is promising in agricultural productivity (Cole et al., 2018). The microorganisms from these habitats may resemble each other based on their morphological features but have different genetic compositions that determine their functions (Adeleke and Babalola, 2020a). The genetic composition and mechanisms exhibited by endophytic microbes are important in eliciting their functions on the host plant as different functions are linked with the specific endophyte domain (Table 3.2).

Harnessing plant-microbe resources is appropriate in ensuring sustainable agriculture. By implication, the exploitation of microbial endophytes is a key factor in addressing environmental challenges derived from soil stressors (Sandargo et al., 2019).

Table 3.2: Bacterial endophytes and their functions

Plant	Bacterial endophytes	Effects/Functions	References
Cowpea (*Vigna unguiculata*)	*Rhizobium, Azotobacter, Azospirillum*	Stimulate plant growth, improve crop yields, nitrogen fixation, and production of phytohormones.	Arafa and El-Batanony, (2018)
Maize (*Zea mays*)	*Pseudomonas, Rhizobium, Xanthomonas, Azospirillum, Bacillus*	Nitrogen fixation, crop improvement, biocontrol of plant pathogens, and enhanced nutrient absorption by plants.	Ke et al., (2019)
Soybean (*Glycine max*)	*Methylobacterium, Rhizobium*	Improve crop yield, enhance plant growth, and metabolites synthesis.	Hamayun et al., (2017)
Rice (*Oryza sativa*)	*Azotobacter*	Nitrogen fixation, siderophores production, and phosphate solubilization.	Banik et al., (2016)
Tomato (*Solanum lycopersicum*)	*Azospirillium Pseudomonas*	Nitrogen fixation, plant growth promotion, and phosphate solubilization	Bergna et al., (2018)
Black pepper (*Piper nigrum*)	*Pseudomonas*	Production of growth hormones, siderophores, hydrogen cyanide, antibiotics, and phosphate solubilization.	Feng et al., (2017)
Ryegrass (*Lolium perenne*)	*Pseudomonas*	Production of growth hormones, indole acetic acid, siderophore, antibiotics, and bioremediation.	
Wheat (*Triticum aestivum*)	*Bacillus circulans, Azotobacter chroococcum*	Plant growth promotion, secretion of lytic enzymes, and production of biocontrol agents.	Larran et al., (2016)

Current findings on plant endophytic communities, diversities, and their functions have addressed several ecological challenges that make them important in sustainable agriculture. Various studies have identified endophytic bacterial phyla, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria from *Arabidopsis thaliana, Brassica chinensis, Glycine max, Vitis vinifera*, and *Zea mays* (Reeve et al., 2015, Truong et al., 2017). The advancement of molecular tools and bioinformatics as applied in microbial genomic studies has revealed information on the genomic content of plant-associated microbial endophytes (Levy et al., 2018). Research using modern molecular tools is still underway to obtain further knowledge of the functions of bacterial endophytes that would aid their commercialization as bioinoculants.

3.4 Plant roots structural complexity and endophytes colonization

Changes in environmental conditions can influence microbial populations in the host plants. The plant roots which are responsible for skeletal support and absorbing and transporting soil nutrients needed for plant growth, also harbor highly diverse numbers of microorganisms due to excess release of exudates. This has resulted in microbial diffusion from the rhizosphere into the endosphere, by vertical or horizontal means (Adeleke and Babalola, 2020a). The colonization tendencies of bacterial endophytes into the plant roots depend on many factors which include the age of the plant, root architecture and environment; plant species, availability of soil nutrients, and plant developmental stages (Afzal et al., 2019). Bacterial endophyte attachment is aided by certain lytic enzymes that disrupt the plant cell wall, thus creating permissible pathways for easy penetration and colonization within the plant tissues by specialized organelles such as flagella or pili (Zheng et al., 2015). However, the presence of harmful microorganisms such as nematodes, viruses, and certain bacteria are factors that negatively affect the colonization tendencies of bacterial endophytes.

The plant-microbe interactions and infiltration into the root zone are complex (Figure 3.1). Endophytic bacteria positively contribute to plant nutrition, facilitate root nutrient absorption, and release metabolites, chelates, or enzymes to mobilize insoluble nutrients (Alawiye and Babalola, 2019). The mutual association between PGPE and mycorrhizal fungi benefits plants, even under stress conditions, thus making them potential candidates in the formulation of bioinoculants. PGPE with known promotional potential for plant growth includes *Bradyrhizobium, Pseudomonas, Bacillus, Paenibacillus, Burkholderia, Agrobacterium, Klebsiella, Streptomyces, Rhizobium, Arthrobacter, Brevibacillus, Alcaligenes, Azotobacter, Enterobacter, Serratia,* and *Azospirillum* (Bashan, 1999, Kaur et al., 2016, Nehra et al., 2016). The bioinoculant produced from PGPE conserves and influences other soil microorganisms in stimulating plant growth and other physiological functions when applied to the soil (Hong et al., 2018b). Arbuscular mycorrhizal (AM) fungi and PGPE are further classified as subgroups of plant growth-promoting microorganisms (PGPM). The PGPR, such as *Chryseobacterium humid, Pseudomonas reactans*, and AM such as *Rhizophagus irregularis* improve crop yields (Moreira et al., 2016). PGPE and AM can be applied in combination with organic manure as a good alternative to agrochemicals to maximize crop yield/productivity and reduce environmental pollution. Bioinoculants containing bacteria or fungi can be applied singularly or combined to agricultural soil for higher crop productivity (Gouda et al., 2018).

The lateral root structure of a plant provides a pathway of entry for bacteria into the host tissue, functioning analogously to the plant root system (Dong et al., 2018). Plant hormones such as auxin initiate biological processes involving lateral and adventitious root formation and elongation (López-Bucio et al., 2003).

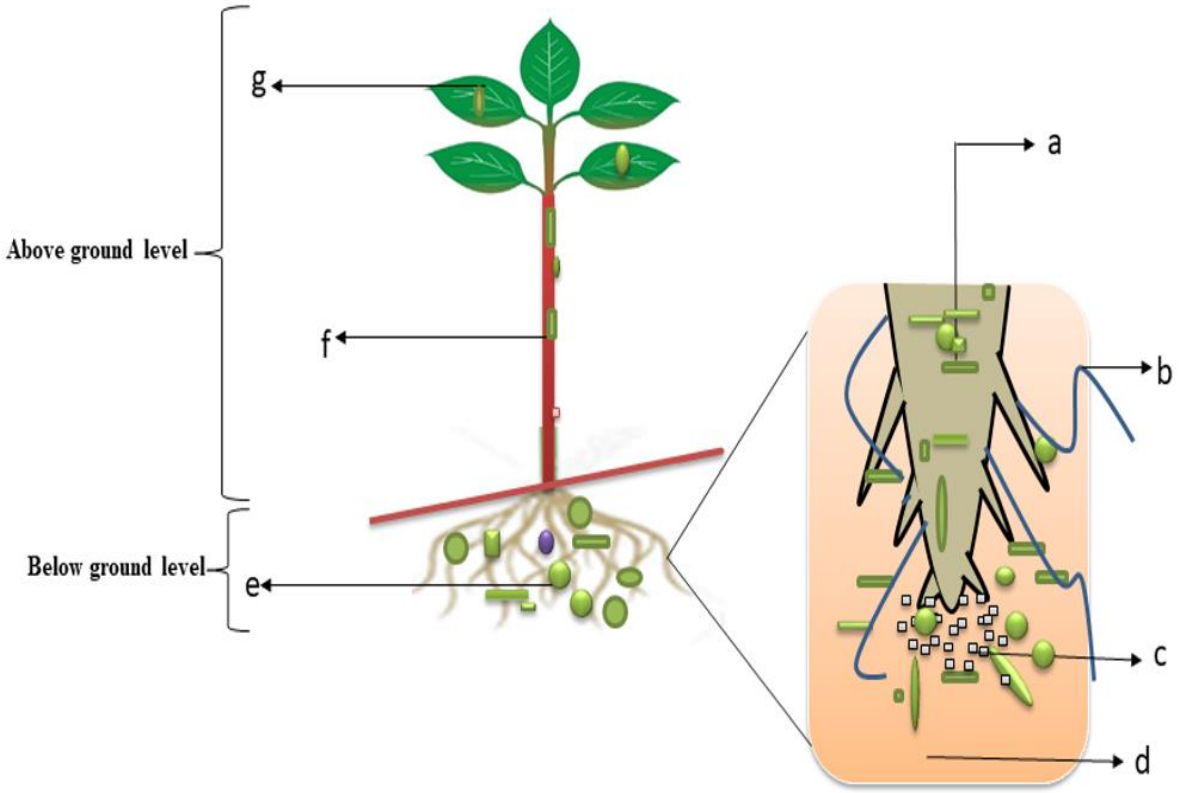


Figure 3.1: A diagram of endophytes interactions and infiltration into plant roots. **a.** endophytes colonizing the intracellular spaces of plant root; **b**. root hair; **c**. exudates secretion to the surrounding root environment; **d**. the rhizosphere region; **e**. microbial attachment and colonization onto the root; **f**. signal networking of plant-endophytic microbes interaction; **g**. leaf associated endophytes

The roots of plants penetrate deeply below ground level, providing mechanical supports needed by plants to absorb nutrients from the soil by capillary networking in channeling nutrients up to the stem and other parts of the plants through plant supporting tissues (xylem and phloem) (Dobbelaere et al., 2003).

Plant roots are naturally surrounded by nutrients, releasing exudates containing monosaccharide sugars, necessitating the build-up of microbial activities entering the plants at the early developmental stage. These nutrients also support the growth of mycorrhizal fungi that enable them to produce spores, promote mycelial growth, accelerate bioremediation of pollutants, and enhance root protection against infection. Also, the secretion of plant hormones that cause root development and supply of nutrients for plant growth contribute to their ability to survive in a nutrient-limiting environment. The secretion of these metabolic compounds, such as sugars, amino acids, and water supports, can greatly contribute to the viability of microbes in the endo-rhizosphere (Alawiye and Babalola, 2019). Hence, plant-root structures influence plant growth and microbial populations to survive external environmental stress (Enebe and Babalola, 2018).

3.5 Plant growth stimulating factors and ecological factor consequences

Diverse groups of microorganisms exist in nature. They are found in different environments such as plants, soil, water, air, etc. Plants stand as a major ecological niche for innumerable microorganisms inhabiting their surrounding soil and plant organs (Taghavi et al., 2009). Their strong affinity in establishing close association makes them inhabit the plant surface (rhizoplane) and colonize intracellular spaces within the plant tissues without causing harm (Adeleke and Babalola, 2020a). These microorganisms stimulate plant growth factors that enable them to exert growth effects on plants, as well as the source of nutrients for other

beneficial microorganisms that might cohabit the plant endosphere (Santoyo et al., 2016). Culturing endophytes in a culture medium under favorable conditions has made possible the exploration of developing unaltered microbial resources; and their production in large quantities to formulate bio-inoculants such as bio-fertilizer that can be adjuvants in enriching soil nutrients for higher crop productivity (Bashan, 1998, Verma and White, 2018). However, to formulate biofertilizers maximally, it is fundamental to have in place suitable biological carriers that can facilitate the proliferation of microbial cells and sustain microbial viability under storage conditions after production.

The survival of endophytic microorganisms in plants is largely affected by the limited supply of nutrients and environmental factors, such as drought, rainfall, temperature, and soil parameters (Edwards et al., 2015). Under certain conditions, plant beneficial endophytes can be opportunistic with the tendency of causing disease symptoms in plants. Therefore, an antagonistic mechanisms approach in the effective control of plant pathogens is crucial. Studies have shown that the biocontrol efficacy of native PGPR against rhizome rot disease that causes light yellowing of leaf in turmeric plants (Chenniappan et al., 2019). Research findings on the disease conditions caused by *Pseudomonas syringae* in olive, green bean (leaf spot), tobacco (leaf spot), and tomato (soft rot) have been linked to their potential to produce toxins and resist antimicrobial substances (Xin et al., 2018). The cause of fire blight diseases in many ornamentals and fruit trees by *Erwinia amylovora* has resulted in yield loss and reduction in crop productivity (Doolotkeldieva and Bobusheva, 2016). *Ralstonia solanacearum, Xanthomonas* spp, and *Xylella fastidiosa* have been classified as the potential etiological agent of wilt in potato and moko disease in bananas (Wubshet, 2018).

Though, endophytes isolated from the same plant cultivar might differ in genotype but share similar effects on plant physiology and biocontrol activities against plant pathogens. The endophytic mechanisms in a host plant can displace pathogens in the same ecosystem by instigating plant defense mechanisms and producing active biological compounds. The cause of disease symptoms in plants depends on host plant susceptibility, suitable growth conditions, plant microflora, and other pathogens (Eljounaidi et al., 2016).

3.6 Roles of plant growth-promoting endophyte in enhancing agricultural crop productivity

The biological and metabolic processes involve the recycling of soil nutrients, biotransformation of environmental waste into soluble form for easy absorption by plants as well as the synthesis of certain biological molecules controlling plant pathogens attack (Ojuederie et al., 2019, Compant et al., 2010). For example, bio-application of PGPE *Azospirillum* spp associated with the root of the Cactus plant and Cyanobacteria from drought-prone areas has contributed to soil quality, aeration, water-retention capacity, and soil elemental carbon to nitrogen ratio (C: N) (Romeh and Hendawi, 2014).

Endophytes can develop vigor to the plant growth promotion effects, resistance to drought, and tolerance to poor soil conditions (Teixeira et al., 2019). The improvement of plant growth can be influenced by compound-like phytohormones produced by microbial endophytes. The IAA is secreted by endophytic fungi, *Colletotrichum alatae*, *Fusarium* spp, *Cylindrocarpon* spp, *Cladosporium tenuissium*, *Trichoderma harzianum*, and *Hypoxylon* spp isolated from *Dendrobium moniliforme* stimulate plant growth and protection against plant pathogens (Glick et al., 2001, Shah et al., 2019). Apart from auxins, cytokinin, gibberellin, and organic acids produced as principal mechanisms by endophytes, other indirect mechanisms that aid plant

growth are known. The cytokinins produced by endophytes isolated from Scots pine exerted beneficial effects by promoting plant growth. Other volatile compounds, such as acetoin and 2,3-butanediol secreted by bacterial endophytes stimulated plant growth (Rath et al., 2018). The modulation effects of polyamines produced by mycorrhiza and *Azospirillum brasilense* on plant growth and development facilitate their cooperation with the host plant (Bashan and Levanony, 1990, Kaushal and Wani, 2016).

Plant growth regulators like auxins vitalized cell division, cell elongation, and cell differentiation. Endophytic bacteria and fungi potentially secrete physiologically active auxins that benefit plants. However, microorganisms colonizing various plant organs influenced the physiological functions and growth of plants by secreting growth parameters (Odelade and Babalola, 2019). Additionally, PGPE exhibits a strong affinity in reducing metal toxicity under stress conditions, thus promoting plant growth (Zhang et al., 2020). Highlights below are mechanisms employed by PGPE in enhancing agricultural crop productivity.

3.6.1 Nitrogen (N_2) fixation

Nitrogen accounts for 78% of atmospheric gases. It forms the main component of chemical fertilizers, and plant biomolecules for the synthesis of nucleic acids and proteins (Ferreira et al., 2016). When combined with other mineral elements such as oxygen or hydrogen, nitrogen produced complex nitrogenous compounds needed for soil amendment in ammonium (NH_4^+) and nitrate (NO_3^-) fertilizers. Nutrient-limiting soils affect the performance of plants in terms of flowering seed formation, growth, etc. PGPE in the genera *Rhizobium*, *Pseudomonas, Sinorhizobium, Bradyrhizobium, Azorhizobium, Enterobacter, Klebsiella, Mesorhizobium*, *Allorhizobium,* and *Azospirillum* symbiotically colonize the tissues of legumes and non-legume plants and fix biological nitrogen in the form of ammonia (NH_3) to the soil without interference

to the soil nutrients supply to the host plant (Bashan et al., 2009, Nohwar et al., 2019). The isolation of endophytic bacterium, *Azospirillum* from the roots of non-leguminous crops and their re-inoculation have contributed to plant growth (Döbereiner, 1992, Mhatre et al., 2019). Availability of nutrients needed for plant survival can enhance mutual patterns of microbes colonizing the root-endosphere in promoting plant growth and fixing nitrogen needed for plant functions. The mechanisms underlying the nitrogen fixation in non-leguminous plants by the potential non-rhizobia fixing bacteria are known, especially in sugarcane upon co-inoculation with endophytic bacteria such as *Azospirillum amazonense, Herbaspirillum rubrisubalbicans, H. seropedicae, Gluconacetobacter diazotrophicus,* and *Burkholderia tropica* (Pedraza et al., 2010, Merasenla et al., 2016).

The ability of endophytic microbes to produce genes coding nitrogenase may underline their nitrogen-fixing potential for improved crop plant performance in terms of yield biomass, root/shoot ratio and height (Knoth et al., 2014). Some groups of root endophytes previously identified as nitrogen fixers include *Rhodotorula graminis, Acetobacter diazotrophicus, Rhizobium tropici* bv *populus, Pseudomonas putida, Herbaspirillum* spp, *Burkholderia vietnasmiensis, Sphyingomonas yanoikuyae,* and *Azoarcus* spp (Glick et al., 2001, Knoth et al., 2014). The nitrogen-fixing capability of these bacterial endophytes might be slightly compared to the *Rhizobium* inhabiting the root nodule of leguminous plants. High efficiency of nitrogen fixating capability of bacterial endophytes *Gluconacetobacter diazotrophicus* on *Picea engelmannii* and *Pinus flexilis* growing in a nutrient-limiting environment have demonstrated the roles of *G. diazotrophicus* strains in nitrogen fixation and other physiological functions in those plants (Carrell and Frank, 2014). An improvement in the seedling growth of canola (*Brassica napus* L.) inoculated with nitrogen-fixing bacterial endophytes *Paenibacillus*

polymyxa P_2b-$_2R$ has been tested with success (Puri et al., 2016). Also, the nitrogen-fixing potential of *Pseudomonas stutzeri* A15 and their growth-promoting effects on inoculated rice seedlings compared to uninoculated rice seedlings improved the plant yield (Pham et al., 2017). Nitrogen fixation by microorganisms can cause metabolic changes in plant growth and fitness, particularly in nitrogen-deficient soil environments. However, the amount of nitrogen fixation to the soil for plant use might vary depending on the number of microorganisms involved and their ability to produce nitrogen fixation genes (*nif*H) (Smercina et al., 2019).

Endophytic microbe interactions with the host plant and their nitrogenase production have efficiently made atmospheric nitrogen available to many plants. The nitrogen-fixing ability of endophytic microbes can ensure plant survival in a limiting nitrogen soil environment, thus contributing to plant growth and immunity (Ke et al., 2019). The nitrogen-fixing potential of some bacterial endophytes such as *Rahnella, Burkholderia, Acinetobacter,* and *Sphingomonas* isolated from the stem part of *Popus trichocarpa* and *Salix sitchensis* have made nitrogen accessible for plants by stimulating their growth in nitrogen-deficient soils (Ma et al., 2016a). The persistence of nitrogen-fixing bacterial endophytes in a nitrogen-limiting soil environment might enhance nitrogen fixation rate and nitrogen accumulation in the host plants (Arora et al., 2019).

3.6.2 Phosphate (P) Solubilization

Phosphorus is one of the essential micronutrients needed for the proper functioning in living organisms, viz; energy transport, root development, plant growth promotion as well as other physiological processes (Lobo et al., 2019). The application of phosphorus to alter soil had resulted in a phosphorus complex formation thereby making it inaccessible for plant absorption. In most agricultural soil, phosphate level ranges between 400 mg/kg and 1200

mg/kg, though, only 1 mg/kg of the soluble form of phosphate (HPO_4^{-1} and $H_2PO_4^{-2}$) is made available to plants (Rasul et al., 2019). Some endophytic microbes undergo several processes to make phosphorus available to plants via solubilization, acidification, chelation, ion exchange, organic acid production, and secretion of extracellular acid phosphatase (Santoyo et al., 2016). Endophytes assimilate soluble phosphate by preventing adsorption and this makes endophytes a reservoir of phosphorus, which enhances their phosphate solubilization potential in a phosphate limiting environment (Vidya et al., 2016).

Phosphate-solubilizing microbes play a major role in unlocking phosphate compounds in the soil, thus making them readily available for plant use (Rasul et al., 2019). The phosphate-solubilizing capability of endophytes enhances microbial survival and promotes plant growth by synthesizing organic acids, such as citric and gluconic acids that chelate the insoluble phosphate compounds at low pH to release protons. Different phosphate-solubilizing bacterial genera: *Achromobacter, Aereobacter, Agrobacterium, Bacillus, Burkholderia, Erwinia, Enterobacter, Flavobacterium, Micrococcus, Pseudomonas, Rhizobium,* and *Streptomyces* have been isolated from the endosphere and phyllosphere regions of plants (Pandey et al., 2016).

The media composition, types of substrates, and growing conditions can contribute to the rate of phosphate production by microbial endophytes. However, not all bacterial isolates from plant rhizoplane, rhizosphere, or endosphere can stimulate plant growth or solubilize phosphate in the soil for plant use. For instance, phytase potential synthesis of endophyte bacteria isolated from *Zea mays* L. contributes to plant functions in acquiring soil nutrients (Hafsan et al., 2018). An increase in maize seedlings inoculated with *Bacillus amyloliquefaciens* FZB45 producing

phytase under phosphorus limiting soil has been observed via phosphorus accessibility and plant uptake (Ramírez and Kloepper, 2010).

3.6.3 Iron (Fe) mineralization

Iron is one of the essential elements that form life and exists in the soil in insoluble ferric form. Like phosphorus, iron exists in various forms such as iron (iii) oxide (ferrihydrite, goethite, and hematite) in the soil, and is not readily available for direct plant absorption. Iron availability in the soil might be due to chelating agents produced by microbial endophytes, thus making iron abundant in iron-deficient soils (Salam and Varma 2019). The low molecular weight siderophores developed a strong affinity for iron (iii) ions, as well as bind to other iron (ii) ions bivalent metal for easy assimilation by plants. The calcareous and alkaline nature of some agricultural soils limits plant access to elemental iron from the soil. Soil-root endophytes produce siderophores chelate iron by transforming them into a soluble form for plant use. Plants acquire iron from siderophores producers via direct mechanisms as determined by the ability of microorganisms to produce siderophores. Uptake of iron (Fe) from the iron-siderophore network across plant root is necessitated by chelate breakdown and solubilization of unavailable forms of iron by phytosiderophore synthesis (Sah et al. 2017).

The siderophore produced by microorganisms (microbial siderophores) generally displays more affinity than plant synthesized siderophores (phytosiderophores). Substructure plant growth in metal-contaminated soil amasses high iron that will support siderophore-producing microbes (Ferreira et al., 2019). The microbial siderophores with high iron affinity are regarded as a significant means of making iron available to plants under metal-stressed conditions. Metal accumulation in the soil is made accessible for plant absorption when siderophore microbes are involved in the iron solubilization process. The actual role of siderophores produced by

endophytic microbes is unknown except by the induction of systemic resistance (Hardoim et al., 2015).

Some endophytes have elicited siderophore genes that modulate iron metabolism and transport in most plants. The siderophore gene potential of *Epichloë festucae* isolated from ryegrass has modulated the effects of siderophores on *Epichloë festucae* infected plants. The induction of resistance due to siderophore synthesis by endophytic *Methylobacterium* strains inhibits pathogenic bacteria *Xylella fastidiosa* causing chlorosis in *Citrus* trees (Bucci, 2018). A high siderophore production rate of 47% and 25% of total endophytic bacteria colonizing the endosphere than rhizosphere of silver birch (*Betula pendula* L.) and black alder (*Alnus glutinosa* L.) has dictated the level of iron in the soil (Złoch et al., 2016). Reduction in Cd^{2+} toxicity in the host plants has also been linked to an increase in iron acquisition. Iron uptake in plants is accelerated by ligand exchange, breaking down organic chelate, and direct formation of Fe (iii) siderophore complexes (Ma et al., 2016b). Bioaccumulation of heavy metals in *Spartina maritima* improves plant growth. The inability of endophytic bacteria in the uptake of Cd concentrates from polluted marshes soils has reduced their resistance ability (Mesa et al., 2015).

The phytosiderophores and siderophores produced by microorganisms can enhance iron-chelating capability in many plants. The activities of root endophytes in response to higher iron uptake in non-sterile calcareous soils than sterile soils due to soil variables enable them to form plant-microbe interactions (Otieno et al., 2015). The iron-chelating ability of *Pseudomonas* spp isolated from growing mung beans (*Vigna radiata*) and their growth effects have yielded positive responses regarding plant performance (Otieno et al., 2015). Besides, iron uptake by plants has contributed to leaf color pigmentation thereby preventing chlorosis. The potential of

bacterial endophytes producing siderophores by inoculation with the indigenous soil microbes to form bacteria-siderophore complexes make iron resources available for the plant. Hence, the siderophore producing PGPE - *Pseudomonas putida* can enhance plant iron nutrition (Sah et al., 2017).

3.6.4 Phytoremediation/Bioremediation

Various waste generated from different sources can serve as excellent nutrient sources for microbial growth. The over-accumulation of recalcitrant xenobiotic compounds has made them resistant to microbial degradation and thus negatively influences plant growth, crop yields, soil nutrients, diversity, and distribution of beneficial microorganisms in response to phytoremediation activities (Uzoh and Babalola, 2018). Heavy metals that contaminate a soil environment include selenium, copper, lead, zinc, cadmium, cobalt, chromium, and mercury. Other contaminants include; (i) inorganic compounds – phosphate, ammonia, nitrate, sodium, arsenic), radioactive compounds (strontium, uranium or cesium); (ii) organic compounds – chlorinated solvents like trichloroethylene; explosives, such as petroleum hydrocarbons including toluene, xylene, and benzene, polycyclic aromatic hydrocarbons; trinitrotoluene and 1,3,5-trinitro-1,3,5-hexahydrotriazine; and (iii) pesticides – atrazine and bentazon (Salam and Varma, 2019).

Screening of essential microbial traits has proven efficient in the degradation of environmental pollutants from contaminated soil (Rostami and Azhdarpoor, 2019). The removal of pollutants from the soil environment can be accomplished by the activities of endophytic microbes involving rhizodegradation, transformation, and stabilization using lytic enzymes in breaking down complex organic molecules. The inability of plant microbes to degrade environmental

pollutants due to their non-remediation properties can result in less biomass production (Enebe and Babalola, 2018).

Some endophytic bacteria grow well under drought stress and remediate environmental pollutants. The presence of recalcitrant and xenobiotic substances in the soil affects the microbial population and their metabolic activities. Inoculation of heavy metal polluted soils with PGPE can facilitate the bioavailability of heavy metals, thus making them available for plant growth (Wang et al., 2019a). Accumulation of various toxic chemicals in the soil can transiently migrate through the plants into the food chain system, causing human health issues and other related disease conditions. Waste removal from agricultural soil using endophytic microorganisms has prompted developing an eco-friendly biological approach in environmental pollution control (Gupta et al., 2020a).

Considerable numbers of bacterial species associated with endosphere degrading environmental pollutants have been isolated and identified (Rani et al., 2019). The use of PGPE in reducing heavy metal induction phytotoxicity enhances plant biomass production in metal-polluted soils (Chen et al., 2010). The role of PGPE on plant improvement and crop yields; and their potential in heavy metals bioremediation on agricultural polluted soil can help in bioremediation processes (Ma et al., 2016b). Some examples of endophytes genera involved in bioremediation processes include *Pseudomonas, Bacillus, Burkholderia, Stenotrophomonas, Pusillimonas, Terribacillus, Acinetobacter, Achromobacter, Staphylococcus, Curtobacteriu, Microbacterium, Arthrobater, Leifsonia, Paenibacillus, Enterobacter, Acinetobacter, Rahnella, Herbaspirillum, Serratia, Flavobacterium, Chryseobacterium, Sphingomonas, Methylobacterium and Variovorax* (Ma et al., 2016b).

3.6.5 Siderophores production

Siderophores are biological molecules produced by microorganisms, with a low molecular weight of 200 Da to 2 KDa. They are primarily involved in iron-chelation in nutrient-limiting environments (Odelade and Babalola, 2019). The activities of PGPE in the synthesis of siderophores are expressed by indirect mechanisms on plants (Omomowo and Babalola, 2019). Many PGPEs reduce plant stress either by synthesizing biological control agents or by systemic induction of plant response against pathogens (Ferreira et al., 2019). Endophytes producing siderophores help control plant pathogens that affect crop yields and act as key determinants for plant-induced systemic resistance. Some endophytic microbes can synthesize substances that exert lethal effects on various pathogens. The substances include metabolites (volatile organic compounds), siderophores antibiotics, and enzymes. The siderophores produced by endophytic microbes are composed of chelating compounds, bind to iron, and improve plants' resistance to environmental stresses (Dimkpa et al., 2009).

Siderophore's production capability of the endophytic bacteria domain in controlling phytopathogens has contributed to plant health and immunity (Omomowo and Babalola, 2019). For example, siderophores production from *Bacillus antiquum* in the control of *Rhizoctonia solani* that cause stem canker and black scurf and charcoal rot disease caused by *Macrophonomia phaseolina* in sorghum have suggested their potential use as a biological control agent (Gopalakrishnan et al., 2011, Aloo et al., 2019). The singularly or combined use of antibiotics in biocontrol systems has been employed in disease control and suppression. Endophytic microbes can potentially out-compete pathogens colonizing the same ecological niche, thus reducing their detrimental effects on crop yield (Card et al., 2015).

3.6.6 Enzyme production

Endophytes produce enzymes primarily to facilitate tissue colonization by utilizing polysaccharide compounds containing xylan and pectin present in the host plants (Fouda et al., 2021). Examples of enzymes produced by endophytes that facilitate tissue colonization include lipase, amylase, tannase, cellulase, glucanase, protease, laccase, xylanase, and chitinase (Sharma, 2019). Like other metabolites, enzymes produced by endophytic microbes perform specific functions against invading plant pathogens and degradation of organic materials such as lignin, complex polysaccharides, cellulose, and hemicellulose (Chu et al., 2021). Despite the actions of lytic enzymes like lipase or cellulase on organic compounds, the host plants permit endophyte association within the tissue but recognize them as an obstacle for tissue colonization (Chen et al., 2016). Other metabolites such as essential oils and alkaloids secreted from plants can facilitate host colonization patterns (Korenblum and Aharoni, 2019). Under favorable conditions, fungal endophytes can complete their life cycle within the host tissues by synthesizing enzymes that can cause alteration in the host plant defense mechanisms and decomposition of organic materials (Tiwari and Rana, 2015, Adeleke and Babalola, 2021a).

3.6.7 Biocontrol activities of plant endophytes

The PGPE colonizing plant endosphere capable of synthesizing biomolecules can enhance plant performance in stimulating plant resistance against pathogens (Adeleke and Babalola, 2020a). Plant protection against pathogens may be achieved directly through antagonistic interactions and induction of host resistance, or indirectly by suppressing plant pathogens and enhancing plant growth through various mechanisms (Orozco-Mosqueda et al., 2018). Such mechanisms include the ability of endophytes to secrete antimicrobial metabolites, enzymes

degrading microbial cell walls, and hydrogen cyanide that represses the growth of microbial pathogens (Adeleke and Babalola, 2020a).

Endophytic microbes associated with plants live and grow inside the cell of the host plant without any disease symptoms. These microorganisms that colonize plant tissues may have evolved from plants and become non-pathogenic (Xu et al., 2019a). During long-term co-evolution of endophytes and plants, the establishment of equilibrium can make beneficial endophytes turn pathogenic in host plants under stress conditions. Over time, plant-microbe forms ecological balancing when microbial activity and plant interactions are achieved (Nefzi et al., 2019). The mechanisms exhibited by endophytes as biocontrol agents against plant pathogens can directly or indirectly be achieved with significant ecological effects. The use of microbial endophytes in plant protection against pathogens may be due to their functional roles in producing phytoalexins (Kollakkodan et al., 2020).

Plant growth-promoting endophyte plays a major role in crop management against microbial pathogens. The ability of endophytes to elicit biocontrol traits, antibiotic production, iron chelator siderophores, extracellular enzymes determine their biocontrol activities (Ye et al., 2019). The biocontrol activities of *A. terreus* and *P. citrinum* isolated from sunflower plants against *Sclerotium rolfsii* with overall performance and improvement in sunflower yield have positively influenced sunflower growth (Potshangbam et al., 2017). The bioprospecting application of PGPE as biocontrol agents against phytopathogens is listed in Table 3.3.

A major challenge in the use of microbes as biocontrol agents can be based on its application. The exploitation of derivative fungal or bacterial resources as biological agents has been employed in the control of pathogenic microbes affecting plants (Pan et al., 2015).

Table 3.3: Endophytic fungi and biocontrol activities

PGPE	Disease caused/activity	References
Wheat (*Triticum aestivum*)	Biocontrol activity of *Trichoderma hamatum, Penicillium* spp, and *Rhodotorula rubra* against *Pyrennophora triticirepentis* and *Drechslera triticirepensis* that cause tan spot of wheat	Larran et al., (2016)
Chinese ginseng (*Panax notoginseng*)	Biocontrol activity of *Fusarium*, *Cladosporium*, and *Penicillium* against *Fusarium oxysporum*, *Phoma herbarum, Mycocentrospora acerina, F. solani* and *Alternaria panax* causing root rot of *Panax notoginseng*	Zheng et al., (2017)
Maize (*Zea mays*)	*Paenibacillus polymyxa, Bacillus subtili* and *Burkholderia gladioli* against *Sclerotinia homoecarpa* causing stem and crown rot of maize	Shehata et al., (2016)
Cocoa (*Theobroma cacao*)	Biocontrol activity of *Paenibacillus polymyxa, Curvularia* spp, *Fusarium* spp and *Tolypocladium* spp against *Phytophthora palmivora* that cause black pod disease of cocoa	Segaran and Sathiavelu, (2019)
Ginger (*Zingiber officinale* Rosc)	Biocontrol activity of *Rhizopycnis vagum* against *Phytophthora infestans, Rhizoctonia solani, Fusarium oxysporum, Colletotrichum acutatum, Sclerotium rolfsii, Corynespora cassiicola,* that cause root-knot disease of ginger	Anisha et al., (2018)
Lettuce (*Lettuce sativa* L) leaves	Biocontrol activity of *Trichoderma asperellum* against *Corynespora cassiicola* and *Curvularia aeria* that cause leaf spot fungi of lettuce	Baiyee et al., (2019)

The recruitment of endophytes in modern agricultural practices has made them a potential candidate in the formulation of biopesticides on application to suppress activities of phytopathogens (Sindhu et al., 2016). Reports on the fast and intense defense mechanisms of endophytic bacteria in the host plant, suitably in the control of entomopathogenic fungi that cause collar rot in plants have revealed their importance in growing pathogen-free plants (Jaber and Ownley, 2018).

Metabolites secreted by *Fusarium* strains, E4 and E5 in controlling plant diseases and for promoting the growth of *Euphorbia pekinensis* depend on increased terpenoid content (Nefzi et al., 2019). *Muscodor albus* isolated from *Cinnamomum zeylanicum* producing 28 volatile compounds have elicited their bactericidal and fungicidal activities on microbial pathogens (Praptiwi et al., 2015). *M. crispansis*, a fungus of Dwarf pineapple (*Ananas ananassoides*) from Bolivian Amazon Basin has also displayed antimicrobial efficacy due to volatile organic compounds secretions against fungal pathogens: *Botrytis cinerea, Fusarium culmorum, Pythium ultimum, Alternaria helianthi, Verticillium dahlia, Phytophthora cinnamomi, Sclerotinia sclerotiorum,* and *Rhizoctonia solani*; and bacteria, *Xanthomonas axonopodis* (Yuan et al., 2017, Hashem et al., 2019).

The use of endophytic bacteria in the control of *Verticillium* wilt and *Fusarium* wilts (wilts that cause vascular coloration, chlorosis, and necrosis in the leaves of herbaceous plants such as vegetables, maize, sunflower, soybean, and cowpea) have contributed to plant health. Studies have shown the application of *Pseudomonas, Clavibacter, Erwinia,* and *Ralstonia* in the control of vascular wilt diseases in infected plants (Eljounaidi et al., 2016).

Several challenges surround the control of pathogens associated with most plants since there are no clear-cut measures in proffering curable treatments to the infected plants except by removing the infected parts from the plant body. The persistence of vascular wilt soil pathogens depends on their resistive structures that ensure their viable living for a long time, invariably making its control difficult (Yadeta and Thomma, 2013). The use of chemical pesticides to control vascular wilt diseases may be cost-effective but inefficient as this negatively poses environmental and health challenges.

Consequently, it is imperative to find an alternative ecological friendly approach to the control of plant diseases. One of such promising approaches is the exploration of biocontrol agents from bacterial or fungal endophytes. For effective use of endophytes as biocontrol agents, it must be; (i) a natural means of exploration to ameliorate over-dependence on the use of fertilizers and pesticides, (ii) self-sustaining with the ability of proliferation and forms cellular biofilm after the establishment in a niche, and (iii) reduce the use of pesticides that permit prevention of long-term diseases in an eco-friendly manner (Eljounaidi et al., 2016).

3.6.8 Phytohormone production

The phytohormones suppress metal stress in plants, thus suggesting that metal stress reduction by endophytic microbes can cumulatively exert combining nutritional and physiological effects on the plants (Afzal et al., 2019). The phytohormones either inhibit or promote plant growth depending on the concentration and increase nutrient uptake and improve crop productivity (Spaepen et al., 2009). The ability to synthesize certain plant growth hormones, which include cytokinin, auxin, IAA, abscisic acid, and gibberellin have been attributed to two predominant endophytic bacteria genera, namely *Bacillus* and *Pseudomonas* (Hashem et al., 2019).

Indole-3-acetic acid (IAA), a major auxin produced by microorganisms partakes in different physiological processes in the plant, such as modulation of plant growth and functions as cell-cell signaling molecules (Crozier et al., 1988). The IAA concentration differs in various parts of the plants (Ismaila et al., 2018). This auxin produced by endophytes modulates plant-endophyte interactions and plant survival in metal-polluted soil. PGPE such as *Enterobacter*, *Staphylococcus, Pseudomonas, Azospirillium*, and *Azotobacter* from rhizosphere soil of faba bean (*Vicia faba*) and root of sugarcane (*Saccharum officinarum*) have been identified as phytohormone producers (Okon and Kapulnik, 1986, Alemu, 2016, Kruasuwan and Thamchaipenet, 2016). Phytohomorne synthesis enhances root development in many plants (Santoyo et al., 2016). The low synthesis of IAA can stimulate primary root development and elongation, while high IAA biosynthesis by endophytic microbes can stimulate adventitious root development with impairment to the primary root growth. However, modification of phytohormones equilibrium through ACC synthesis can accelerate plant growth (Dobbelaere et al., 2003).

Ethylene is another form of phytohormones that contributes significantly to the growth and survival of plants under drought stress. Its functions include cell elongation, leaf development (senescence), root initiation, root nodulation, fruit ripening, and abscission as well as auxin transport (Ji et al., 2020). Ethylene synthesis in plants is achieved through the production of enzyme S-adenosyl-L-methionine (SAM) synthetase, which catalyzes the conversion of methionine and adenosine triphosphate (ATP) to SAM; then ACC synthase mediating the hydrolysis of SAM to ACC and methylthioadenosine (MTA), and finally, oxidization of ACC by enzyme ACC oxidase to its products form, carbon dioxide, ethylene, and hydrogen cyanide (He et al., 2019). Growing plants in a metal stress environment stimulate ethylene production,

thus inhibiting root elongation, root hair formation, and lateral root growth (Glick et al., 1998, Wu et al., 2020). Similarly, some endophytic microbes reduce the stress-regulated influence in plants by enzyme reaction in the hydrolysis of ACC and subsequently reduce ethylene production stress by plants (Santoyo et al., 2016).

3.6.9 Induced systemic resistance and metabolite secretion

Induce systemic resistance (ISR) is a systemic induction process responsible for plant stimuli in developing resistance against plant pathogens. The modern application of chemical and PGPBE metabolites as biocontrol measures is promising in crop pathogen control and agricultural sustainability (Alvin et al., 2016). The plant-associated endophytes can develop multiple mechanisms to stimulate plant defense systems through the expression of oxidative and nitrosative genes that are required for the activation of mechanisms such as superoxide dismutase, peroxidases, guaiacol, catalase, b-1,3-glucanases, and chitinase activity (Okon and Labandera-Gonzalez, 1994). Consequently, it can instigate reactive oxygen species production in protecting cell organelles con cell oxidative shock and physiological stress that may have preset these actions due to endophytic microbe activity (Asghari et al., 2020).

The life cycle of endophytes in host plants is said to be shorter than the host plant itself; hence, endophytes proliferate rapidly and form microbial biomass within the host plants for selective antagonism in contributing to the resistance against other endophyte-like pathogens (Sahu et al., 2019). The induction of systemic resistance in endophytes enhanced their tolerance to other camouflaged endophyte pathogens. The mutualistic association between endophytes and host plants prompted the systemic responses in plants. The ability of endophytes to bypass host responses makes them successfully colonize the plants without suppressing the activity of the host plants (Xu et al., 2019a).

Endophytic bacteria genera *Bacillus* and *Pseudomonas* are regarded to have caused systemic induction resistance in plants based on key attributes and their ability to possess attachment cell organelles, organic acids (salicylic acid, jasmonic acid) production, synthesis of volatile compounds and other biomolecules siderophores, lipopolysaccharides and *N*-acyl-homoserine lactones (Hardoim et al., 2015). The induction resistance of *Methylobacterium* spp strain IMBG290 isolated from potato shoot against potato pathogen *Pectobacterium atrosepticum*, owing to the vicissitude in the microbial community structure and diversity, boosts plant immunity against phytopathogens (Ardanov et al., 2016). The corresponding changes in the endophytes community to pathogen resistance significantly repress disease activity.

Less information is available on fungal endophytes plant protection through ISR. Like bacterial endophytes, fungal endophytes also produce certain metabolites compounds that inhibit plant pathogens. Some compounds include phenols, polyketones, peptides, steroids, flavonoids, quinols, alkaloids, terpenoids, and chlorinated compounds (Hardoim et al., 2015). The antibiotic substances 2-phenylethanol and 4-hydroxybenzoate produced by *Enterobacter* spp strain 638, and munumbicins, kakadumycins, and coronamycin produced by actinomycetes stimulate resistance against pathogens (Braga and Faria, 2020). The antibacterial activity of chemical compounds multicyclic indolosesquiterpenes produced by *Streptomyces* spp isolated from mangrove tree and spoxazomicins A to C produced from *Streptosporangium oxazolinicum* isolated from orchid plants, importantly stimulate plant defense against pathogens (Chakravorty et al., 2020). The array of chemical compounds produced by bacterial and fungal endophytes are found applicable in agriculture; nevertheless, elucidation on the specific functions in plant-microbe interactions is required.

3.7 Conclusion

The diverse endophytic microbes occupying the external and intracellular compartments of plants play an important role in plant ecology and physiology. These endophytic microbial diversities inhabiting different plant organs might exhibit genetic relatedness. Their exploration and applications in modern agriculture sustainability can guarantee maximum crop production and food safety. Though, crop production is mostly influenced by rainfall, drought, temperature, salinity, as well as pathogens, the conventional application of agrochemicals (chemical fertilizers and pesticides) to improve agricultural productivity has been associated with various environmental or ecological effects.

The complex assemblage of microbial communities inhabiting the endosphere was reviewed in the present study. The survival and diversity of plant endophytes are influenced by environmental conditions and plant phenotype or genotype. Sometimes, the impact of endophytes on plant growth and crop productivity is monitored through laboratory routines involving isolation, identification, and inoculation of desirable microorganisms and monitoring under controlled greenhouse pots experiments.

In an ecosystem, the understanding of the genetic composition of plant endophytes is vital in developing environmentally friendly agriculture by harnessing microbial bioproducts in formulating bioinoculants, such as biopesticides or biofertilizers. The roles of endophytes in agriculture are numerous and include plant growth promotion, improving plant health and immunity, enhancing crop and agricultural productivity, plant protection against pathogens, plant tolerance to environmental stresses, bioremediation of organic pollutants, etc. Microbial endophytes have been used in the formulation of biopesticides toxic to plant pathogens as well as the secretion of metabolite compounds such as antibiotics and hydrogen cyanide.

Therefore, agricultural productivity can be enhanced using modern agricultural techniques that involve the synthesis of organic or microbial-based products in promoting plant growth and control of pathogens as the best alternative to pesticides and chemical fertilizer usage in sustaining plant growth and health, using microbial resources for improved agriculture in a sustainable manner.

4.0 CHAPTER FOUR

Screening of plant growth-promoting bacterial endophytes isolates from sunflower endosphere

Abstract

Harnessing endophytic bioproducts as an alternative to chemical fertilizer usage is promising in agricultural biotechnology to maximally boost food production. Here, we isolated and screen agriculturally important bacterial endophytes from the sunflower root and stem, and experiments to determine their effects on sunflower yield under greenhouse conditions were conducted. The bacterial isolates were identified by culturing, biochemical, and molecular techniques. Fifty endophytic bacterial isolates were screened and twenty were selected based on indole-3-acetic acid and ammonia synthesis, hydrogen cyanide production, phosphate solubilization, and enzyme production. The identified bacterial isolates are rod-like, with 30% Gram-negative and 70% Gram-positive. The bacterial genera identified include *Bacillus, Pseudomonas,* and *Stenotrophomonas*. *B. cereus* SFR35 exhibited high phosphate solubilizing index of 4.09±0.08 mm from the plate assay. The indole-3-acetic acid quantitative test yielded a maximum IAA activity of 16.94 µg/ml from *S. maltophilia* JVB5 at 6% L-tryptophan. The below-and-above-ground sunflower yield parameters showed a significant difference among the treatments compared to the control. Hence, plant growth-promoting attributes exhibited by endophytic bacteria suggest their promising use in agriculture as potential candidates in formulating bioinoculants with a multifunctional application for an enhanced sunflower yield.

Keywords: Bioinoculants, crop improvement, *Helianthus annuus*, root colonization, soil fertility

4.1 Introduction

There is a need to source alternative measures in mitigating the environmental threats and soil degradation posed by agrochemical use on farmlands. To achieve this, bioinoculant synthesis from bacterial endophytes becomes important in developing eco-friendly agriculture (Basu et al., 2021). The inclusion of bio-incentives, such as bionoculants from microorganisms into the agricultural-food value chain aims to alleviate the negative impacts of chemical fertilizer on the ecosystem (Maheshwari et al., 2019a). It is promising that devising suitable methodologies for isolating agriculturally important endophytic microbes from economic crops would contribute immensely to avert future food insecurity (Kuźniar et al., 2019b, Adeleke and Babalola, 2021a).

The endosphere represents a complex environment within the plant tissues colonized by diverse microbiomes found above and below ground level (Dong et al., 2018, Tonial et al., 2020). The viability of the endosphere community can be linked to their close interactions within the plant tissue (Khamwan et al., 2018b). Also, the impacts of endophytic microbes in the ecosystem have systematically brought new insights into microbial research and a possible understanding of plant-microbe ecology, as many bacteria colonizing the plant endosphere contribute to plant growth and crop yield (Harrison and Griffin, 2020). However, the functions of microbial endophytes in plant growth promotion and tolerance to stress adaptors are influenced by soil type, limiting nutrient supply, plant genotype, soil parameters, plant developmental stages, and root exudates (Korenblum et al., 2020).

The use of plant growth-promoting endophytic bacteria (PGPEB) as bioinoculants has played a vital role in plant survival and adaptation in nutrient-limiting environments, improving soil fertility, and sustaining plant growth and health (Bibi et al., 2017, Alkahtani et al., 2020,

Compant et al., 2020). The plant growth-promoting attributes of endophytes microbes have been linked to their multiple functions via various mechanisms to enhance plant growth, including biofertilization, biocontrol, and phytostimulation (Adeleke and Babalola, 2021c). Notably, PGPEB directly contributes to plant growth by producing phytohormones, such as gibberellins, cytokinins, and IAA (Fouda et al., 2021). Bacterial IAA can form a synergy with the endogenous plant IAA to stimulate plant growth. Also, PGPEB promotes plant growth by phosphate solubilization and nitrogen fixation, ammonia production, and production of extracellular enzymes, such as amylase, mannanase, catalase, protease, pectinase, cellulase, and chitinase (Dubey et al., 2021, Toghueo and Boyom, 2021). In addition, the ability of bacterial endophytes to colonize plant endosphere can induce biocontrol activity against phytopathogens through the synthesis of exopolysaccharides, antibiotics, siderophore, and hydrogen cyanide, thus improving plant immunity and reducing yield loss (Chowdhury and Bae, 2018).

Consequently, reports have shown that endophytic bacteria upon inoculation contribute to plant performance in terms of quality and quantity (Alkahtani et al., 2020, Knežević et al., 2021). For example, some root-associated endophytic bacterial genera, such as *Pseudomonas, Rhizobium, Stenotrophomonas, Bacillus, Burkholderia,* and *Pantoea* have been found to exert a positive influence on plant growth based on their ability to produce plant growth-promoting traits, hence, improving crop productivity (Adeleke et al., 2021, Mowafy et al., 2021, Preyanga et al., 2021). Furthermore, inoculation of oilseed crops, such as soybean, sunflower, and rapeseed with plant growth-promoting bacteria have boosted plant tolerance to stress adaptors and phytohormone synthesis for yield enhancement (Jiménez-Gómez et al., 2020, Ahmad et al., 2021, Dubey et al., 2021). Interestingly, an increase in the yield of oilseed crops inoculated

with endophytic, *Bacillus amyloliquefaciens*, *B. megaterium* M3, and *B. subtilis* OSU142, *Pseudomonas fluorescens* LBUM677 have been recently reported (Jiménez et al., 2020, Keshavarz, 2020, Sheteiwy et al., 2021).

Promisingly, characterization of agriculturally important endophytic bacteria from the endosphere of oilseed crops, and their inoculation efficacy under greenhouse and field experimental conditions have yielded success in enhancing plant performance (Lally et al., 2017, Abdel Latef et al., 2021), but less information is available on PGPEB isolated from sunflower cultivated in Southern Africa. Research into sunflower bacterial endophytes would ensure maximizing their full potential for improved crop productivity sustainably.

Sunflower is an economic crop ranked fourth among the most cultivated oilseed crops in North America and other continents (Bashir et al., 2020, Adeleke and Babalola, 2020b). Sunflower cultivation is a promising method to ensure food security and supply of nutritional and healthy food for both livestock and humans (Seiler et al., 2017). To maximally improve sunflower yield, seed inoculation with PGPEB before and after planting is necessary to optimize their effects on plant physiological functions, growth, and yield (Khare et al., 2018). Furthermore, harnessing endophytic microbes based on their multifunctional traits in agricultural management on large-scale cultivation of oilseed sunflower would advance food security and sunflower oil production (Adeleke and Babalola, 2020b, Adeleke and Babalola, 2021c). Therefore, this research is designed to isolate, characterize and screen agriculturally important root and stem associated bacterial endophytes for improved sunflower yield.

4.2 Materials and methods

4.2.1 Sampling and isolation of root and stems endosphere bacteria

The roots and stems of *H. annuus* cultivar PAN 7160 CLP were sourced from organic farmland located in Lichtenburg, North West Province, South Africa in February 2020. The climatic conditions of this region are characterized by an annual rainfall of 600 mm and a temperature range of 3 to 21^{o}C during winter and 22 to 34^{o}C during summer. The healthy sunflower samples were carefully uprooted, placed inside sterile zip-lock bags, and transported to the Microbial Biotechnology Research Group laboratory, North-West University, South Africa at 4^{o}C. In all, a total of 24 sunflower samples were randomly collected in triplicates from 4 points within a field for isolating sunflower endosphere bacteria.

4.2.2 Root and stem surface sterilization and isolation of endophytic bacteria

The roots and stems were cut into small sizes with a sterile scalpel and then washed in sterile distilled water. To ensure complete removal of the epiphytic bacteria, surface sterilization was achieved by soaking the samples in 70% ethanol for 3 minutes, followed by 3% sodium hypochlorite for 3 minutes, 70% ethanol for 30 sec., and lastly rinsed 5 times with sterile distilled water. The level of sterility of the samples was assessed by plating the last rinse on Luria Bertani (LB) media, Miller (Sigma Aldrich, USA), nutrient agar (NA), and tryptic soy agar (TSA) (Wiratno et al., 2019, Wang et al., 2020a). One gram (1 g) of plant material was weighed (Radwag weighing machine; Lasec; Poland), suspended in 1 M phosphate-buffered solution (FBS), and manually macerated in a mortar and pestle until a smooth suspension was obtained. Sample suspensions were dispensed into test tubes containing measured sterile distilled water (9 ml) as a stock solution and then transferred from tubes 10^{-1} up to 10^{-9}

dilutions. An aliquot of 0.1 ml from dilutions 10^{-5} and 10^{-6} was pipetted into Petri dishes and poured plated with sterilized LB agar, NA, and TSA prepared as stipulated in the manufacturer's guide. Inoculated Petri plates were incubated at 28°C for 24 hours. Distinct bacterial colonies formed on the plates were counted and selected based on morphological appearance. The representative of bacterial colonies formed is expressed as colony forming units (CFU) per gram. Pure cultures were obtained by repeated streaking on the fresh LB, NA, and TSA agar.

4.2.3 Gram staining and biochemical characterization of pure bacterial cultures

Gram staining and various biochemical tests were performed to characterize bacterial isolates. Oxidase, urease, starch hydrolysis, citrate, and sugar fermentation tests were performed following the modified methods described by Majeed et al. (2018). Other biochemical tests performed include Voges-Proskauer, hydrogen sulfide production, citrate test, and nitrate utilization, methyl red test, and indole production test (Duhan et al., 2020).

4.2.4 Screening of bacterial isolates for plant growth-promoting (PGP) traits

4.2.4.1 Ammonia production test

The ability of bacterial isolates to produce ammonia was tested according to the methods described by Alkahtani et al. (2020). Briefly, ammonia production test was performed as follows: 0.1 ml of 24-hour old bacterial culture (10^6 CFU/ml) was aseptically dispensed into test tubes containing 10 ml sterile peptone broth (peptone 0.2 g, 10 ml sterile water) by inoculation and incubated on a rotary shaker incubator (SI-600, LAB Companion, Korea) at 120 rpm, at ambient temperature for 96 hours. Consequently, Nessler's reagent (500 µl) was gently dispensed into each test tube, then allowed to stand for 5 minutes for color change

detection. The development of yellow to dark brown after Nessler's reagent was recorded positive for ammonia production. A tube without bacterial inoculation serves as a control.

4.2.4.2 Phosphate solubilization (PS) test

The phosphate solubilization potential of the bacterial isolates was evaluated according to the methods described by Premono et al. (1996). The qualitative test was performed on modified Pikovskaya agar composed of (g/L; tricalcium phosphate ($Ca_3(PO_4)_2$) 5, glucose ($C_6H_{12}O_6$) 10, manganese sulfate ($MnSO_4 \cdot H_2O$) 0.002, sodium chloride (NaCl) 0.2, potassium chloride (KCl) 0.2, magnesium sulfate ($MgSO_4$) 0.1, ammonium sulfate ($(NH4)_2SO_4$) 0.5, yeast extract 0.5, agar 15 with adjusted pH 7. Twenty-four (24) hour-old bacterial cultures grown in Petri plates were inoculated by spotting directly at the center of each Pikovskya's agar (PA) plate. Afterward, plates were incubated at 28^{o}C for 5 days for the visible zone of clearance (ZOC). The ZOC (mm) around the colony on the cultured plates indicated positive results for phosphate solubilization while the uninoculated plate serves as a control. The ZOC (mm) around the colony was measured, and colony diameter measurements (mm) were summarized as low (+), medium (++), and high (+++). The phosphate-solubilizing index (PSI) was enumerated as

$$PSI = \frac{\text{colony diameter (mm) + ZOC (mm)}}{\text{colony diameter (mm)}}$$

PSI was grouped as low (PSI < 2.00), intermediate (2.00 ≤ PSI < 4.00), or high (PSI ≥ 4.00) based on Marra et al. (2011) methods.

For the quantitative assay, phosphate solubilization tendencies of the bacterial isolates were performed by inoculating 10 ml sterile Pikovskaya broth in 50 ml Falcon tubes with 0.1 ml

(10^6 CFU/ml) freshly grown bacterial culture, incubated at 30°C for 5 days at 180 rpm on a rotary shaker machine. Bacterial supernatant was obtained after cold centrifugation of 10 ml bacterial cultures at 10,000 rpm for 5 minutes. Four milliliters (4 ml) of the color reagent (1:1:1:2 ratio of 3M H_2SO_4, 10% (w/v) ascorbic acid, 2.5% (w/v) ammonium molybdate, and distilled water) were added to 10% (w/v) of 5 ml trichloroacetic acid inside test tubes. The tubes were allowed to stand for 15 minutes upon incubation at room temperature. The quantity of phosphate content was measured according to phosphomolybdate, a blue method at an absorbance of 820 nm. The phosphate solubilization potential by the bacterium in the Pikovskaya broth was determined from the phosphate (KH_2PO_4) standard curve. Medium without bacterial inoculation served as control.

4.2.4.3 Siderophore production

The siderophore production ability of the bacterial isolates was performed according to the methods of Khan et al. (2020) with little modifications. Each bacterial isolate was aseptically inoculated into a sterilized medium amended with CAS, i.e., chrome azurol S. Preparation of CAS solution was performed by weighing 60.5 mg CAS into 10 ml of 1 mM iron (III) solution ($FeCl_3.6H_2O$) (a). The iron (III) solution was diluted in 10 mM HCl. The solution (a) was gently mixed with 0.0729 g i.e., 72.9 mg hexadecyltrimethylammonium (HDTMA, Merck, SA) bromide suspended in 40 ml sterile distilled water (b) on the magnetic stirrer. From the mixture of "a" and "b," solution, 100 ml was measured and added to 900 ml sterilized LB medium with adjusted pH 6.8. The medium was sterilized at 121°C for 15 minutes, allowed to cool, and pour plated into sterile Petri dishes. The bacterial culture was spot-inoculated at the mid-point of the solidified agar plates and incubated at 28°C for 5 days. The development of yellow ring

coloration around the bacterial colonies on the plates was reported positive for siderophore production. Un-inoculated plates served as control.

The quantity of siderophore production was determined by inoculating LB broth solution containing CAS with 0.1 ml of 24-hour old bacterial culture and incubated at 180 rpm on a rotary shaker for 7 days. Centrifugation of bacterial culture suspension was achieved at 8000 x g for 10 minutes. Five hundred microliters of the cell filtrate were added to 0.5 ml CAS reagent and properly mixed, then incubated for 2 minutes at room temperature. The quantity of siderophore released was measured at 630 nm using a spectrophotometer (Thermo Spectronic, Merck Chemicals, SA). The siderophore estimate was obtained from the regression equation of the standard curve. The experiment was carried out in triplicate.

4.2.4.4 Hydrogen cyanide production

The test for HCN production by bacterial endophytes was determined according to the modified method described by Igiehon et al. (2019). Nine milliliters of LB broth amended with 0.4% (w/v) glycine was aseptically dispensed into test tubes, sterilized, allowed to cool, and then inoculated with 0.1 ml (10^6 CFU/ml) fresh bacterial inoculum. Whatman filter paper No.1 was first dipped in 0.5% (w/v) picric acid and subsequently in 2% (w/v) sodium carbonate. After that, the filter paper was plugged into each test tube (without touching the broth solution), then screw-cap and stop-up with parafilm and incubated at 28^{o}C for 5 days. The test tubes were examined daily for the observed changes in the color of the filter paper. A color change from yellow to brown showed a positive result. The uninoculated tube served as control. The experiment was carried out in triplicate.

4.2.4.5 Indole acetic acid production

Notably, one major attribute of PGP bacteria is their ability to produce plant hormone, IAA, and was tested according to the modified method of Gutierrez et al. (2009). LB broth with or without L-tryptophan was used for the test. With L-tryptophan, 0.1% was amended into 100 ml LB broth inside test tubes, aseptically inoculated with 1 ml bacterial culture (10^6 CFU/ml), and then incubated at 28^{o}C for 7 days at 120 rpm in a rotary shaker incubator (SI-600, LAB Companion, Korea). The bacterial cell suspension pipetted into 2 ml tubes was subjected to cold centrifugation (4^{o}C) at 8,000 rpm for 10 minutes. IAA from the crude extract was measured by transferring 1,000 µl of the supernatant crude extract into a clean tube on addition with one drop of orthophosphoric acid (10 mM) and Salkowski reagent (2 ml) (1:30:50 ratio of 0.5 M $FeCl_3$ solution: 95% w/w sulfuric acid: distilled water). The mixing solution was incubated for 10 minutes at ambient room temperature. A positive result was based on the pink coloration in the tubes after incubation in the dark. The uninoculated plate served as control. Color development by the bacterial strains was grouped as low, average, and high. The IAA activity of the reacting mixture after incubation was estimated at 530 nm using UV-spectrophotometer (ThermoFisher Scientific, USA). IAA concentration was evaluated from the IAA gradient standard curve (SC). The experiment was performed in triplicate for mean value calculation.

4.2.5 Enzymes production

Screening for enzyme production was performed by plate assay techniques. The enzymes screened include, mannanase, cellulase, amylase, xylanase, and protease.

4.2.5.1 Mannanase

Screening of bacterial isolates for mannanase production was performed as described by Blibech et al. (2020) with little modifications. Briefly, media composition of g/L; Locust bean gum (3), dipotassium hydrogen phosphate (K_2HPO_4) 1, iron sulfate ($FeSO_4$) 0.001, ammonium chloride (NH_4Cl) 1, sodium chloride (NaCl) 0.5, calcium chloride ($CaCl_2$) 0.1, magnesium sulfate ($MgSO_4$) 0.5, and agar 13 was prepared at pH 7.2 before sterilization at 121^oC for 15 minutes and allow to cool. The media were pour plated and allowed to solidify. A 24-hour old bacterial culture was gently inoculated at the middle of the agar plates and then incubated at 28^oC for 2 days. Then, each cultured plate was observed after flooding with iodine solution for 15 minutes. The staining solution was poured off and further treated by flooding with 1 M sodium chloride (NaCl) for 15 minutes for the visible ZOC around the colonies. The colonies with a ZOC (mm) indicated mannanase production. The uninoculated plate served as control. Each experiment was carried out in triplicate for each bacterial isolate.

4.2.5.2 Cellulase

The qualitative screening of endophytic bacteria for cellulase production was performed using plate assay techniques according to Alkahtani et al. (2020) with little modifications. Freshly grown pure cultures of bacterial isolates were inoculated by single streaking on carboxymethyl cellulose amended media composed of g/L; dipotassium hydrogen phosphate (K_2HPO_4) 1, sodium nitrate ($NaNO_3$) 3, iron sulfate ($FeSO_4$) 0.01, carboxymethyl cellulose (CMC) 1, potassium chloride (KCl) 0.5, magnesium sulfate ($MgSO_4$) 0.5, and agar 20 at pH 7.0. The inoculated CMC plates were incubated at 28^oC for 48 hours and then flooded with 1% (w/v) Congo red (CR) for 10 minutes. The CR on the plates was gently washed off and plates were further washed with 1 M NaCl for 15 minutes. The ZOC (mm) encircling the colonies indicated

a positive result for cellulase production. The uninoculated plate served as control. Negative result plates were further flooded with 5% acetic acid solution for 2 minutes and then washed with sterile distilled water. A clear ZOC (mm) around the colony was determined and recorded. The experiment was carried out in triplicate for each bacterial isolate.

4.2.5.3 Amylase

The amylase production was tested on starch agar according to the methods of Alkahtani et al. (2020) with little modification. A 24-hour bacterial culture was spot-inoculated on sterilized starch agar medium composed of peptone 5 g, magnesium sulfate ($MgSO_4$) 0.5 g, yeast extract 5 g, iron sulfate ($FeSO_4$) 0.01 g, soluble starch 10 g, agar 15 g, and sodium chloride (NaCl) 0.01 g in 1 Liter of sterile water and then incubated at 37^oC for 48 hours. After that, Lugol's iodine solution (iodine 0.4%, potassium iodide 0.8%, distilled water – 200 ml) was poured on the plates for 10 minutes. The formation of a ZOC (mm) around each bacterial isolate on the plate indicated amylase production. The uninoculated plate served as control. The experiment was carried out in triplicate for each bacterial isolate.

4.2.5.4 Xylanase

Screening of bacterial isolates for xylanase production on mineral salt medium (MSM) supplemented with 0.5% xylan (beechwood) was performed according to the methods described by Alkahtani et al. (2020) with minor modifications. The MSM compositions include, agar 2%, peptone 0.5%, yeast extract 0.3%, and sodium chloride (NaCl) 0.5%. The final pH of the media solution was adjusted to 9 before sterilization at 121^oC for 15 minutes. The media were allowed to cool and pour plating. Plates were inoculated with fresh (24-hour old) bacterial culture by straight streak at the mid-point of the plates and then incubated at 28^oC

for 24 hours. After that, plates were flooded with 0.4% Congo red and incubated for 10 minutes, then washed with 1 M NaCl to evaluate the ZOC. The ZOC around the bacterial isolates on each plate is considered positive for xylanase production. The uninoculated plate served as control. The experiment was carried out in triplicate.

4.2.5.5 Protease

The primary screening for each endophytic bacterium for protease synthesis was performed on LB agar plates supplemented with skim milk powder according to the methods described by Alkahtani et al. (2020) with little modifications. The media composition (g/L) includes, skim milk powder 28, dextrose 1, casein 5, yeast extract 2.5, and agar 15 at pH 7. The media were prepared, sterilized, and then allowed to cool before pouring plating. After that, fresh bacterial culture was inoculated on each plate and incubated at 28^{o}C for 2 days. The bacterial isolates exhibiting a circular ZOC (mm) indicated a positive result for protease production. An un-inoculated plate served as control. The experiment was performed in triplicate.

4.2.6 Molecular identification of plant growth-promoting bacterial endophytes

4.2.6.1 DNA extraction

The genomic content of pure bacterial isolates was extracted using a commercial Quick-DNA^{TM} Miniprep Kit specific for fungi or bacteria (Zymo Research, Irvine, CA, USA; Cat. No. D6005), following the manufacturer's guide. The quantity of the extracted DNA (ng/µl) was measured using a NanoDrop ND- 2000 UV-Vis spectrophotometer (ThermoFisher Scientific, USA) and stored at -80^{o}C.

4.2.6.2 PCR-amplification and sequence analysis

The determination of 16S rRNA nucleotide sequences of the identified bacterial isolates was achieved using the amplified PCR products. The specific forward and reverse primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) purchased from Inqaba Biotechnological Industrial (Pty) Ltd, Pretoria, South Africa. A total of 25 µl reaction volume for each bacterial isolate composed of 12.5 µl OneTaq 2X MasterMix with the Standard Buffer, 1 µM for each primer, approximately 5 ng genomic DNA, and 9.5 µl nuclease-free water were used for PCR amplification on DNA Engine DYAD^{TM} Peltier Thermal Cycler (BIO-RAD, USA, C1000 Touch^{TM}). The PCR cycle parameters were programmed as follows: initial denaturation at 94°C for 5 minutes; 35 cycles of amplification. Also, the denaturation for 30 seconds at 94°C, annealing for 30 seconds at 50^{o}C, and extension for 1 minute at 68°C; and a final overall extension for 10 minutes at 68°C. After running PCR, the PCR product was determined on agarose gel electrophoresis. Subsequently, the gel was carefully removed, and confirmation of the expected size of the product was visualized on a UV trans-illuminator. The resulting outcome was captured in a Chemidoc^{TM} imaging system (BIO-RAD Laboratories, California, USA). Finally, 20 µl of the PCR product for each bacterial isolate was placed in an ice-box pack and sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria of South Africa. 16S rRNA gene sequences for each bacterium were submitted to GenBank on the NCBI online server and were assigned with accession numbers. The strain code and accession numbers of the 16S rRNA gene sequence data of the identified bacterial endophytes deposited in the GenBank include the SFR35, MW265416; FTL29, MW265418; CAL14, MW265422; T4S, MW265423; BS27, MW265425; BOVIS40, MW265419; JVB5, MW265431. Also, PK60, MW265415; VS52, MW265429; BAAG44,

MW265424; SFS19, MW265430; OLT2020, MW265417, BSA123, MW265426, TSN29, MW265420; BLBS20, MW265427; FOBS21, MW261910; SFL02, MW265413; LS11, MW265414; J4R, MW265421; and VEJU7080, MW265428.

4.2.7 Inoculum preparation and seed treatment

Based on the *in vitro* screening tests of the isolated bacteria, three bacterial isolates namely, *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 with most promising plant growth-promoting traits, such as ammonia, exopolysaccharide, siderophore, IAA, HCN, etc were selected for seed treatment and effect of mono-inoculation (1 bacterial strain), co-inoculation (2 bacterial strains) and combined inoculation (3 bacterial strains) were tested under greenhouse experimental conditions. A seed inoculation assay was used to facilitate bacterial adherence to the disinfected sunflower seeds. Surface sterilization of the seeds was performed as described in section 4.2.2 above. The effectiveness of sunflower seed inoculation was performed following the methods of Ullah et al. (2017). The bacterial inoculum size in LB broth at 24-hour incubation was standardized to 0.5 (10^6 CFU/ml) at OD_{600}. Prepared LB broth inoculated with fresh bacterial culture was incubated on shaker incubator machine (SI-600, LAB Companion, Korea) at 180 rpm for 24 hours. The bacterial cells in the broth culture were harvested by cold centrifugation at 8,000 x g for 10 minutes to obtain the pelletized cells and then washed in 0.85% normal saline solution. The centrifugation and washing of the pellets were performed under sterile conditions. The sterilized seeds were suspended in a bacterial liquid medium (suspension) containing 1% (v/w) carboxymethyl cellulose (CMC) in a 250 ml flask and agitated for 60 minutes (Arkhipova et al., 2019, Konappa et al., 2020). The CMC serves as a sticker that causes the adhesion of bacterial mass to the seeds. The seeds suspended

in sterile distilled water containing 1% (v/w) CMC without bacterial inoculum served as control.

4.2.8 Greenhouse experimental study

The soil used for the experiment was sourced from agricultural farmlands in North-West University, Mafikeng Campus. Soil debris and other plant materials were removed, air dry, and sieved with a 2 mm micro stainless steel mesh sieve, and the soil was placed inside autoclavable plastic bags and sterilized at 121^{o}C for 15 minutes. Sterilized soil was allowed to cool for 2 days after which 15 kg of soil was transferred into plastic pots. The inoculated and non-inoculated pots were arranged in a complete randomized design (CRD) in triplicates for each treatment at a 10 cm distance apart in a greenhouse under natural light. The plastic pots measured 34 cm diameter and 29 cm tall were washed with sterile water and sterilized with 15% sodium hypochlorite solution before filling with 15 kg dry-sterilized loamy soil. The pots containing the sterile soil were moistened with 500 ml sterilized water before sowing. Ten seeds were sown per pot at a depth of 1.5 cm. After seed emergence, i.e., at 8 days, thinning was performed, leaving one sunflower seedling in a pot. Growing sunflower seedlings were maintained on a day-night cycle of 13-14 hours natural light, a temperature of 30±2^{o}C, and relative humidity of 85%. The pots were moistened with an equal amount of water and maintained daily. Pots containing seedlings without inoculation serve as control. The plants were harvested at maturity, 132 days after planting.

4.2.8.1 Sunflower morphological parameters below-and-above-ground level

The data collected based on sunflower morphological parameters after harvesting include the number of lateral roots, root number, fresh weight, root and shoot weight (dry), taproot length,

etc. These parameters were considered following the method described by Igiehon et al. (2019). After harvesting from a greenhouse, sunflower plants were taken to the laboratory. The soil adhering to plant roots were thoroughly washed under running sterile water. The fresh weight of roots and shoots was measured using a weighing balance (Wagi Elektroniczne, Poland). Also, lateral roots and root numbers were determined. Furthermore, plant roots and shoots were oven-dried at 60°C for 24 hours and re-weighed on a weighing balance for determining roots and shoot dry weights.

4.2.8.2 Determination of sunflower yield parameters

The sunflower yield parameters were obtained above plant level. The parameters considered include seed weight (fresh and dry), and head weight (fresh and dry). After drying, sunflower seeds were manually separated from the sunflower head containing seeds. Seeds were dried in an oven at 60°C for 24 hours, and dry weight was measured on a weighing balance. The plant samples were kept in plastic bags for further analysis. The whole experiment was repeated for each treatment.

4.2.9 Data analysis

The analysis of data from this study was analyzed using Statistical Package for the Social Sciences (SPSS) version 16.0. One-way analysis of variance (ANOVA) was performed for the data, followed by the Duncan test at a 5% level of significance to determine differences between mean. For each treatment, generated data were presented as arithmetic mean±standard deviation.

4.3 Results

4.3.1 Bacterial endophytes isolation and identification

The bacteria associated with the root and stem endosphere of sunflower collected at Lichtenburg were isolated by culture-dependent techniques. Bacterial count (CFU/g) was successfully recovered after serial dilution and plating methods from all media. The total bacterial counts (CFU/g x 10^6) of the stem and roots of sunflower on different media is presented in Figure 4.1. From the isolation step, the selection of the bacterial isolates was based on the observed differences in their morphological characteristics, and further biochemical characterization was conducted. The results of bacterial characterization based on cultural, biochemical characterization, and sugar fermentation tests are presented in Tables 4.1a and 4.1b. Based on Gram reaction, 70% of the bacterial isolates were identified as Gram-positive while 30% were Gram-negative. The Gram-negative bacterial endophytes identified include *Pseudomonas* sp. FOBS21, *Stenotrophomonas maltophilia* PK60 and JVB5, *S. indicatrix* BOVIS40, *P. saponiphilia* J4R, and *P. lini* BS27. All the bacterial isolates are rod in shape when viewed under a light microscope. Also, they show positive results for oxidase, hydrogen sulfide production, Voges-Proskauer, and methyl red.

Bacillus thuringiensis BSA123 is positive for citrate test, while nine were positive for gelatin liquefaction, *B. cereus* SFR35 can utilize indole, *B. thuringiensis* BSA123 were positive for casein hydrolysis. For nitrate utilization, 8 of the bacterial isolates produce gas (N_2) while 16 reduce nitrate. All the isolates were urease negative. For sugar fermentation tests, all of the isolates ferment glucose, fructose, arabinose, mannitol, xylose, and maltose, respectively. Based on molecular identification, *Bacillus* species were the most common identifiable bacteria in the stem and root samples (Table 4.2).

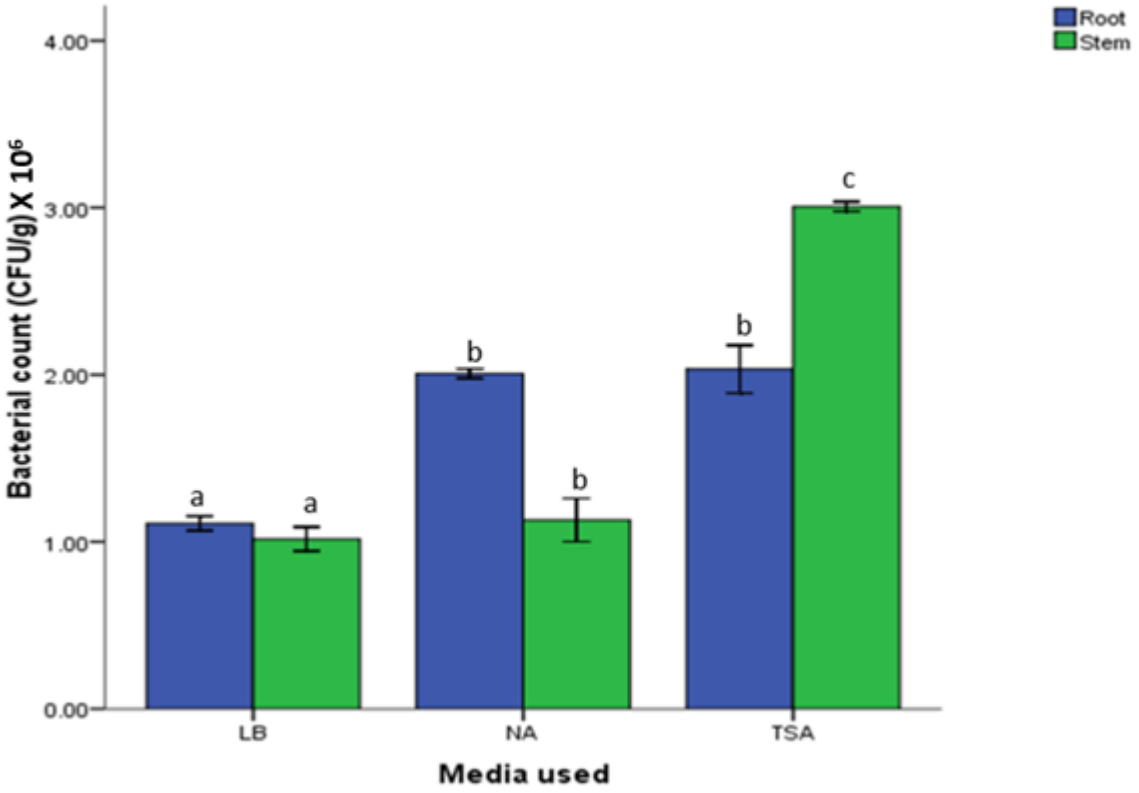


Figure 4.1: Total bacterial counts (CFU/g) of the stem and roots of sunflower on different media. Different alphabets with the root and stem samples indicate a significant difference of the triplicate readings

Table 4.1a: Biochemical characterization of bacterial isolates from sunflower

Strain	Shape	GR	CT	Gel	OX	SH	H_2S	VP	MR	MT	NT		IND	CS	UR
											Clr	Gas			
SFR35	Rod	+	+	+	+	+	+	+	+	+	+	-	+	-	-
FTL29	Rod	+	-	+	+	+	+	+	+	+	+	-	-	+	-
CAL14	Rod	+	+	-	+	+	+	+	+	+	-	+	-	+	-
T4S	Rod	+	+	-	+	+	+	+	+	+	+	-	-	+	-
BS27	Rod	-	+	-	+	+	+	+	+	+	+	-	-	+	-
BOVIS40	Rod	-	+	-	+	-	+	+	+	+	+	-	-	-	-
JVB5	Rod	-	+	-	+	+	+	+	+	+	+	-	-	+	-
PK60	Rod	-	+	+	+	+	+	+	+	+	+	-	+	+	-
VS52	Rod	+	+	+	+	+	+	+	+	+	+	-	-	+	-
BAAG44	Rod	+	+	+	+	-	+	+	+	+	+	-	-	-	-
SFS19	Rod	+	-	-	+	-	+	+	+	+	+	-	-	+	-
OLT2020	Rod	+	-	-	+	-	+	+	+	+	+	+	+	+	-
BSA123	Rod	+	+	+	+	+	+	+	+	+	-	+	-	+	-
TSN29	Rod	+	+	-	+	+	+	+	+	+	+	+	-	+	-
FOBS21	Rod	-	+	-	+	+	+	+	+	+	+	-	-	+	-
BLBS20	Rod	+	+	-	+	+	+	+	+	+	-	+	-	-	-
SFL02	Rod	+	+	-	+	+	+	+	+	+	+	+	+	+	-
LS11	Rod	+	-	+	+	+	+	+	+	+	-	+	+	+	-
J4R	Rod	-	-	+	+	+	+	+	+	+	+	+	-	+	-
VEJU7080	Rod	+	+	+	+	-	+	+	+	+	+	-	-	-	-

Key: + - positive, - - negative, GR - Gram reaction, Clr – color, CT- citrate, Gel – gelation, OX – oxidase, SH – starch hydrolysis, H2S – hydrogen sulfide, VP – Vogue Proskauer, MR – methyl red, NT – nitrate, IND – indole, CS – casein, UR – urease, MT - motility

Table 4.1b: Sugar fermentation test

Isolate code	**Sugars used**								
	Mal	Gal	Glu	Arab	Suc	Fru	Raff	Mann	Xyl
SFR35	+A	+AG	+Ag	+A	+Ag	+A	+A	+A	+A
FTL29	+A	+Ag	+Ag	+A	+Ag	+A	-a	+A	+A
CAL14	+A	+Ag	+AG	+A	+Ag	+A	+A	+A	+A
T4S	+A	+AG	+AG	+A	+Ag	+A	+A	+A	+A
BS27	+A	+Ag	+AG	+A	+Ag	+A	+A	+A	+A
BOVIS40	+A	+Ag	+Ag	+A	+Ag	+A	-a	+A	+A
JVB5	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A
PK60	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A
VS52	+A	+AG	+AG	+A	+AG	+A	-a	+A	+A
BAAG44	+A	-ag	+AG	+A	+Ag	+A	+A	+A	+A
SFS19	+A	-ag	+Ag	+A	-ag	+A	+A	+A	+A
OLT2020	+A	+AG	+AG	+A	+AG	+A	-a	+A	+A
BSA123	+A	+AG	+AG	+A	+Ag	+A	+A	+A	+A
TSN29	+A	+Ag	+AG	+A	+Ag	+A	-a	+A	+A
FOBS21	+A	+AG	+Ag	+A	+Ag	+A	+A	+A	+A
BLBS20	+A	+AG	+Ag	+A	-ag	+A	+A	+A	+A
SFL02	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A
LS11	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A
J4R	+A	-ag	+Ag	+A	+Ag	+A	+A	+A	+A
VEJU7080	+A	+AG	+AG	+A	+Ag	+A	+A	+A	+A

Key: ag = no acid and gas production, AG - acid and gas production, + = positive, - = negative, Suc – sucrose, Glu – glucose, Fru – fructose, Arab – arabinose, Mann – mannose, Xyl – xylose Mal – maltose, Raff - raffinose

Table 4.2: Taxonomic classifications of the 16S rRNA gene of the identified bacterial isolates

Strain	Identity	% SIM	GBAN	HA	INS	AM	CL	XL	MN	PR
SFR35	*Bacillus cereus*	100	MW265416	MW092893	R	-	+	-	+	+
FTL29	*B. wiedmannii*	99	MW265418	MZ292345	R	+	+	+	+	+
CAL14	*Bacillus* sp.	95	MW265422	MK554656	R	-	+	-	-	+
T4S	*B. cereus*	100	MW265423	MW115619	R	-	+	+	+	+
BS27	*Pseudomonas lini*	94	MW265425	JQ833637	R	-	+	+	-	-
BOVIS40	*Stentotrophomonas indicatrix*	100	MW265419	MW116366	R	+	+	+	+	+
JVB5	*S. maltophilia*	100	MW265431	MT605498	R	-	+	-	-	+
TSN29	*B. albus*	99	MW265420	MT636856	R	-	+	-	+	-
BLBS20	*B. cereus*	100	MW265427	MT543036	R	-	+	-	-	-
SFL02	*B. thuringiensis*	99	MW265413	MK743981	R	-	+	+	+	-
PK60	*S. maltophilia*	97	MW265415	MK588914	S	+	+	-	+	+
VS52	*B. subtilis*	100	MW265429	MT613731	S	+	+	+	+	+
BAAG44	*B. thuringiensis*	98	MW265424	MK743981	S	-	+	-	+	+
SFS19	*B. pseudomycoides*	99	MW265430	MK999393	S	-	+	-	-	-
OLT2020	*B. toyonensis*	100	MW265417	MT605503	S	+	+	+	+	-
BSA123	*B. thuringiensis*	100	MW265426	JX994096	S	+	+	+	+	+
LS11	*B. paramycoides*	100	MW265414	MW090883	S	+	+	-	+	+
J4R	*P. saponiphilia*	100	MW265421	MT501808	S	-	+	-	+	+
VEJU7080	*B. cereus*	99	MW265428	MH231418	S	-	+	+	+	+
FOBS21	*Pseudomonas* sp.	100	MW261910	MT561438	S	-	+	-	+	-

Key: SIM – similarity, GBAN - GenBank accession number, HA - homologous accessions, ISN - isolation source, AM – amylase, CL – cellulase, XL – xylanase, MN – mannanase, PR – protease, R – root, S – stem, + - positive reaction, - - negative reaction

The zero E-v value was recorded for all the isolates. Each bacterial strain was designated as FOBS21, SFL02, LS11, PK60, SFR35, OLT2020, FTL29, BOVIS40, TSN29, J4R, CAL14, T4S, BAAG44, BS27, BSA123, BLBS20, VEJU7080, VS52, SFS19, and JVB5. Ten each were obtained from the roots and stems of the sunflower (Table 4.2).

4.3.2 Bacterial identification by molecular method and 16S rRNA gene amplification

Ten (10) bacterial each from the stem and roots of sunflower was molecularly identified, as shown in Table 4.2. The 16S rRNA gene sequences were analyzed on the NCBI-BLAST server. The sequence results from GenBank showed a high percentage identity of 95 to 100% of the bacterial isolates except for *P. lini* BS27 with 94% identity. The sequence analysis shown in Table 4.2 revealed that Gram-negative bacterial strains of FOBS21, PK60/JVB5, BOVIS40, J4R, and BS27 belong to *Pseudomonas* sp., *Stenotrophomonas maltophilia*, *S. indicatrix, P. saponiphilia* and *P. lini,* respectively, while Gram-positive bacterial isolates in the genus *Bacillus* were also identified.

4.3.3 Plant growth-promoting (PGP) screening

The functional characterization of plant growth-promoting traits performed is presented in Table 4.3. The qualitative screening revealed the ability of the bacterial isolates to produce HCN based on the color change observed in the filter paper. The ability of bacterial isolates to produce ammonia has been attributed to enhancing plant growth. All bacterial isolates screened indicated a positive reaction for HCN production.

Table 4.3: Plant growth-promoting screening of endophytic bacteria

Strain	HCN	AM	SDR	IAA	EPS	PS	PS (%)	PSI (mm)	SDR (%)
B. cereus SFR35	++	+	+	+	++	+	29.96±0.03^{e}	4.09±0.08^{l}	64.93±0.04^{k}
B. wiedmannii FTL29	+	+	++	+	+++	+	30.96±0.03^{e}	2.65±0.04^{e}	15.30±0.04^{d}
Bacillus sp. CAL14	++	+	++	+	+++	+	33.83±0.04^{g}	3.14±0.05^{j}	52.96±0.04^{i}
B. cereus T4S	++	+	+++	+	+++	+	30.54±0.05^{e}	2.62±0.02^{e}	87.73±0.05^{q}
Pseudomomas lini BS27	++	+	+	+	++	+	30.62±0.03^{e}	3.07±0.12^{hij}	36.90±0.02^{f}
Stenotrophomonas indicatrix BOVIS40	++	+	+++	+	+++	+	32.81±0.18^{g}	2.10±0.10^{a}	77.33±0.05^{n}
S. maltophilia JVB5	++	+	+++	+	+++	+	32.20±0.17^{g}	2.95±0.05^{g}	79.81±0.17^{o}
B. albus TSN29	++	+	+	+	+	+	26.11±0.03^{d}	3.50±0.02^{k}	80.50±0.02^{p}
B. cereus BLBS20	+	+	+	+	+	+	31.82±0.14^{f}	2.50±0.04^{d}	0.53±0.03^{a}
B. thuringiensis SFL02	+	+	+++	+	+	+	26.48±0.03^{c}	2.41±0.01^{c}	77.72±0.04^{n}
S. maltophilia PK60	++	+	+++	+	+++	+	33.48±0.03^{g}	3.03±0.06^{ghi}	58.50±0.02^{j}
B. subtilis VS52	++	+	+++	+	+	+	33.43±0.04^{g}	2.82±0.01^{f}	17.73±0.04^{e}
B. thuringiensis BAAG44	++	+	+++	+	++	+	15.76±0.21^{a}	2.43±0.03^{cd}	70.12±0.03^{m}
B. pseudomycoides SFS19	+	+	+++	+	+++	+	27.64±0.04^{d}	2.17±0.03^{a}	68.89±0.09^{l}
B. toyonensis OLT2020	+	+	+	+	++	+	22.64±0.03^{b}	3.01±0.02^{gh}	5.72±0.02^{b}
B. thuringiensis BSA123	++	+	++	+	++	+	24.95±0.04^{c}	2.79±0.03^{f}	64.92±0.04^{k}
B. paramycoides LS11	++	+	++	+	+	+	29.75±0.22^{e}	2.80±0.03^{f}	44.53±0.03^{g}
P. saponiphilia J4R	++	+	+	+	+	+	33.24±0.05^{g}	3.11±0.02^{ij}	12.91±0.01^{c}
B. cereus VEJU7080	++	+	+++	+	+	+	24.55±0.04^{c}	2.66±0.02^{e}	46.10±0.02^{h}
Pseudomonas sp. FOBS21	+	+	++	+	+	+	31.32±0.02^{b}	2.31±0.02^{b}	53.34±0.04^{i}

Key: IAA = indole-3-acetic acid, AM = ammonia, HCN = hydrogen cyanide, EPS = exopolysaccharide**,** PS = phosphate solubilization, SDR = siderophore. The values of triplicate readings represented as mean±standard deviation with different alphabets down the column show a significance difference

Also, all the bacterial isolates screened solubilize phosphate on phosphate agar plates by displaying a circular zone of clearance around the cultured bacterial isolates after incubation and the phosphate solubilizing indices are presented in (Table 4.3). The quantitative results revealed a high siderophore value of 87.73 % in strain T4S (Table 4.3). Also, isolated bacterial species displayed varied IAA activities at different L-tryptophan (L-TRYP) concentrations. As mentioned in the literature, L-TRYP serves as a precursor for IAA biosynthesis by different bacterial and other microorganisms (Ngoma et al., 2013). In this study, varying concentrations of L-TRYP were supplemented in LB broth.

The confirmation of IAA synthesis by the bacterial isolates was observed based on color development on the addition of Salkowski reagent to the cultured tubes after incubation. The rate of IAA production in LB broth without L-TRYP was low compared to the LB broth supplemented with varying concentrations of L-TRYP (Figure 4.2). At a 6% concentration, bacterial strains, BOVIS40, T4S, and JVB5 displayed the highest IAA activity of 10.44 µg/ml, 11.85 µg/ml, and 16.94 µg/ml of all isolates (Figure 4.2). The lowest IAA activity was observed in strain SFR35 compared with the control. Also, the indication of siderophore production based on the circular zone of clearance surrounding the colony on CAS agar plates after incubation and a total of 9 isolates exhibited high, while 5 exhibited medium and 6 exhibited low activity for the siderophore production (Table 4.2).

4.3.4 Enzyme screening

Each bacterial isolate was screened for five important enzymes, namely, amylase, cellulase, xylanase, mannanase, and protease. Enzyme production for each bacterial isolate was revealed after plate screening.

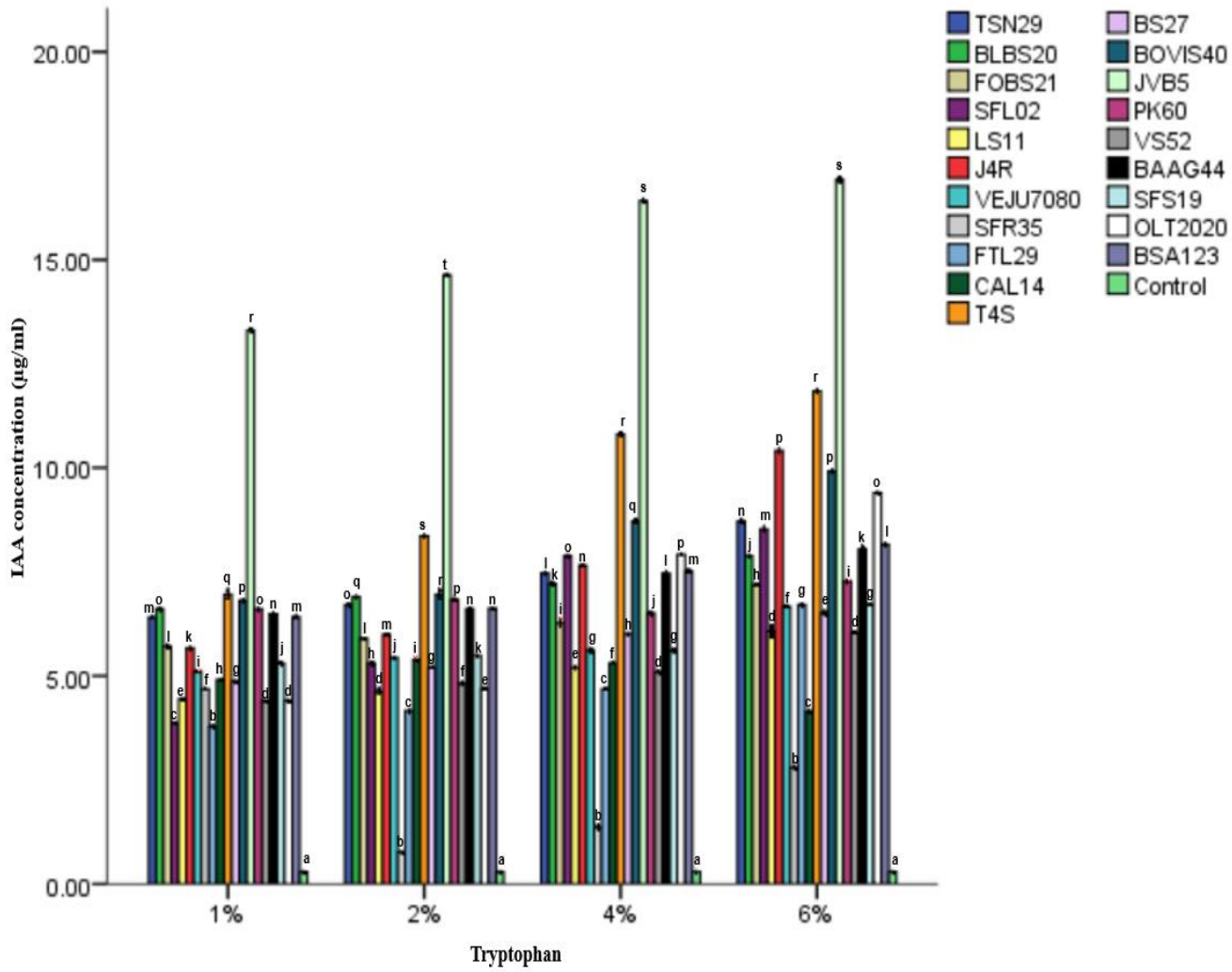


Figure 4.2: Qualitative screening of indole acetic acid production by bacteria endophytes. Bacterial isolate codes are represented in Table 4.2. Different alphabets with the root and stem samples indicate significant differences of triplicate readings

Some bacterial isolates utilize substrate in the medium, thus showing positive results. Briefly, bacterial strains, BOVIS40, FTL29, VS52, and BSA123, exhibited a positive reaction to all enzymes assayed (Table 4.2). Similarly, the bacterial isolates were positive for cellulase production. Except for amylase, bacterial isolates VEJU7080 and T4S were positive for other enzymes screened. In summary, bacterial isolates designated FTL29, TSN29, BSA123, and BAAG44 displayed amylase, xylanase, mannanase, and protease production tendencies, respectively. The qualitative screening of enzyme production by the bacterial isolates is presented in Table 4.4.

4.3.5 Plant growth-promoting screening

Below-and-above-ground sunflower yield parameters were recorded from this study and results showed significant differences among the treatments compared with control. Interestingly, multiple bacterial inoculations yielded significant results compared to mono-and-co-inoculation. The shoot and taproot lengths of 161.67 mm and 253.67 mm were recorded from the sunflower inoculated with multiple bacterial strains (Figure 4.3). Similar results were also observed in the seed wet weight (0.07 g), seed dry weight (0.043 g), head wet and dry weights (179.93 g and 56.94 g), shoot wet and dry weights (227.95 g and 93.14 g), and plant wet weight of 454.86 g as presented in Figure 4.4.

Table 4.4: Qualitative screening of enzyme production by the bacterial isolates

Isolate	**Zone of clearance (mm)**				
	AM	XL	PR	CL	MN
B. cereus SFR35	0.00±00^{a}	0.00±00^{a}	36.24±0.40^{m}	44.03±0.06^{a}	43.06±0.05^{g}
B. wiedmannii FTL29	20.05±0.05^{c}	40.02±0.03^{f}	18.03±0.03^{e}	47.06±0.05^{b}	42.04±0.04^{f}
Bacillus sp. CAL14	0.00±00^{a}	0.00±00^{a}	10.06±0.06^{b}	59.05±0.05^{g}	0.00±00^{a}
B. cereus T4S	0.00±00^{a}	41.02±0.04^{g}	35.05±0.04^{l}	57.02±0.02^{e}	45.00±0.01^{h}
Pseudomonas lini BS27	0.00±00^{a}	32.96±0.06^{c}	30.00±0.11^{i}	58.07±0.11^{f}	18.02±0.55^{b}
Stenotrophomonas indicatrix BOVIS40	44.05±0.05^{e}	35.04±0.04^{e}	16.03±0.02^{d}	50.10±0.10^{d}	43.05±0.05^{g}
S. maltophilia JVB5	0.00±00^{a}	0.00±00^{a}	25.06±0.05^{d}	58.08±0.11^{f}	0.00±00^{a}
B. albus TSN29	0.00±00^{a}	0.00±00^{a}	0.00±00^{a}	48.04±0.05^{c}	54.04±0.05^{k}
B. cereus BLBS20	0.00±00^{a}	0.00±00^{a}	0.00±00^{a}	69.10±0.10^{i}	0.00±00^{a}
B. thuringiensis SFL02	0.00±00^{a}	47.05±0.05^{i}	0.00±00^{a}	60.03±0.03^{h}	30.05±0.06^{e}
S. maltophilia PK60	18.04±0.04^{b}	0.00±00^{a}	31.01±0.03^{j}	59.03±0.06^{g}	18.04±0.55^{b}
B. subtilis VS52	20.05±0.04^{c}	35.04±0.04^{d}	22.03±0.06^{f}	59.02±0.07^{g}	51.06±0.06^{j}
B. thuringiensis BAAG44	0.00±00^{a}	0.00±00^{a}	30.06±0.06^{i}	58.06±0.06^{f}	18.02±0.04^{b}
B. pseudomycoides SFS19	0.00±00^{a}	0.00±00^{a}	0.00±00^{a}	58.02±0.02^{f}	0.00±00^{a}
B. toyonensis OLT2020	0.00±00^{a}	43.05±0.06^{h}	0.00±00^{a}	60.03±0.03^{h}	20.05±0.05^{c}
B. thuringiensis BSA123	18.01±0.05^{b}	40.06±0.06^{f}	35.02±0.03^{l}	47.04±0.05^{b}	42.02±0.04^{f}
B. paramycoides LS11	25.04±0.04^{d}	0.00±00^{a}	29.04±0.04^{h}	58.04±0.05^{f}	45.03±0.04^{h}
P. saponiphilia J4R	0.00±00^{a}	0.00±00^{a}	33.10±0.10^{l}	59.08±0.10^{g}	18.06±0.06^{b}
B. cereus VEJU7080	0.00±00^{a}	32.03±0.03^{b}	15.03±0.05^{c}	48.04±0.04^{c}	50.01±0.02^{i}
Pseudomonas sp. FOBS21	0.00±00^{a}	0.00±00^{a}	32.03±0.03^{k}	50.24±0.40^{d}	25.02±0.03^{d}

Key: AM – amylase, CL – cellulose, XL – xylanase, MN – mannanase, PR – protease. The values of triplicate readings represented as mean±standard deviation with different alphabets down the column show a significant difference

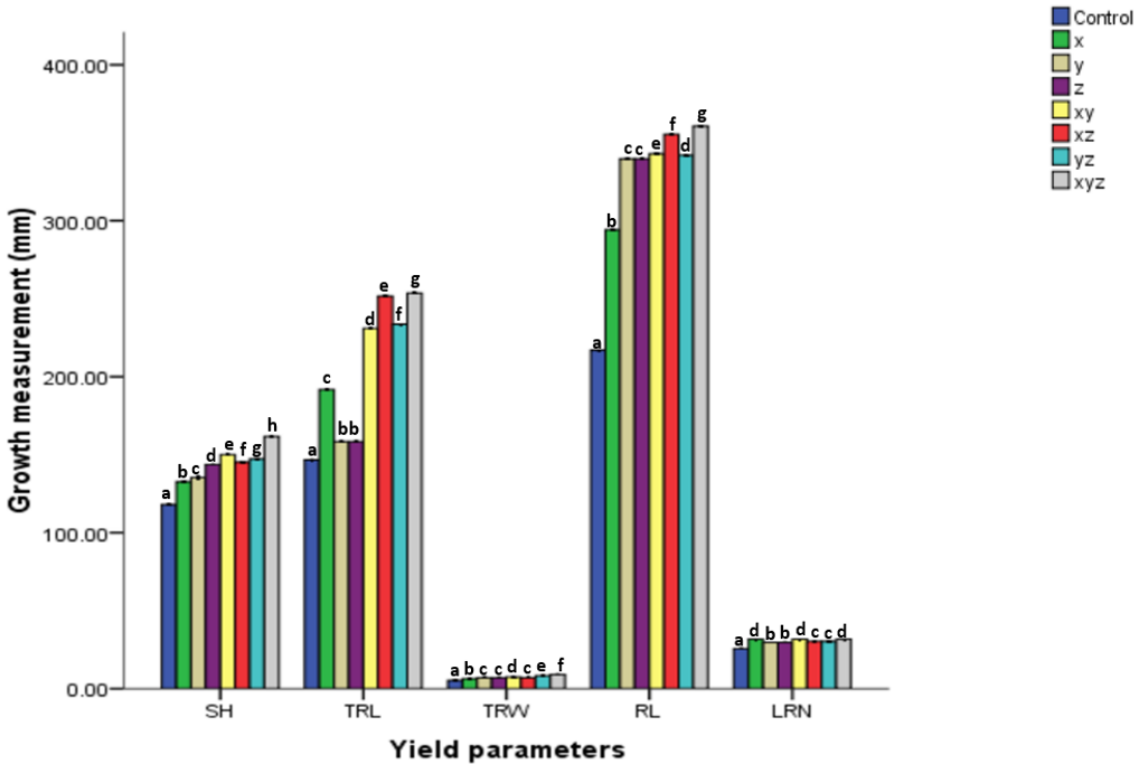


Figure 4.3: Effect of plant growth-promoting endophytic bacteria treatment on sunflower shoot and belowground parameters under greenhouse experiment. x - *S. indicatrix* BOVIS40, y – *B. cereus* T4S, x – *S. maltophilia* JVB5, xy – *S. indicatrix* BOVIS40 + *B. cereus* T4S, xz - *S. indicatrix* BOVIS40 + *S. maltophilia* JVB5, yz - *B. cereus* T4S + *S. maltophilia* JVB5, xyz - *S. indicatrix* BOVIS40 + *B. cereus* T4S + *S. maltophilia* JVB5. Data presented include, shoot height (SH), taproot length (TRL), taproot width (TRW), root length (RL), and lateral root number (LRN) relative to the non-treated control. Different letters along with the yield parameters and control indicate significant differences in triplicate readings

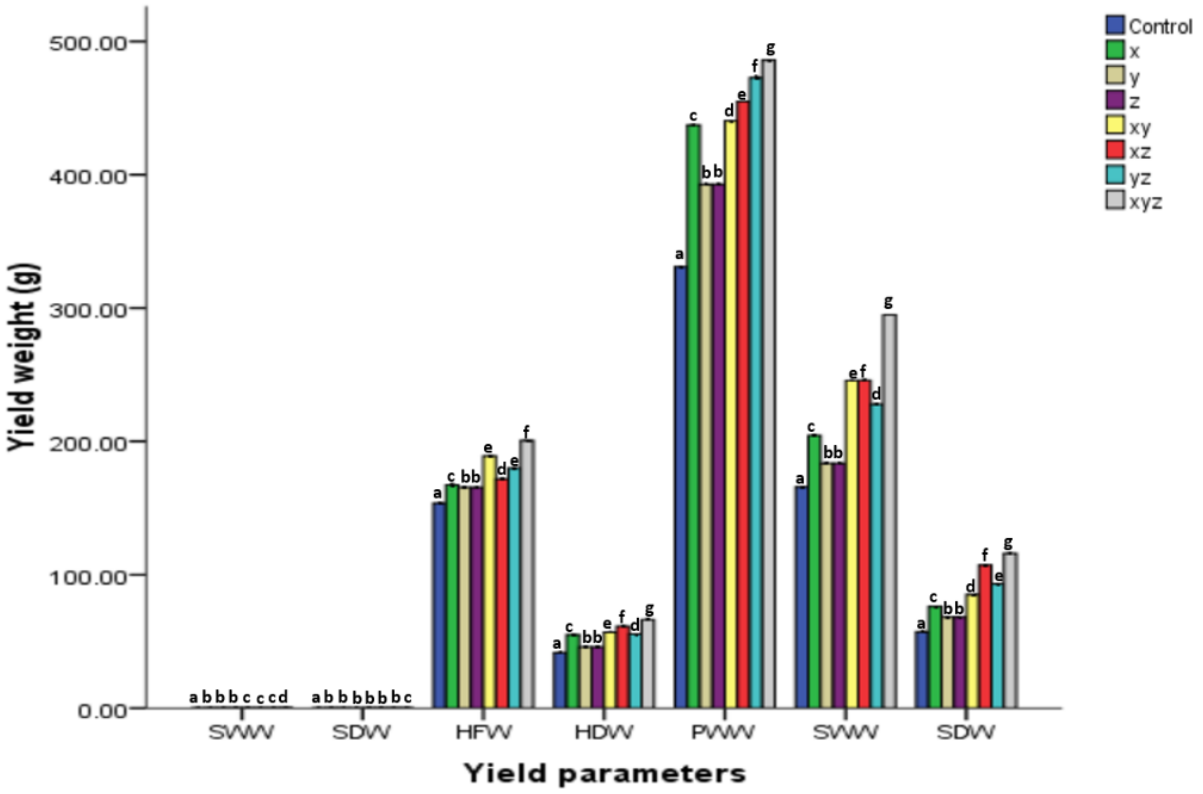


Figure 4.4: Effect of plant growth-promoting endophytic bacteria treatment on sunflower aboveground parameters under greenhouse experiment. x - *S. indicatrix* BOVIS40, y – *B. cereus* T4S, z – *S. maltophilia* JVB5, xy – *S. indicatrix* BOVIS40 + *B. cereus* T4S, xz - *S. indicatrix* BOVIS40 + *S. maltophilia* JVB5, yz - *B. cereus* T4S + *S. maltophilia* JVB5, xyz - *S. indicatrix* BOVIS40 + *B. cereus* T4S + *S. maltophilia* JVB5. Data presented include, seed wet and dry weight (SWW and SDWH), head fresh and dry weight (HFW and HDW), shoot wet and dry weight (SHWT and SHDW), and plant wet weight (PWW) relative to the non-treated control. Different letters along with the yield parameters and control indicate significant differences in triplicate readings

4.4 Discussion

Plants harbor diverse agriculturally important microbial endophytes with notable plant growth-promoting traits (Alkahtani et al., 2020). The isolation, characterization, and identification of bacterial endophytes from plants and their survival tendencies under different climatic conditions can underline their exploration as a suitable candidate in formulating bioinoculants for improved crop yield sustainably (Khalil et al., 2021). The biostimulatory effects of some endophytic microbes upon application have contributed to general plant performance in terms of drought tolerance, production of volatile compounds, root development, and antibiosis against phytopathogens (Fouda et al., 2021). The growth of plants is being modulated by the amount of phytohormone produced by microbial endophytes inhabiting the endosphere (Passari et al., 2016). For instance, IAA production significantly enhances seedling growth, development, and elongation of lateral roots and cell differentiation (Shahzad et al., 2017). Hence, harnessing beneficial microbial endophytes can be promising in agricultural biotechnology.

In this study, we report the combination of culturing and molecular techniques in the characterization of bacterial colonizing roots and stem endosphere and their effect on sunflower growth. The biochemical characterization of the isolated bacteria reflected the most identifiable Gram-positive endophytes compared to Gram-negative members. Twenty bacterial isolates were studied and screening tests confirm their multifunctional attributes to produce plant growth biostimulants. Previously, the bacteria identified in this study have been isolated from oilseed crops with plant growth promotion attributes (Ambrosini et al., 2016, Schmidt et al., 2021). For example, the genus *Bacillus* and *Stenotrophomonas* have been reported to efficiently colonize plant tissue with biocontrol and plant growth-promoting activities (Borah

et al., 2021). Also, findings have revealed identifiable *Bacillus* from sunflower plants (Forchetti et al., 2007). As identified in this study, the *Stenotrophomonas* species are known as opportunistic bacteria inhabiting the rhizosphere and the endosphere of plants (Ulrich et al., 2021), nevertheless, recent genomic findings of endophytic *Stenotrophomonas* strain 169 have revealed their ability in enhancing plant growth and stress tolerance (Ulrich et al., 2021). Also, the synergistic interaction of endophytic *S. rhizophila* and *S. maltophilia* BJo1 with soybean have been reported to partake in modulating plant physiology, biochemical activities, inducing salt tolerance, and plant growth promotion (Egamberdieva et al., 2016, Alexander et al., 2019). However, from this study, the identifiable bacterial endophytes corroborate earlier reports of Ambrosini et al. (2012), who isolated similar PGPB, such as *Stenotrophomonas* sp., *Burkholderia* sp., *Pseudomonas* sp., *Rhizobium* sp. from the root of sunflower.

Different bacterial endophytes have been documented for their ability to produce IAA as well as their involvement in IAA biosynthetic pathways (Annapurna et al., 2018, Shastry et al., 2020). IAA synthesis by the bacterial endophytes can suggest their immense contribution to the root formation by increasing root surface area to facilitate plant access for uptake of essential nutrients and water directly from the soil (Kumari et al., 2018). In this study, it was revealed that all the screened bacterial isolates exhibited IAA synthesis with varied activity. The highest IAA activity of 16.95 µg/ml was obtained in *S. maltophilia* JVB5 while the lowest activity of 2.79 µg/ml was produced by *B. cereus* SFR35 at 6% L-tryptophan supplementation compared with the control. The increase in the amount of IAA produced by the bacterial isolates in the IAA production medium (IPM) might be due to the presence of L-tryptophan and this conforms with earlier findings of Chukwuneme et al. (2020), who reported that the secretion of IAA can be enhanced by the addition of tryptophan to the IPM. IAA production

ability of *B. amyloliquefaciens* FZB42 in a tryptophan-dependent medium and effect on plant growth promotion has been reported (Idris et al., 2007). Promisingly, inoculating sunflower plants with IAA producing endophytic bacteria can contribute to plant growth promotion and increased tolerance to environmental stressors. The IAA potential displayed by the bacterial isolates corroborates the findings of Bashir et al. (2020), who reported the IAA production potential of *Bacillus* spp isolated from the endosphere of sunflower. Furthermore, versatile PGPEB, such as *P. stutzeri, B. subtilis, S. maltophilia, B. cereus,* and *B. thuringiensis* native to the sunflower with IAA production potential have caused an improvement in sunflower growth, seed germination, root elongation, and crop yield (Pandey et al., 2013, Singh et al., 2019).

Phosphate solubilization is one of the major mechanisms employed by microbial endophytes in plant growth promotion (Ambrosini et al., 2012). Several endophytic bacterial genera, such as *Stenotrophomonas, Bacillus,* and *Pseudomonas* have been reported as phosphate solubilizers (Pandey et al., 2013). Phosphate-solubilizing bacteria (PSB) are free-living beneficial bacteria that help in the transformation process of insoluble phosphate in soil into absorbable form for plant benefits (Haile et al., 2016). In this study, all the bacterial isolates were tested positive for phosphate and considered PSB. This PSB significantly play an important role in the bioavailability of phosphate into soluble form for plant use, thus reducing the over-dependence on chemical fertilizers on the soil (Valetti et al., 2018). These bacteria can be harnessed with greater potential and a promising tool in future agricultural production. Diverse PSB associated with plant endosphere has been reported to increase phosphate levels in the soil (Alkahtani et al., 2020, Varga et al., 2020). Shahid et al. (2015) had identified phosphate solubilizing bacterial *Bacillus* sp. from the endosphere of sunflower with the potential of enhancing crop yield. Also, the characterization of mineral phosphate-solubilizing bacteria, *Bacillus* sp. Ps-5

and *Alcaligenes faecalis* Ss-2 for enhanced sunflower growth and yield-attributing traits have been reported (Shahid et al., 2015). The results obtained from this study corroborate with (Pandey et al., 2013) and Vandana et al. (2021), who reported phosphate-solubilizing *Stenotrophomonas*, *Bacillus*, and *Pseudomonas* from the root endosphere of oilseed crops, sunflower, and soybean. The phosphate solubilization potential of bacterial isolates might depend on the optimum growth conditions, genetic make-up, and limited nutrient supply (Youseif, 2018). Furthermore, the results obtained in this study compared to other studies confirmed the phosphate-solubilizing potential of sunflower-associated PGPEB, thus showing the potential of making soluble phosphate minerals available in the soil for plant nutrition.

Harnessing siderophore-producing microbes can be promising in agriculture in lowering the stress imposed on plants growing in heavy metals contaminated soils (Ferreira et al., 2019). The siderophore-producing bacterial endophytes associated with economic plants have been reported to stimulate plant growth promotion, high iron acquisition, and antibiosis against plant pathogens (Maheshwari et al., 2019b). In this study, differences in siderophore production by the isolated bacterial endophytes were observed and this may be due to the varied siderophore-producing ability and genetic make-up of the organism (Maheshwari et al., 2019a). Siderophore producing ability of endophytic bacteria associated with *Vitis vinifera* has been reported to increase mineral elements in the soil (Andreolli et al., 2016). Plant responses against toxic metals may suggest a strong linkage to the siderophore-producing ability of endophytes associated with medicinal plants (Passari et al., 2016, Maheshwari et al., 2019b). Pourbabaee et al. (2018) had reported the potential contribution of siderophore-producing bacteria on growth and Fe ion concentration of sunflower under water stress.

The action of endosphere colonizing bacteria to secrete metabolic compounds against plant pathogens is known, and this may include the secretion of volatile compounds like hydrogen cyanide (HCN), enzymes, ammonia, and siderophores (Alkahtani et al., 2020). HCN production by bacteria can inhibit cell metabolism and electron transport chain, thus causing cell death. The production of HCN and siderophores by the bacterial isolates can offer them a competitive advantage to be used as biocontrol agents in plant disease suppressiveness due to the limited available nutrients for the bacterial metabolism in the natural econiches (Maheshwari et al., 2019b). Also, the induction of biosynthesis in the bacterial isolates may cause the secretion of antimicrobial compounds by the tested bacterial isolates (Alkahtani et al., 2020). The results obtained in this study revealed that the bacteria screened displayed a positive reaction for HCN production. Also, the ability of plant growth-promoting endophytic bacteria to produce ammonia as a secondary metabolite, importantly, in stimulating antibiosis effects has been reported (Khan et al., 2020). All the bacterial isolates produced ammonia and HCN, thus suggesting their use as a biocontrol agent. In this study, the HCN and ammonia production by the bacterial isolates conforms to the findings of Pandey et al. (2013) and Moin et al. (2020) reported the production of HCN, ammonia, and volatile antifungal metabolites by an endophytic bacterium, *Pseudomonas* isolated from healthy sunflower plants. Similarly, the survival of bacteria in the genera *Bacillus* in diverse ecological systems have been linked to their ability to produce, exopolysaccharide, signal molecules, multilayered cell wall structure, extracellular enzymes, and the formation of stress-resistant endospores (Lyngwi et al., 2016). Microbes serve as a potential source of enzymes and their use in industries is promising because they can easily be obtained from low-cost organic substrates. They are easy to isolate, with high catalytic activity, relative thermostability, production, and optimization. Screening of

extracellular enzymes, cellulases, proteases, xylanases, and chitinases from plant microbes have been documented (Alkahtani et al., 2020, Blibech et al., 2020), and their profound demand for various industrial applications (Toghueo and Boyom, 2021), although the novel attributes of some of these enzymes from bacteria inhabiting the roots and stem of sunflower endosphere have not been documented.

The substrate level and growth conditions may influence the enzyme production ability of the bacterial isolates in the growth medium (Yadav, 2017). Regarding the enzyme activity and biotechnological view, the endophytic bacteria associated with sunflower can be employed as bioinoculants and upon application in degrading complex waste compounds to enhance the bioavailability of nutrients in the soil for plant use.

In this study, bacterial endophytes with promising *in-vitro* phytostimulant activities, i.e., *B. cereus* T4S, *S. maltophilia* JVB5, and *S. indicatrix* BOVIS40 were selected to assess their effect on sunflower growth performance under greenhouse experiment. The bacterial isolates were proportionately applied; singularly, dually, and combined compared to the control. From the pot experiment, the combined treatment (xyz) showed a higher yield compared to other treatments (x, y, z, xy, xz, and yz) and the control. At the initial stage of inoculation, the effects of the bacterial isolates on the plant might be slow due to environmental adjustment (acclimatization) and colonization, nevertheless, once established the PGPEB contributes to plant growth and health (Khamwan et al., 2018a). The difference in the yield parameters, below and above ground may be due to the bacterial treatment and nutrient availability between plants and endophytic bacteria (de Aquino et al., 2018). Plant inoculation with endophytic microbes has been reported to contribute to plant health, nutrition, and resistance against phytopathogens (Etesami and Alikhani, 2016a, Fouda et al., 2021, Arif et al., 2021). The results obtained in this

study are in agreement with the findings of Shahid et al. (2015), who observed an improvement in sunflower yield inoculated with endophytic bacteria *Alcaligenes faecalis* Ss-2 and *Bacillus* sp. Ps-5 in a pot experiment. Furthermore, inoculation of sunflower with copious PGPEB under controlled experiment and their contributions in plant growth promotion can suggest maximizing their potential in agricultural biotechnology.

4.5 Conclusion

This study provides information on bacterial strains associated specifically with one plant species, i.e. *H. annuus*, and the three most copious plant growth-promoting strains contributing to sunflower yield. Interestingly, the efficacy of strains, *Stenotrophomonas indicatrix* BOVIS40, *S. maltophilia* JVB5, and *Bacillus cereus* T4S to produce ammonia, yield high IAA at 6% tryptophan supplementation and solubilize phosphate can underline the nutrient acquisition, overall root development in plants, and plant growth. Also, the differential effects of singularly, dually or combined inoculation were profound on the sunflower yield compared to the control, thus suggesting the promising use of these strains as bioinoculants and alternative to agrochemicals. Hence, this present study provides insights into the possible exploration of sunflower root-associated bacterial endophytes as bioinoculants and applications in agriculture for improved sunflower yield in a sustainable manner.

5.0 CHAPTER FIVE

Optimization of process parameters for indole acetic acid production ability of sunflower associated bacteria

Abstract

Indole acetic acid (IAA) is one of the key phytohormones produced by diverse endosphere microbes that essentially partake in various cellular metabolisms for plant growth promotion. Phytohormone synthesis by microorganisms serves as an important indicator in the development of plants. Here, we optimized various process parameters of the IAA-producing culture medium by considering the effect of incubation time, temperature, pH, carbon, and nitrogen sources at different concentrations. The plant growth-promoting bacterial strains, namely, *Stenotrophomonas indicatrix* BOVIS40, *S. maltophilia* JVB5, and *Bacillus cereus* T4S from the root endosphere of sunflower (*Helianthus annuus*) were employed in this study. The maximum IAA activity was attained at 164 hours of incubation. The IAA medium amended with yeast extract yielded high IAA activity of 46.43 µg/ml by strain BOVIS40. Optimum IAA production of 23.36 µl/ml at 5% sucrose by JVB5 and 20.72 µl/ml at 3% glucose by T4S was recorded upon incubation. Also, at pH 7.0, maximum IAA activity of 25.36 µl/ml was obtained from strain BOVIS40. All the isolates produced considerable high IAA activity at temperatures 25^{o}C, 30^{o}C, and 37^{o}C. The seed inoculation assay by bacterial strains enhances root length, lateral root number, and shoot development in sunflower seedlings. The results from this study validate the ability of bacteria associated with sunflower to produce indole acetic acid under different growth conditions. Therefore, harnessing their potential in agriculture as bioinoculants can contribute to root development for nutrient and water absorption from the

soil with great effects on plants' survival under nutrient-limiting soil and drought, hence adding value to agricultural productivity.

Keywords: Crop improvement, endo-rhizobacteria, *Helianthus annuus*, phytohormone synthesis, seed inoculation

5.1 Introduction

Sunflower stands as one of the most cultivated crops after maize, cowpea, wheat, and sorghum in the North West Province (NWP) of South Africa (Adeleke and Babalola, 2020b). As an important agricultural crop, its economic value is high and sunflower oil is readily available in South African markets. An increase in crop yield depends on the bioavailability of essential nutrients in the soil environment for plant growth (Adeleke and Babalola, 2020b). Nutrients are made available to plants through the activities of rhizosphere and endosphere microbes, thus contributing to plant growth for crop improvement to meet the high demand for food production to the growing population (Rashid et al., 2016). Diverse endophytic microbes modulate plant growth via the synthesis of plant growth hormones such as auxin, gibberellin, cytokinins, ethylene, etc. Many reports on plant growth-promoting activities of microorganisms inhabiting the root endosphere of plants such as sunflower, rice, turmeric, kidney bean, etc. have been documented (Kumar et al., 2016, Bertani et al., 2016, Majeed et al., 2018, El-Attar et al., 2019).

In recent times, research focusing on the screening of microorganisms capable of producing phytohormones and their application in agriculture for crop improvement and stable rhizosphere biology is on the increase. Like rhizosphere rhizobacteria, many bacteria inhabiting the internal tissue of plants produce indole acetic acid as a plant inducer (Babalola and Akindolire, 2011, Adeleke and Babalola, 2020a). Endosphere represents the entire internal tissues of plants colonized by microorganisms, most especially the dominant beneficial bacterial type (Wang et al., 2020a). Environmental stressors can influence the survival of microbes in the plant endosphere, thus limiting their ability to produce IAA (Shymanovich and Faeth, 2019, Enebe and Babalola, 2019). Interestingly, the endosphere houses microbial

domains, namely, bacteria, fungi, or archaea, with bacteria commonly found in abundance compared with other microbes (Duhan et al., 2020). The secretion of exudates from the root of plants can be a factor responsible for the selection of endo-rhizosphere beneficial bacteria in the host plants. Nevertheless, the ability of rhizosphere bacteria to utilize metabolic compounds released from plant roots enhances their infiltration capacity into plants to become endophytes (Omomowo and Babalola, 2019).

Studies on the screening and efficacy of beneficial bacterial communities from the roots of economic plants and their effects on crop yield have been documented (Chandra et al., 2018). To mention a few, many endophytic bacterial genera *Pantoea, Paenibacillus, Bacillus, Stenotrophomonas, Mimosa, Rhizobium, Methylophilus, Pseudomonas,* and *Aneurinibacillus* recognized as plant growth-promoting endophytes (PGPE) have been widely applied in agriculture for improved crop production (Muresu et al., 2019, Sánchez-Cruz et al., 2019, Duhan et al., 2020).

Indole acetic acid (IAA) helps in plant growth promotion with main functions ranging from abscission, plant response to stimuli (geotropism and phototropism), root elongation, development, leaf formation, fruits, and embryo development (Khan et al., 2017, Shahzad et al., 2017). Auxin production enhances root development, increases the number of root hairs and lateral roots, thus supporting the plant in the absorption of nutrients from the soil. Reports have shown that the addition of sugars to the growth medium inoculated with *Rhizobium* yielding a maximum IAA production have contributed to the activities of endophytic microbes in the supply of required energy (Ullah et al., 2017, Chandra et al., 2018). The importance of IAA production from endophytic bacteria in plant development is known, and further discovery

of other plant hormones can be applied synergistically for improved crop yield (Bhutani et al., 2018).

The vast information on the ability of plant microbes to produce a substantial amount of IAA can further help in understanding specific pathways undergone by these microorganisms (Baral et al., 2018, Ahmad et al., 2020). At the developmental stage of plants, a large amount of water-soluble exudates such as fatty acids, amino acids, organic acids, and sugars are released from the root of plants. These compounds provide essential metabolic compounds required by plants and microbes to produce IAA (More et al., 2019). IAA synthesis by PGPBE may function less as a hormone in microbial cells, but may rather ensure plant-microbe interactions (Liu et al., 2016b). Here, the optimization of process parameters, for IAA synthesis by endophytic bacteria was conducted. This study focused on IAA production from endophytic bacteria using different substrates under different growth conditions.

5.2 Materials and methods

5.2.1 Source of plant-associated bacteria

The bacterial strains (BOVIS40, T4S, and JVB5) used for this study were isolated from the roots of sunflower plants. The isolates were identified based on cultural identification and molecular studies as described by Duhan et al. (2020). 16S rRNA sequences for the bacterial isolate were submitted to GenBank on the NCBI online server and were assigned accession numbers. The strain code and accession numbers of the 16S rRNA gene sequence data of the identified bacterial endophytes deposited in the GenBank are T4S, MW265423; BOVIS40, MW265425; JVB5, MW265419, respectively. Sunflower roots were collected from commercial farmlands in Lichtenburg, NWP of South Africa. The plants were carefully dug

out, labeled, and placed in sterile zip-lock bags for transportation to the laboratory for microbial analysis. Sunflower roots were cut into small sizes and surface sterilized and macerated using a mortar and pestle in sterile form. One gram (1 g) of the samples was weighed and aseptically prepared in sterile tubes filled with 9 ml sterile distilled water to form a stock solution. After that, 1 ml of the aliquot from the stock solution was pipetted from one tube to the next up to 10^{-9} dilutions. From dilutions 10^{-5} and 10^{-6}, 100 µl of the suspension were gently dispensed into Petri plates. The plates were pour-plated into molten sterilized Luria Bertani (LB) media and incubated at 28±2^{o}C for 24 hours. Colonies from the plates were observed and selected based on morphological characteristics for further purification by streaking on fresh LB agar plates. Pure bacterial colonies were stored on agar slants at refrigeration temperature. Based on the high IAA activity exhibited by the bacterial strains (results presented below in this study), they were further selected for optimization by considering growth parameters such as nitrogen source, carbon source, pH, and temperature.

5.2.2 Bacterial screening for IAA production

Indole acetic acid synthesis by the bacterial isolates was conducted according to Gutierrez et al. (2009). Ten milliliters (10 ml) LB broth supplemented with tryptophan were aseptically inoculated with 100 µl freshly grown bacterial culture (10^{6} CFUml^{-1}) and incubated at 28^{o}C for 7 days at 120 rpm in a rotary shaker incubator (SI-600, LAB Companion, Korea). The bacterial cell suspension was cold centrifuged (4^{o}C) at 8,000 rpm for 10 minutes to obtain a free cell supernatant layer. From the crude extract, 1 ml of the culture supernatant was measured and transferred into a clean tube and 2 ml Salkowski reagent (1:30:50 ratio of 0.5 M $FeCl_3$ solution: 95% w/w sulfuric acid: distilled water) was added. Then, 1 drop of 10 mM orthophosphoric acid was also added to the mixture. The mixing solution was incubated for 10

minutes at room temperature for color development. The appearance of pink coloration in the tubes after incubation in the dark indicated a positive result. An un-inoculated tube served as control. From the reacting mixture and control tube after incubation, the absorbance was determined at 530 nm using UV-spectrophotometer (ThermoFisher Scientific, USA). IAA concentration was evaluated from the IAA gradient standard curve (SC).

5.2.3 IAA assay

Different concentrations of L-tryptophan (5, 10, 20, 50, and 100 mg/ml) were weighed and prepared according to standard assay techniques in sterile distilled water. To each of the concentrations, Salkowski reagent was added to the dilution (1:2), followed by 1 drop of orthophosphoric acid, and then incubated for 10 minutes to observe color formation. After incubation, the absorbance for each solution was measured at 530 nm. The readings obtained were used to plot the standard curve.

5.2.4 Media preparation and optimization process

Varied concentrations of carbon and nitrogen sources were supplemented into IAA production medium (IPM) composed of (g/L); yeast extract 6, L-tryptophan 1, peptone 10, and NaCl 5 at pH 7.6 (Chandra et al., 2018). Other optimized conditions include incubation time, temperature, and pH. A 24-hour grown bacteria strain BOVIS40, T4S, and JVB5 with 0.5 optical density at 630 nm was inoculated into 200 ml IPM inside 500 ml conical flasks and then incubated at 37^{o}C, 180 rpm for 11 days. One ml of the medium was constantly withdrawn at 24 hours intervals and assayed for IAA production by Salkowski's reagent as described in section 5.2.2. Furthermore, similar incubation conditions were used to monitor the effects of other parameters under the same IAA assay conditions. The ability of bacterial isolates to

utilize carbon as substrate and their effects on IAA production was tested using 5 sugars, namely maltose, fructose, sucrose, glucose, and galactose, at different concentrations (1%, 3%, and 5%) as described by Khan et al. (2020). Additionally, the ability of bacterial isolates to utilize nitrogenous-base compounds such as peptone, potassium nitrate, casein, yeast extract, and urea as a substrate was tested at different concentrations of 1%, 3%, and 5% for IAA production (Chandra et al., 2018). The pH of the medium ranging from 4 to10 was examined for IAA production. pH was adjusted using 1 M of NaOH or HCl. IPM was optimized at varied temperatures of 25^{o}C, 30^{o}C, 37^{o}C, 45^{o}C, and 60^{o}C. After incubation, the supernatant was subjected to IAA assay and IAA concentration was measured at 630 nm for pH and temperature, and 590 nm for carbon and nitrogen, respectively, using a spectrophotometer (Thermo Spectronic; Meck, South Africa).

5.2.5 Sunflower seed inoculation and effects on seedling growth

A seed inoculation assay was used to facilitate bacterial adherence to the disinfected sunflower seeds. The effectiveness of sunflower seed inoculation was performed based on the methods described by Ullah et al. (2017). The bacterial inoculum size in LB broth at 24-hour incubation was standardized to 0.5 (10^{6} CFUml^{-1}) at OD_{600}. The 3 strains used for this study were selected based on their high plant growth-promoting characteristics. Cleaning of the seeds was performed by washing in sterile distilled water to remove floating - unhealthy seeds and dirt and disinfected in 70% ethanol for 3 minutes, followed by 3% hypochlorite for 1 minute, then immersed in 70% alcohol for 2 minutes with final washing with sterile distilled water (5 times). Prepared LB broth inoculated with fresh bacterial culture was incubated at room temperature in a rotary incubator machine at 180 rpm for 24 hours. The broth culture was centrifuged to obtain the pelletized cells after washing in 0.85% normal saline and re-suspended in the same

solution. The surface-sterilized seeds were suspended in a bacterial suspension containing 1% (v/w) carboxylmethyl cellulose (CMC) as an adhesive (binder) in a 250 ml flask for 60 minutes. The seeds suspended in sterile distilled water without bacterial inoculum served as control. Under sterile conditions, 10 coated seeds (of each bacterial strain) were placed inside Petri dishes lined with moistened sterile absorbent cotton and sealed with parafilm. The plates were kept at 28^{o}C in the growth chamber and seedling growth was monitored daily for 5 days. Each treatment was performed in triplicates. The plates were carefully taken out for seedling growth assessment. The fresh and dry weight of seedlings after oven drying at 60^{o}C were measured as described by Igiehon et al. (2019). The seedling's fresh and dry weight obtained was expressed in gram (g) per triplicate.

5.2.6 Statistical analysis

The analysis of data from this study was performed using SPSS - Statistical Package for the Social Sciences (v.6.0) and Microsoft Excel. Data obtained were presented as mean±standard deviation. A significant difference among the treatment groups was calculated using ANOVA - one-way analysis of variance, and DMRT - Duncan's Multiple Range Test was used to compare the treatment means. When the *p*-value obtained is < 0.05, it is considered significant.

5.3 Results

In this study, three bacterial strains, *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 from the roots of sunflower with high IAA activity were selected for the optimization assay. The IAA production ability of the bacterial endophytes was observed to be higher in L-tryptophan amended medium (Figure 5.1).

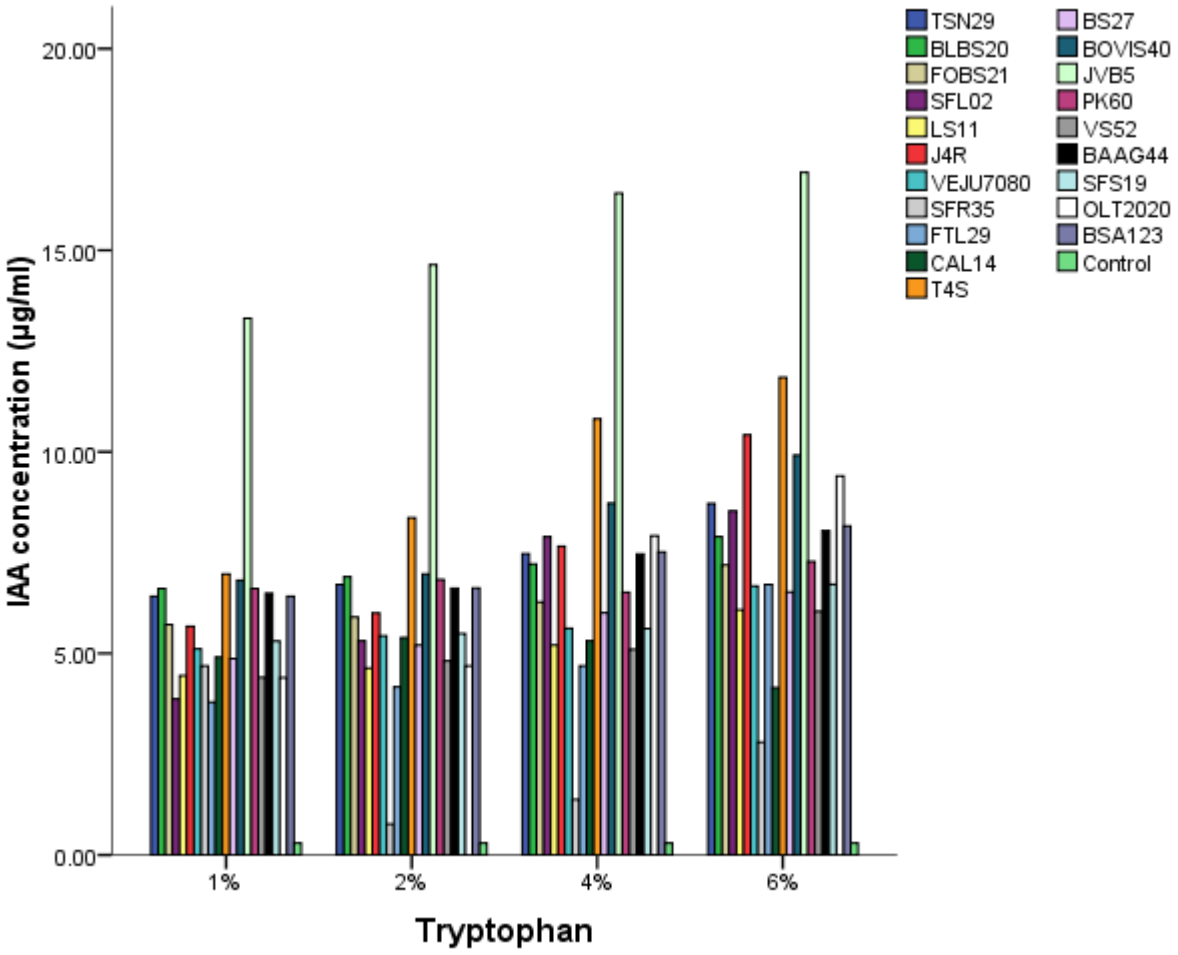


Figure 5.1: Qualitative screening of indole acetic acid production by bacterial endophytes. Key: SFR35 - *Bacillus cereus,* FTL29 - *B. wiedmannii,* CAL14 - *Bacillus* sp., T4S - *B. cereus,* BS27 - *Pseudomonas lini,* BOVIS40 – *Stenotrophomonas indicatrix,* JVB5 – *S. maltophilia,* TSN29 - *Bacillus albus,* BLBS20 - *B. cereus,* SFL02 - *B. thuringiensis,* PK60 – *S. maltophilia,* VS52 - *B. subtilis,* BAAG44 - *B. thuringiensis,* SFS19 - *B. pseudomycoides,* OLT2020 - *B. toyonensis,* BSA123 - *B. thuringiensis,* LS11 - *B. paramycoides,* J4R - *Pseud. saponiphilia,* VEJU7080 - *B. cereus,* FOBS21 - *Pseudomonas* sp.

5.3.1 Effect of incubation time and pH

The time course for IAA production by each bacterial strain in the IAA growth medium is presented in Figure 5.2. The IAA activity recorded increased progressively with an increase in incubation time (0-264 hours). Optimum IAA activity was attained at 168 hours and beyond this point, IAA activity declined. The optimum IAA activity of 16.94 µg/ml, 11.76 µg/ml, and 9.92 µg/ml at 168 hours were recorded for strain JVB5, T4S, and BOVIS40, respectively. The results of IAA produced by the bacterial isolates monitored between pH 4-10 are represented in Figure 5.3. IAA activity of bacterial strain increased from pH 4 to pH 7, and beyond this point, a decline in IAA activity was observed from pH 8 to pH 10. Bacterial isolates produced varying amounts of IAA at pH 6, 7, and 8, where strain BOVIS40 showed maximum IAA production of 25.36 µg/ml, followed by strain T4S, 12.34 µg/ml and TVB5, 5.46 µg/ml at pH 7. Similarly, maximum IAA activity (11.83 µg/ml) by strain JVB5 at pH 6, 10.74 µg/ml at pH 8, with the least activity of 2.83 µg/ml at pH 4 were obtained. At pH 9 and 10, no significant difference was observed in the IAA activity of strain T4S and JVB5.

5.3.2 Effect of temperature

The effect of incubation temperature could provide information on whether the bacterial strains can produce IAA at high or low temperatures. The IPM was subjected to a temperature range of 25^{o}C - 60^{o}C (Figure 5.4). Maximum IAA activity of 34.40 µg/ml was exhibited by isolate T4S at 37^{o}C. The amount of IAA produced at temperatures 45^{o}C and 60^{o}C was low compared with other temperatures. Bacterial strains show an increase in IAA activity from temperature 25^{o}C to 37^{o}C before it declined, although, at 30^{o}C and 37^{o}C, IAA activity displayed by JVB5 was not significantly different from each other.

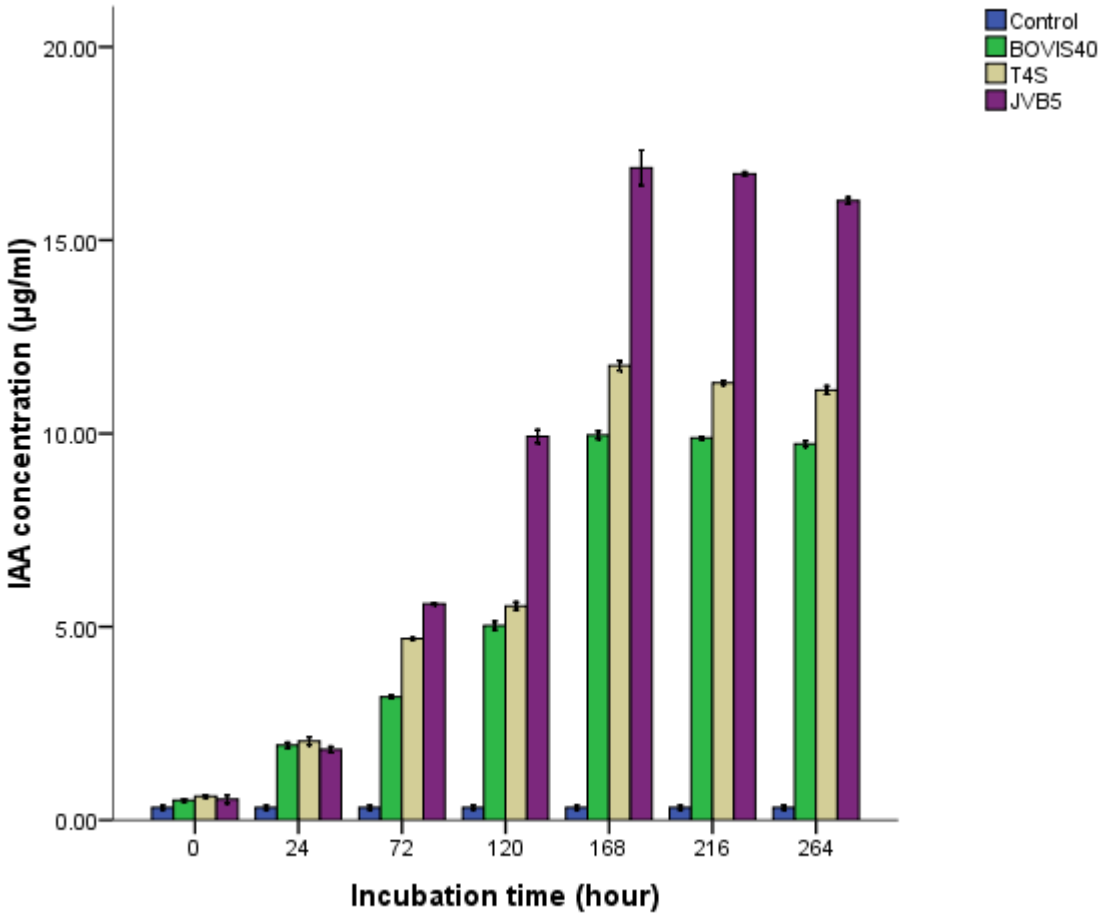


Figure 5.2: IAA activity by the most copious bacterial isolates in the growth medium over 11 days of incubation

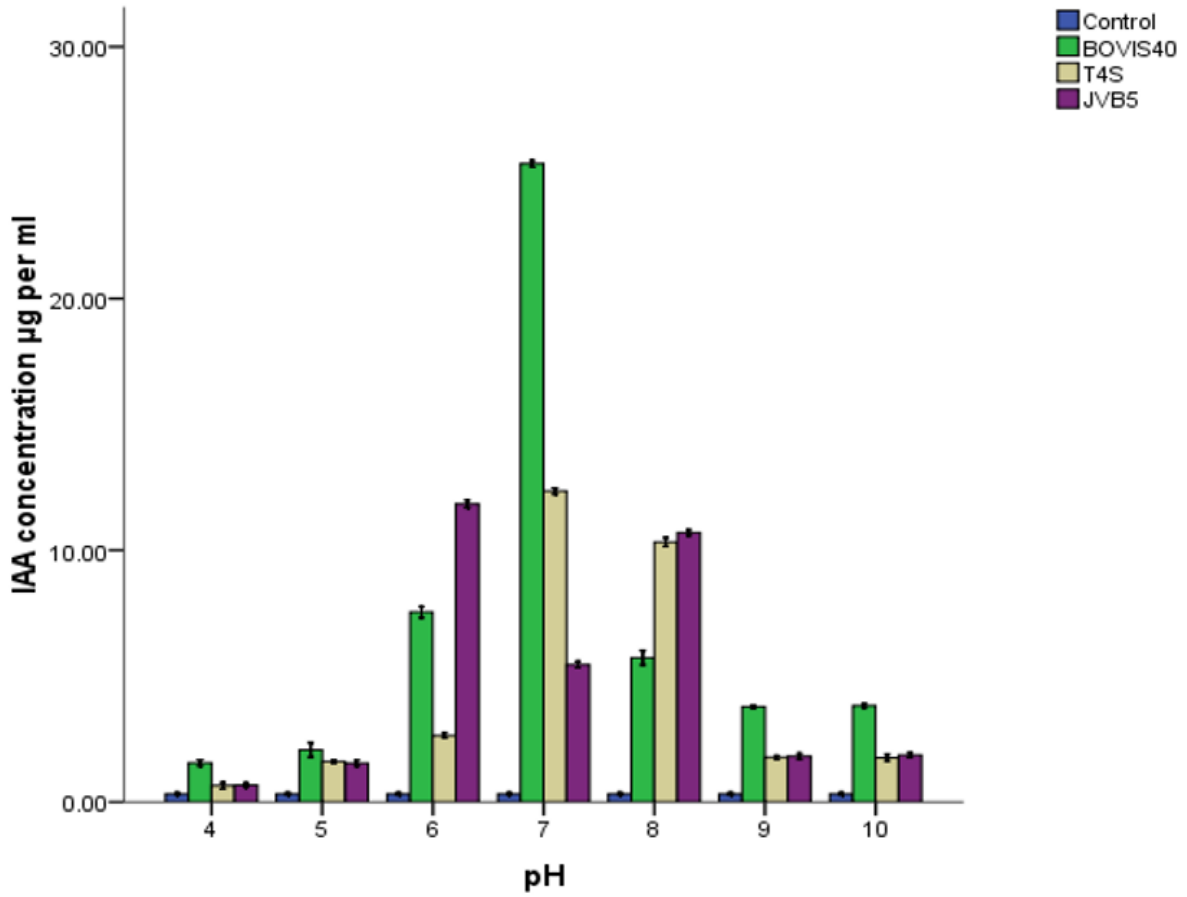


Figure 5.3: Effect of pH on IAA production

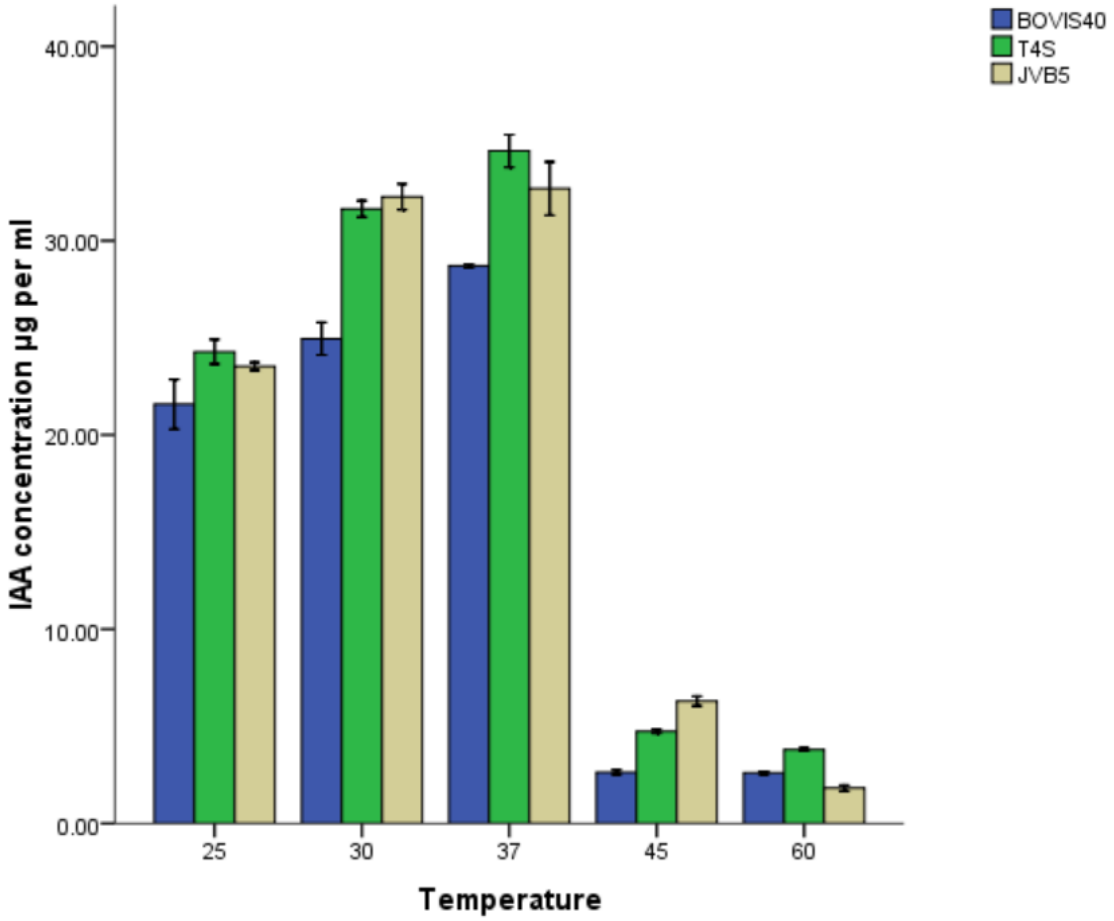


Figure 5.4: Effect of temperature on IAA production

5.3.3 Effect of carbon sources

Figure 5.5 shows the effect of carbon sources on IAA production. The amount of IAA produced in the growth medium varied with the varied sugar concentration in the IPM medium. Out of sugars tested, a maximum IAA activity of 23.36 µg/ml was produced by isolate JVB5 at 5% in sucrose supplemented medium, followed by isolate T4S with a maximum yield of 20.72 µg/ml at 3% glucose.

5.3.4 Effect of nitrogen sources

IAA production under varied nitrogen sources was monitored to ascertain the best concentration for IAA production (Figure 5.6). In an IAA medium amended with casein and yeast extract, all the isolates produced IAA activity > 20 µg/ml concentration compared with other substrates used. Maximum IAA activity of 19.31 µg/ml and 17.70 µg/ml was exhibited by strain JVB5 at 3% and 5% peptone concentration. Similarly, strain T4S exhibited a maximum IAA activity of 17.94 µg/ml at 3% peptone. A high IAA activity of 14.97 µg/ml was obtained from strain T4S at 5% potassium nitrate. IAA activity by strain JVB5 at 5% yeast extract was not significantly different. Similar results were obtained for strain BOVIS40 and T4S at 1% and strain T4S and JVB5 at 3% yeast extract respectively. However, an increase in the amount of IAA activity with an increase in yeast extract concentration was recorded (Figure 5.6). Strain BOVIS40 exhibited IAA activity increase from 21.71 µg/ml to 45.34 µg/ml, while T4S exhibited an IAA activity increase from 21.00 µg/ml to 42.89 µg/ml and JVB5 from 25.58 to 45.82 µg/ml respectively. There was no significant difference in the amount of IAA produced by JVB5 at 1% and 5% urea while strain T4S displayed the best IAA activity of 5.30 µg/ml at 1%.

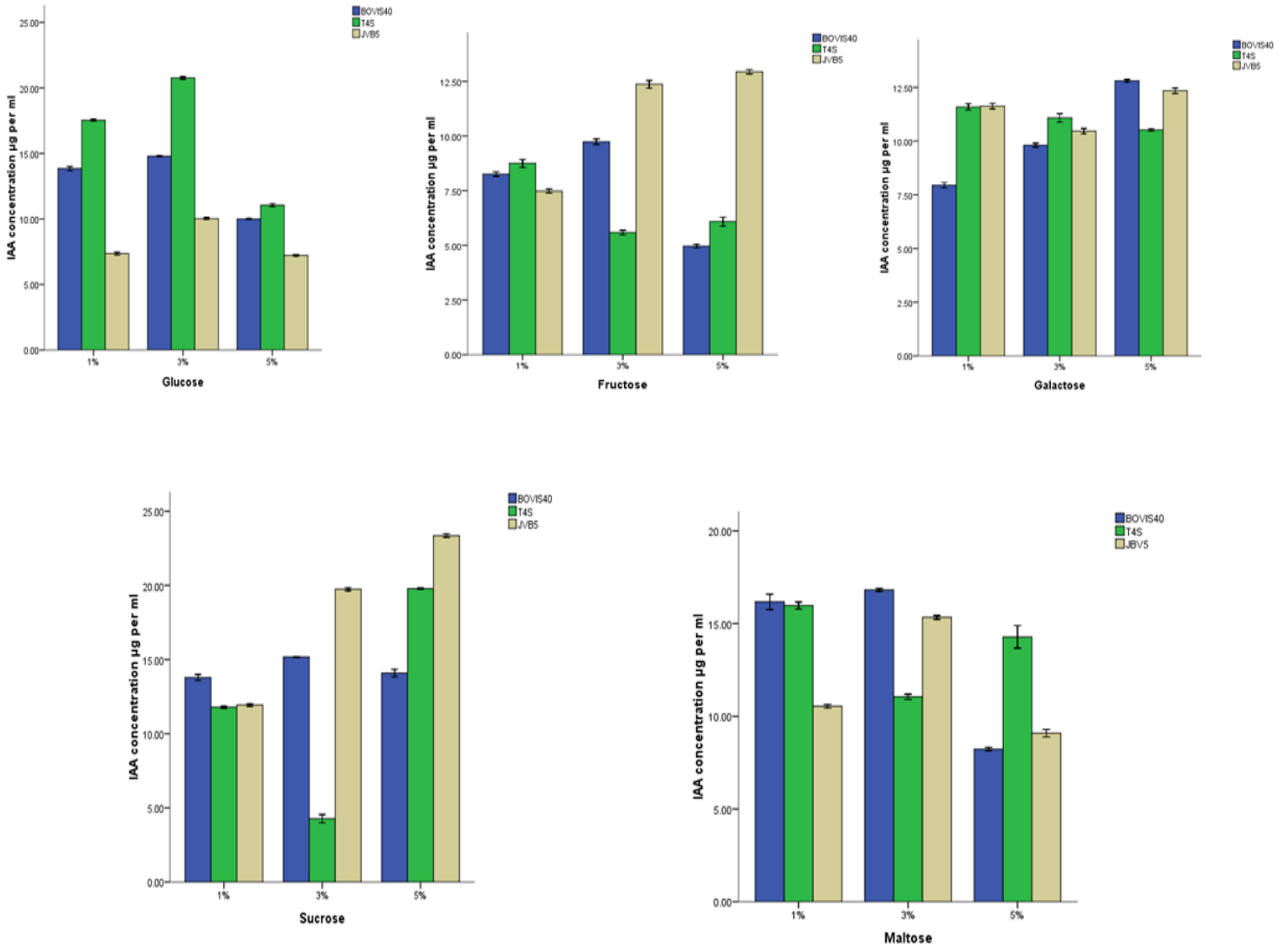


Figure 5.5: Effect of the various carbon sources on IAA production by the bacterial strains

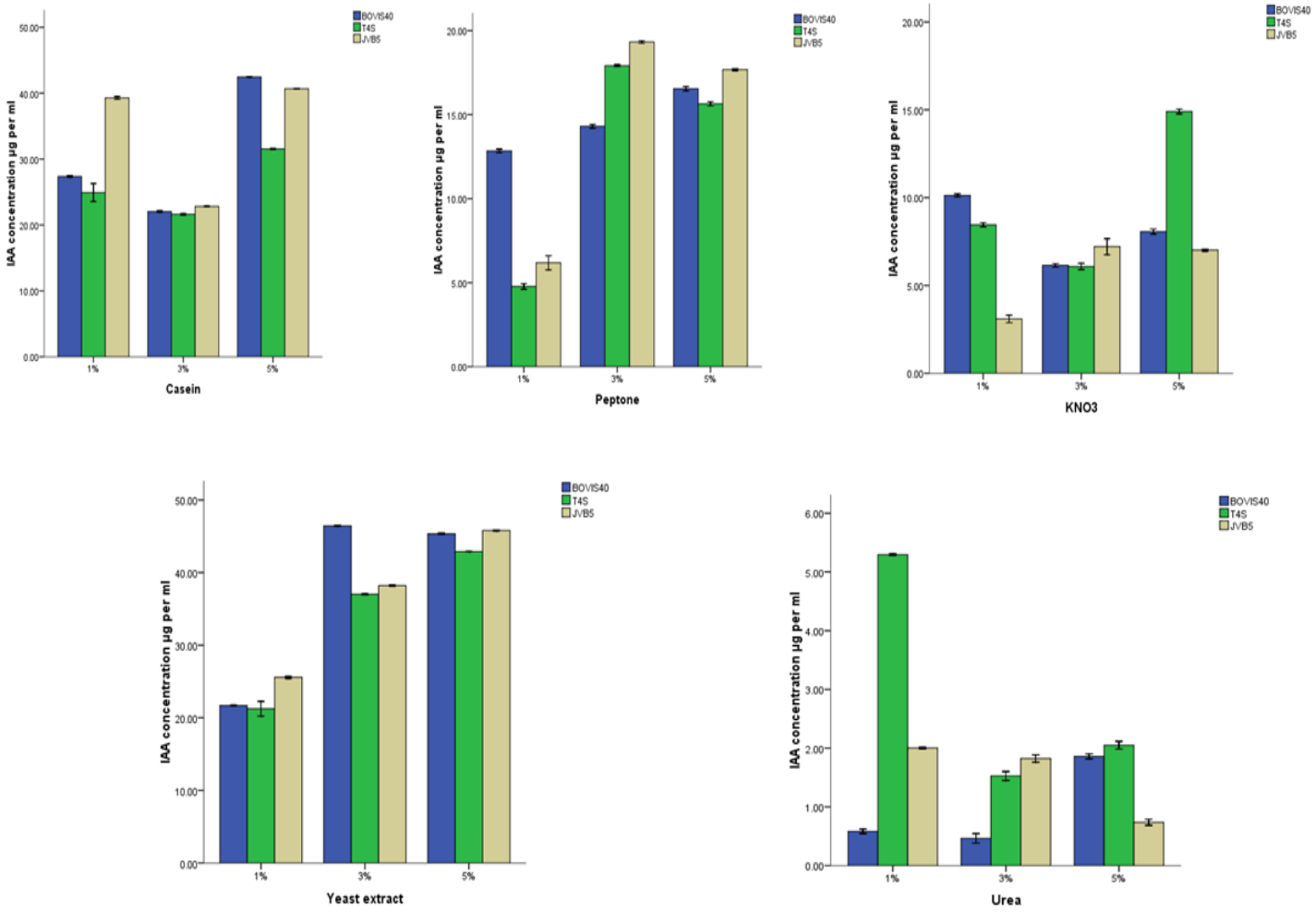


Figure 5.6: Effect of different nitrogen sources on IAA production by the bacterial strains

5.3.5 Seedling growth

The effect of IAA-producing bacterial strains BOVIS40, T4S, and JVB5 on sunflower seeds by inoculation was tested (Table 5.1). Fresh weight of 0.15g, 0.18 g, and 0.17 g was obtained from the sunflower seedling inoculated with bacterial strain BOVIS40, T4S, and JVB5 compared to the control. Correspondingly, seedling dry weight of 0.06 g for BOVIS40, 0.04 g for T4S, and 0.05 g for JVB5 were obtained. A significant difference in the sunflower seedling parameters compared with the control was recorded and presented in Table 5.1.

Table 5.1: Growth parameters of inoculated sunflower seedlings

Bacterial strain	**Root length (mm)**	**Shoot length (mm)**	**Number of lateral roots**	**Fresh weight**	**Dry weight**
Control	16.00±0.0a[a]	22.03±0.06[b]	5.09±0.19[a]	0.14±0.00[b]	0.03±0.00[a]
BOVIS 40	25.00±0.02[c]	27.98±0.08[c]	10.17±0.17[c]	0.15±0.00[b]	0.06±0.00[a]
T4S	19.66±0.05[b]	26.99±0.02[c]	11.99±0.01[c]	0.17±0.00[b]	0.04±0.00[a]
JBV5	36.67±0.02[d]	24.66±0.03[a]	6.65±0.05[b]	0.18±0.00[b]	0.05±0.00[a]

Values are represented as means ± standard deviation of triplicate readings

5.4 Discussion

The need to ensure a safe environment for improved crop production has been a major concern, as insight into the endosphere bacterial community remains a hotspot in developing eco-friendly agriculture (Adeleke and Babalola, 2020a). Endosphere represents an important intracellular region within the plants that harbors diverse microbial domains in the below-and-above ground level (van Overbeek and Saikkonen, 2016). Due to complex root architecture, the most predominant endophytic bacteria are selected by plants based on their colonization pattern in establishing bacterial biomass. The presence of endophytic bacteria in host plants and their ability to synthesize growth hormones, for example, IAA, has contributed to plant growth and immunity in diverse ways (Santoyo et al., 2016).

IAA is considered the most important phytohormone that enhances plant root development and the rate of nutrient absorption for plant growth promotion (Ahmad et al., 2020). In combined forms, the ability of microbes to produce growth hormones has underlined their multifunctional effects for improved agricultural productivity (Igiehon et al., 2019). Different bacterial species have been implicated in the synthesis of IAA depending on their ability to utilize the precursory substance L-tryptophan in the growth medium, thus figuring out the exploration of most IAA-producing bacterial endophytes and their application in agriculture (Mustafa et al., 2018). Harnessing bio-incentives from *Bacillus* spp as a suitable candidate and use for bioinoculant formulation on a commercial scale has been the desire by researchers (Akinrinlola et al., 2018, Kushwaha et al., 2020).

In this study, the ability of bacterial strains to synthesize IAA was similar to research findings by different authors (Chandra et al., 2018, Minakshi et al., 2020). Nevertheless, optimization of process parameters for IAA production by bacteria colonizing the sunflower root endosphere

has not been documented. Hence, optimization of screened bacterial strains with high plant growth-promoting traits and *in vitro* assays on sunflower seedlings germination was studied. To determine IAA concentration produced by bacteria, it is important to grow them in a culture medium containing appropriate nutrients and substrate suitably for optimal yield. Either low or high IAA activity, their effects on plant growth remain fundamental. At low IAA, the primary root formation is enhanced while at high IAA, adventitious and lateral root development is enhanced, respectively (Shahzad et al., 2017). Isolation of plant growth-promoting bacterial endophytes inhabiting the leguminous and non-leguminous plants endosphere with the ability to synthesize IAA is promising, as their use in greenhouse and field trials has been experimented (Abd-Alla et al., 2019).

Importantly, the time monitoring in a culture medium for metabolite synthesis is crucial in determining the biological activity of bacterial endophytes in the fermentation medium. Growing bacterial isolates in a medium amended with L-tryptophan as a precursor has enhanced the IAA activity of the bacterial strains based on the ability to utilize substrate in the medium through diverse IAA metabolic pathways (Hoseinzade et al., 2016). The geometric increase in IAA concentration with incubation time has been attributed to the ability of bacterial isolates to adjust and metabolize the substrate in the growing medium for maximum productivity. At low concentrations, a limited supply of substrate in the growth medium may affect the IAA-producing ability of the endophytic bacteria. In this study, a strong correlation between bacterial biomass and IAA production was found to exist. A decrease in IAA concentration beyond the optimum level might be due to the reduction in the amount of substrate or synthesis of lytic enzymes such as IAA peroxidase and oxidase in the growing medium by the endophytic bacteria (Lebrazi et al., 2020). Nevertheless, bacterial growth under

shaking conditions may influence IAA production due to agitation that allows free flow of oxygen in the medium. Oxygen availability in the medium facilitates the conversion of tryptophan into auxins. Research findings on sunflower root endophytic bacterial strains and their optimization with incubation time have not been documented, although higher IAA potential of *Providencia* spp from tomato plants upon incubation has been reported (Rushabh et al., 2020). Research findings on IAA production by bacterial isolates with varying activities at different incubation times with underlining insights in determining the time course (i.e. either shorter or longer) to minimize the cost of IAA production has been documented (Rushabh et al., 2020). The results obtained corroborate the findings of Myo et al. (2019) who reported IAA activity of 82.36 µg/ml by *Streptomyces fradiae* NKZ-259 after 6 days of incubation. Interestingly, results on the root-associated bacteria such as *Rhizobium* spp, *Bacillus subtilis* KA(1)5r, and *Pseudomonas mosselii* with high IAA concentration at an incubation time of 216 hours and 96 hours, have underlined their ability in the development of medicinal herb *Aconitum heterophyllum* and wheat (*Triticum* spp) (Minakshi et al., 2020, Emami et al., 2019, Lebrazi et al., 2020). However, high IAA activity produced by the bacteria could be an indication of the presence of tryptophan as a precursor in IPM. Furthermore, IAA synthesis by actinomycetes in an IPM supplemented with suitable precursor L-tryptophan has been reported to occur via a tryptophan-dependent pathway or other similar pathways (Samaras et al., 2020). pH is an important factor that influences growth and microbial activity. At low or high pH, various microbial metabolic activities might be affected, such as enzymes involved in IAA production (Alkahtani et al., 2020). Adjustment of pH in the growth medium to suitably favor bacterial growth can facilitate IAA biosynthesis. The differences observed in IAA activity could be attributed to the pH of the medium and media composition (Widawati, 2020).

Research findings and reports on maximum IAA production by *Pantoea glomerans* PVM, *Klebsiella pneumoniae* K8, and *Streptomyces viridis* CMU-H009 at pH ranged between pH 7 and pH 8 have been documented (Myo et al., 2019). Here, the results were similar to the earlier findings of Widawati (2020), who reported optimum IAA activity by *B. siamensis* at pH 7 and pH 8 respectively. Emami et al. (2019) had reported an optimum IAA concentration of 22.63 µg per ml from *Pseudomonas mosselii* isolated from wheat endosphere at pH 7.

Environmental factors influence the activity of endophytic microbes in plants. Changes in temperature of the growth medium may influence IAA synthesis. Here, the results obtained corroborate the previous findings of Emami et al. (2019), who reported optimum IAA production of 23.62 µg per ml by *Pseudomonas mosselii* isolated from the root endosphere of wheat at 32^{o}C.

Different carbon sources amended in IAA-producing medium could serve as an energy source and enhance the overall efficiency of recycling co-factor in the cells for IAA biosynthesis (Myo et al., 2019). The differences observed in the IAA activity by the bacterial isolates can be attributed to the carbon source, concentration, and utilization of the substrate. Carbon sources have been reported to affect the rate of IAA production by bacteria (Khan et al., 2020). Usually, a growing medium amended with monosaccharide sugar compared to di-or-polysaccharides could contribute to high IAA activity based on the ability of endophytic microbes to easily assimilate. In addition, utilization of monosaccharide sugars by most bacteria has been linked to high IAA production (Emami et al., 2019). However, the results from this study revealed high IAA concentration in IPM amended with sucrose, thus suggesting sucrose as a sole carbon source (Widawati, 2020). The differences observed in the IAA concentrations, as suggested in most findings could depend on the sugar source and ability of the bacteria to utilize them for

growth (Oliveira et al., 2021). An increase in IAA production in the IPM amended with sucrose agrees with the findings of Huu Dat et al. (2015). Payel et al. (2017), who reported an increase in IAA production by *Pantoea agglomerans* on sucrose amended media. Also, results from this study corroborate the findings of Bharucha et al. (2013) who reported maximum IAA production by *P. putida* UB1 in a medium amended with sucrose, while lactose and glucose have been reported to be preferred sugars for maximum IAA production by *Enterobacter* sp. and *Rhizobium* (Basu and Ghosh, 2001, Nutaratat et al., 2017). Similarly, reports on maximum IAA production by endophytic bacteria such as *Rhizobium* P2, *Bacillus* spp, *Pantoea* spp, and *Pseudomonas mosselii* colonizing the root endosphere in a medium amended with sucrose, glucose, and maltose have been documented (Apine and Jadhav, 2011, Kucuk and Cevheri, 2016, Emami et al., 2019). However, results obtained from this study were dissimilar to the findings of Widawati (2020) who reported a high IAA concentration of 34.5 µg/ml by endophytic bacteria grown IPM amended with 1% sucrose.

The addition of various nitrogen sources tends to increase IAA concentration compared to the control medium. The soluble nitrogen source as substrate in the growth medium remains a key factor for bacterial growth in the synthesis of metabolites (Khan et al., 2017). The addition of various nitrogen sources to the IPM may influence the rate of IAA production (Shahzad et al., 2017). Out of the nitrogen sources employed casein and yeast extract yielded high IAA activity (> 20 µg/ml) compared to other nitrogen sources. Like other parameters tested, varying concentrations of nitrogen source added to the growing medium can influence the amount of IAA biosynthesis (Emami et al., 2019). IAA production by rhizobacteria inhabiting the root of leguminous plants in a growing medium amended with glutamic acid and L-asparagine as a nitrogen source has been reported (Zhao et al., 2020). The results from this study corroborate

the findings of Balaji et al. (2012), who reported yeast extract as the best nitrogen source for *Pseudomonas* species with an IAA concentration of 210 µg/ml. Emami et al. (2019) has also reported an IAA concentration of 23.66 µg/ml by *Pseudomonas* in a yeast extract amended medium. Also, the addition of varied concentrations of tryptone, beef extract, and peptone with varied IAA activity could contribute to the bacterial lifestyle in the synthesis of phytohormones (Widawati, 2020).

In this study, the effect of bacterial inoculation on sunflower seedling growth has demonstrated their potential in plant growth promotion and this confirms the reports from previous findings on the use of endophytic bacteria in enhancing plant growth for improved crop yield (Haidar et al., 2018). Inoculation of *Sesbania aculeate, Brassica campestris, Vigna radiate,* and *Pennisetum americanum* by endophytic bacteria such as *Azotobacter* spp, *Bacillus* spp, *Azospirillum brasilense* and *Pseudomonas putida* with positive effects on adventitious root formation, increase in shoots and root length, and enhanced chlorophyll pigmentation has been found (Latif Khan et al., 2016). Previous research has affirmed the linkage in plant root development due to IAA production by bacterial isolates. The cause of variation in the weight and length of roots and shoots of rice and maize inoculated with *Bacillus* and *Pseudomonas* is presumed to be influenced by IAA produced by strain T4S (Karnwal, 2017, Karnwal, 2018). Most *Bacillus* spp isolated from the root endosphere has been implicated in nitrogen fixation in legumes with a positive influence on their yield parameters (Bertani et al., 2016). For example, the increase in shoot, root, and leaf length of rice, relative to the phytohormone synthesis by the bacterial isolates has enhanced crop production. Furthermore, the rooting potential of *Agrobacterium rhizogenes* in jujube's root has been reported (Lebrazi et al., 2020).

5.5 Conclusion

In conclusion, the results from this study revealed the IAA potential of *S. indicatrix* strain BOVIS40, *B. cereus* strain T4S, and *S. maltophilia* strain JBV5 under different growth conditions. The optimization of process parameters showed varied IAA activity with optimum IAA potential of bacterial strains attained at 164 hours of growing. The supplementation of the culture medium with nitrogen and carbon sources suggested a considerable amount of IAA production by the bacterial isolates at varying concentrations. Hence, the ability of strains BOVIS40, T4S, and JVB5 to secrete IAA revealed their potential as plant growth-promoting bacterial endophytes, considering their ability to enhance root development for nutrient absorption needed for plant growth. This might be a contributing factor revealing a significant difference in the inoculated seedling lateral root number, fresh and dry weight, root and shoot length compared to un-inoculated seeds. Hence, harnessing these bacterial strains can be promising based on their multifunctional attributes in the synthesis of bioinoculants for easy application in agriculture.

6.0 CHAPTER SIX

Genomic assessment of *Stenotrophomonas indicatrix* for improved sunflower plant

Abstract

Diverse agriculturally important microbes have been studied with known potential in plant growth promotion. Providing several opportunities, *Stenotrophomonas* species are characterized as promising plant enhancers, inducers, and protectors against environmental stressors. The *S. indicatrix* BOVIS40 isolated from the sunflower root endosphere possessed unique features, as genome insights into the *Stenotrophomonas* species isolated from oilseed crops in Southern Africa have not been reported. Plant growth-promotion screening and genome analysis of *S. indicatrix* BOVIS40 were presented in this study. The genomic information reveals various genes underlining plant growth promotion and resistance to environmental stressors. The genome of *S. indicatrix* BOVIS40 harbors genes involved in the degradation and biotransformation of organic molecules. Also, other genes involved in biofilm production, chemotaxis, and flagellation that facilitate bacterial colonization in the root endosphere and phytohormone genes that modulate root development and stress response in plants were detected in strain BOVIS40. IAA activity of the bacterial strain may be a factor responsible for root formation. A measurable approach to the *S. indicatrix* BOVIS40 lifestyle can strategically provide several opportunities in their use as bioinoculants in developing environmentally friendly agriculture sustainably. The findings presented here provide insights into the genomic functions of *S. indicatrix* BOVIS40, which has set a foundation for future comparative studies for a better understanding of the synergism among microbes inhabiting plant endosphere. Hence, this study highlights the potential of *S. indicatrix* BOVIS40 upon

inoculation under greenhouse experiment, thus suggesting its application in enhancing plant and soil health sustainably.

Keywords: Agricultural sustainability, bacterial endophytes, crop improvement, plant growth-promoting genes, whole-genome sequencing

6.1 Introduction

In recent years, initiatives to increase agricultural food production for the global population by utilizing bioincentives derived from endophytic microorganisms rather than agrochemicals are on the increase (Singh et al., 2011, Mahanty et al., 2017, Adeleke and Babalola, 2021a). The indiscriminate use of agrochemicals on farmlands has affected biodiversity and human health (Adeleke and Babalola, 2020a). Nevertheless, using microbial bioincentives to boost crop production is a viable way to achieve a desirable sustainable food supply (Babalola et al., 2021). The application of bioinoculants has been shown to improve plant development and protection, increase nutrient bioavailability to plants and induce systemic resistance in plants against environmental stresses (Mashiane et al., 2017). Importantly, microbes associated with economic crops have demonstrated multifunctional characteristics in stimulating plant growth through solubilization of soil minerals (phosphorus, potassium, and zinc), nitrogen fixation, enzyme, and siderophore production (Nascimento et al., 2020a, Glick, 2020).

Plant growth promotion depends on natural bioactive compounds, which are inherent or produced by its associated microorganisms (Palanichamy et al., 2018). Their enormous contributions in the formation of ecological balance between plants and microbes, whether from microorganisms or plants, remain fundamental (Fadiji et al., 2021a). The importance of plant growth-promoting (PGP) genes and secondary metabolites from microorganisms are not only applicable in agriculture or restricted to plant improvement, but with wider therapeutic importance (Manganyi et al., 2019, Adeniji et al., 2021).

The necessity for their separation, characterization, and analysis using bioinformatics techniques is critical in order to understand the genetic makeup of bacteria (Mamphogoro et al., 2020). Endophytic investigations utilizing various bioinformatics methods have recently

uncovered various PGP genes and secondary metabolites in bacteria, thanks to current advances in biotechnological findings (Mohotti et al., 2020, Nascimento et al., 2020b). Each of these gene clusters codes for different metabolic compounds, such as siderophore, arypolyene, and lanthipeptide-class-II with specific functions. The siderophore genes found in the genomes of bacteria, such as *Burkholderia*, *Bacillus*, *Pseudomonas*, and *Rhizobium* associated with the plant, provide a powerful arsenal for suppressing pathogens and plant growth promotion (Ludwig-Müller, 2015, Bhattacharyya et al., 2017). Similarly, the detection of several other secondary metabolite gene clusters from endophytic bacteria colonizing medicinal plants has been documented (Ludwig-Müller, 2015, Dinesh et al., 2017).

The survival and adaptive mechanism of bacteria in extreme environments can be linked to their ability to produce vital PGP genes and the synthesis of secondary metabolites, thus making them suitable candidates for biotechnological, agricultural, and industrial applications (Nascimento et al., 2020a, Chukwuneme et al., 2021). The genomic analysis of sequenced bacterial genome revealing conservative and functional genes have been reported (Mitter et al., 2013, Zeng et al., 2018, Akinola et al., 2021), but information regarding novel plant growth-promoting and secondary metabolite genes of *S. indicatrix* isolated from sunflower root endosphere has not been documented, thus necessitating this study. *S. indicatrix* is a Gram-negative, non-spore-forming, rod-like bacterium belonging to the *Stenotrophomonas* genus. Recent findings have shown the identification and characterization of *S. indicatrix* from the root nodules and rhizosphere soils (Malisorn et al., 2020). Other species, such as *S. maltophilia* isolated from the rhizosphere soil of peanut (*Arachis hypogea*) and maize (*Zea mays*) have significantly aided plant growth in nitrogen-deficient soil (Alexander et al., 2019, Perez-Perez et al., 2020). Few species of the genus *Stenotrophomonas*, however, are known to be found in

soils and plants, and they play a variety of roles in plant physiology and metabolism (Kumar and Audipudi, 2015, Singh and Jha, 2017). For example, *S. rhizophilia* strains have been isolated from the soil, rhizosphere, and endosphere of many plants with active participation in plant defense against phytopathogens due to their ability to secrete osmoprotective substances and degradation of lignocellulose compounds (Wolf et al., 2002, Berg et al., 2010, de Lima Brossi et al., 2016). In addition, Maela and Serepa-Dlamini (2020) reported a draft genome sequence of endophytic bacteria *S. pavanii* strain MHSD12 associated with fever bush (*Dicoma anomala*). Despite this, evidence on the *S. indicatrix* from the oilseed crop is uncommon, hence suggesting its promising application in agricultural biotechnology.

The detection of important genes in the genome of *Stenotrophomonas* spp can significantly enhance their functions in the establishment of root-soil, soil-bacterial, and root-bacterial interactions for improved plant adaptation mechanisms to soil stressors (Vurukonda et al., 2018). These genes can affect the lifestyle and bacterial functions in the host plants. For instance, the detection of chemotaxis and biofilm production genes can contribute to root colonization and plant defense mechanisms. *S. indicatrix* can be a potential biological tool in the formulation of bioinoculants for improved crop productivity based on these functional premises. In this study, the genomic insights into endophytic *S. indicatrix* BOVIS40 were obtained from the sunflower root endosphere with significant effects on sunflower yield. Whole-genome sequencing (WGS) analysis unraveled vital genes involved in various biological functions and plant growth promotion. To the best of our knowledge, this is the first report on the WGS of *S. indicatrix* BOVIS40 sourced from sunflower root in Southern Africa. Hence, this study provides insights into *S. indicatrix* BOVIS40 about (i) plant growth-

promoting activities, (ii) whole-genome sequencing, (iii) functional and secondary metabolite genes, and (iv) sunflower improvement under greenhouse experiment.

6.2 Materials and methods

6.2.1 Source of plant growth-promoting endophytic bacterium *S. indicatrix* BOVIS40

The healthy sunflower roots used in this study were sourced from Lichtenburg, South Africa (26°4′31.266″S, 25°58′44.442″E). The plant surface-cleaning, isolation, and characterization of the bacterial isolates were performed according to the modified methods of Shishido et al. (1995). Bacterial isolates were screened for various PGP traits. Phosphate solubilization (PS) assay was performed according to the methods described by Khan et al. (2020) with little modifications, while indole acetic acid synthesis by bacterial strains was conducted following the methods of Gutierrez et al. (2009). Similarly, methods described by Igiehon et al. (2019) and Khan et al. (2020) were employed for exopolysaccharide test and siderophore screening on chrome azurol S (CAS) medium, respectively.

6.2.2 Molecular identification and whole-genome sequencing

The genomic content of strain BOVIS40 was extracted using a commercial Quick-DNA™ Miniprep Kit specific for fungi or bacteria (Zymo Research, Irvine, CA, USA; Cat. No. D6005), as stipulated in the manufacturer's guide. Determination of quantity and quantity of the extracted DNA was achieved using a NanoDrop spectrophotometer (Thermo Fischer Scientific, CA, USA). The molecular identification of bacterial isolate as *S. indicatrix* based on 16S rDNA sequence data analysis was according to the method described by Araújo et al. (2002). The WGS of strain BOVIS40 was performed at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

The WGS of endophytic bacterial strain BOVIS40 was performed according to the standard Illumina method. Succinctly, fragmentation of bacterial genomic DNA was performed using the NEB Ultra II FS kit enzymatic approach. AMPure XP beads were employed for the selection from the resulting DNA fragments based on size range (200 - 700 bp). Subsequently, DNA end-repaired were achieved by fragmentation, and each fragment was ligated to Illumina-specific adapter sequences. Furthermore, the indexing of each sample and selection based on the size in the second step was performed. The quantity of samples at dilution of standard concentration to 4 nM was determined using a fluorometric method. After that, sequencing was performed using a NextSeq mid-out kit (300 cycles) on Illumina's NextSeq platform, following a guideline as described by the manufacturer. The resulting 400 Mb of data (2x150 bp long paired-end reads) were obtained for each sample.

For WGS analysis, each sequence (FASTQ file) was uploaded to the KBase (https://kbase.us/) (Arkin et al., 2018). The quality assessment of the sequence reads was evaluated by FastQC (version 0.11.5) (Bioinformatics, 2011), while the removal of sequence adaptor and low-quality bases was processed with trimmomatic (version 0.36) (Bolger et al., 2014). Furthermore, sequence reads were assembled by SPAdes (version 3.13.0) (Nurk et al., 2013), then annotated by employing RASTtk (Rapid Annotations using Subsystems Technology toolkit – version 1.073) and SEED online server to categorize the distribution and functions of the predicted genes into subsystems (https://rast.nmpdr.org). The prediction of functional protein-coding genes (PCG) was obtained from the genomic protein output after processing in NCBI (https://www.ncbi.nlm.nih.gov/). All analyses were performed using default parameters unless otherwise specified. Secondary metabolites were determined by antiSMASH (version 6.0.0) (Weber et al., 2015). The circular genome visualization with the well-informed genomic feature

was generated on kbase by uploading the genome assembly fasta file (Arkin et al., 2018), while the phylogeny analysis was performed using MrBayes (http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes) (version 3.2.6) (Huelsenbeck and Ronquist, 2001).

6.2.3 Accession number of *S. indicatrix* BOVIS40

From the NCBI results, the Bioproject number, PRJNA706595; BioSample number, SAMN18138830; and whole-genome accession number, JAGENA000000000 were assigned to the *S. indicatrix* BOVIS40.

6.2.4 Inoculum preparation and seed treatment

A seed inoculation assay was used to facilitate bacterial adherence to the disinfected sunflower seeds. The effectiveness of sunflower seed inoculation was performed following the methods of Gholami et al. (2009). The bacterial inoculum size in LB broth at 24-hour incubation was standardized to 0.5 (10^6 CFU/ml) at OD_{600}. Surface sterilization of the seeds was performed by washing in sterile distilled water to remove floating-unhealthy seeds and dirt, and disinfected in 70% ethanol for 3 minutes, followed by 3% hypochlorite for 1 minute, then immersed in 70% alcohol for 2 minutes with final washing with sterile distilled water (5 times). Bacterial inoculated in LB broth was incubated on a rotary incubator machine at 180 rpm for 24 hours. Broth culture was centrifuged to obtain the pelletized cells and then washed in 0.85% normal saline solution. The sterilized seeds were suspended in LB bacterial culture containing 1% (v/w) carboxymethyl cellulose (CMC) as an adhesive in a 250 ml flask for 60 minutes. The seeds suspended in sterile distilled water without bacterial inoculum served as the control.

6.2.5 Greenhouse experimental study

The soil used for planting in the greenhouse was sourced from agricultural farmlands in North-West University, Mafikeng Campus (Figure 6.1). Soil debris and other plant materials were removed, air dry, and sieved with a 2 mm micro stainless steel mesh sieve, and the soil was placed inside autoclavable plastic bags and sterilized at 121^{o}C for 15 minutes. This step was repeated three times to ascertain all spore formers, vegetative cells, and forms of life were eliminated. Sterilized soil was allowed to cool for 2 days after which 15 kg of soil was transferred into plastic pots. The inoculated and non-inoculated pots were randomly arranged by employing a complete randomized design (CRD) approach with 8 replicates for each treatment at a 10 cm distance apart in a greenhouse under natural light. Bacterial strains inoculated in 1 L LB broth were incubated in a rotary shaker (SI-600, LAB Companion, Korea) at 180 rpm for 2 days for optimal growth density. The broth was centrifuged (8000 x g for 10 minutes) and pelletized bacterial cells obtained were suspended in saline solution (0.85%). The centrifugation and washing of the pellets were performed under sterile experimental conditions. The plastic pots measured 34 cm in diameter and 29 cm tall were washed with sterile water and sterilized with 15% sodium hypochlorite solution before filling with 15 kg dry-sterilized loamy soil. The surface-sterilized sunflower seeds were inoculated with 150 ml standardized bacterial inoculum, agitated in a shaker incubator at 120 rpm at 30^{o}C for 2 hours. Then, the filtrate suspension was gently poured out by decanting. Seeds were placed on a foil paper under sterile laminar flow (Filta Matix Laminar Flow Cabinet) and air-dried before being taken to the greenhouse. Air-drying was performed to ensure the sticking of the bacterial inoculum to the surface of the seeds. The sterile plastic pots containing 15 kg sterile autoclaved soil were

moistened with 500 ml sterilized water before sowing. Ten seeds maximum were sown per pot (at 1.5 cm deep).

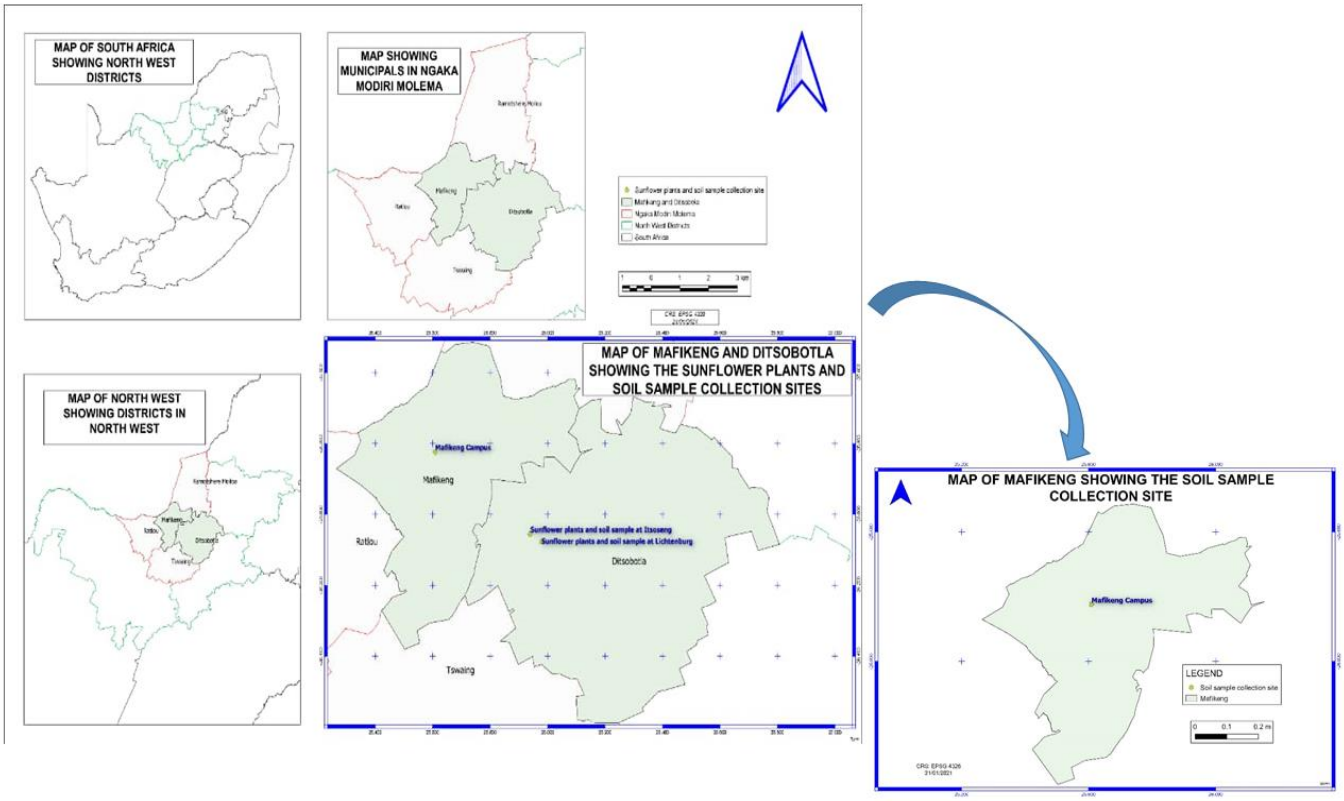


Figure 6.1: Map showing sample collection sites

After seed emergence, i.e., at 8 days, thinning was performed, leaving one seedling in a pot. Growing seedlings were maintained at a temperature of 30±2^{o}C, a day-night cycle of approximately 14 hours under natural light, and relative humidity of 85%. The pots were moistened with an equal amount of water and maintained daily. Pots containing seedlings without inoculation served as the control. The plants were harvested at maturity, 132 days after planting.

6.2.5.1 Sunflower morphological parameters

The data collected based on sunflower morphological parameters after harvesting include the root weight (dry), number of lateral roots, root number, shoot weight (dry), taproot length, root weight (fresh), etc. These parameters were considered following the methods described by Igiehon et al. (2019). After harvesting from a greenhouse, sunflower plants were taken to the research laboratory. The soil adhering to sunflower roots was thoroughly cleansed/washed under running sterile water. The fresh weight of roots and shoots was measured using a weighing balance (Wagi Elektroniczne, Poland). Also, lateral roots and root numbers were determined. Furthermore, plant roots and shoots were oven-dried at 60°C for 24 hours and re-weighed on a weighing balance for the determination of roots and shoot dry weights.

6.2.5.2 Determination of sunflower yield parameters

The sunflower yield parameters such as seed weight (fresh and dry), and total seed weight (dry) were determined. After drying, sunflower seeds were manually separated from the sunflower head containing seeds. Seeds were oven-dried at 60°C for 24 hours, and seed weight (dry) was measured on a weighing balance. The plant samples were kept in plastic bags for further analysis. The whole experiment was repeated for each treatment.

6.2.6 Soil variable analysis

Approximately 0.5 kg of sieved soil used in the greenhouse experiment was used for soil physical and chemical parameters determination (Ali et al., 2009). The chemical parameters such as silt, clay, and sand, and chemical parameters such as organic matter, magnesium, iron, pH, manganese, potassium, phosphorus, total nitrogen, and organic carbon were evaluated.

6.2.7 Data analysis

The analysis of data from this study was analyzed using Statistical Package for the Social Sciences (SPSS) version 16.0. One-way analysis of variance (ANOVA) was performed for the data, followed by the Duncan test at a 5% level of significance to determine differences between mean. For each treatment, generated data were presented as arithmetic mean±standard deviation.

6.3 Results

6.3.1 Biochemical and cultural features

The biochemical and cultural characterization and PGP features of *S. indicatrix* BOVIS40 from the sunflower root endosphere are shown in Table 6.1 The results revealed that strain BOVIS40 is rod-like, catalase-positive, and Gram-negative. Also, strain BOVIS40 utilizes all the sugars tested and grows between pH ranging from 4 to 10, temperature from 25 to 40°C, and 3.5% normal saline. Plant growth-promoting screening showed a significant difference in siderophore value of 77.54%, phosphate content of 32.93 µg/ml, and IAA of 16.15 µg/ml, respectively (Table 6.1). Bacterial endophytes exhibited significant reactions to cellulase, xylanase, and protease compared to amylase and protease production with no significant difference from the plate assay experiment (Table 6.1).

Table 6.1: Biochemical, cultural characterization and plant growth-promoting features of *S. indicatrix* BOVIS40

Characteristics	**Result**	**Plant growth-promoting tests**		
		Isolate screening	**Qualitative**	**Quantitative**
Gram reaction	-	IAA (µg/mL)	+	16.15±0.15[a]
Shape	Rod	Siderophore (%)	++	77.54±0.42[c]
Catalase	+	Phosphate (µg/mL)	+	32.93±0.08[b]
Starch hydrolysis	+	Exopolysaccharide	+	ND
Nitrate	+	**Enzyme assay**		**ZOC (mm)**
Oxidase	+	Amylase	+	33.97±0.06[c]
Citrate	+	Cellulase	+	41.00±0.01[d]
Casein hydrolysis	+	Xylanase	+	20.00±0.01[b]
Salt tolerance (% v/v) (max.)	3.5	Mannanase	+	34.00±0.01[c]
Temperature range (^{o}C)	25-40	Protease	+	3.00±0.01[a]
pH range	4-10			
Glucose	+A			
Fructose	+A			
Galactose	+A			
Sucrose	+A			
Mannitol	+A			
Xylose	+A			
Raffinose	+A			
Maltose	+A			

Arabinose	+A

Key: - = negative reaction, + = positive reaction, +A = positive reaction (acid production), ZOC - zone of clearance measurement, ND – not determined. Values are represented as means ± standard deviation of triplicate readings. Mean±standard deviation values with different superscripts (small letters) within the same column represent a significant difference

6.3.2 Whole-genome sequence information of *S. indicatrix* BOVIS40

The WGS analysis of *S. indicatrix* BOVIS40 yielded a sequence read count of 7,301,524, the genome size is 4,427,090 bp, G+C content of 66.4%, and 252 contigs. The read length mean was 151 bp while L_{50} and N_{50} values were 44 and 29,344 bp, respectively (Table 6.2). The genome analysis revealed 4,160 coding sequences, 2 rRNA, and 62 tRNA. The circular genome visualization of *S. indicatrix* BOVIS40 is presented in Figure 6.2. The subsystem statistics show 27 subsystem feature counts of the coding protein into functional groups based on the annotated genome classification by SEED in RAST. The functional basis of 3,957 protein-coding genes (PCG) was assessed from the KEGG database and RAST online server. The 1,476 genes annotated by the SEED viewer were grouped into molecular function, cellular components, and biological processes. The topmost five groups were protein metabolism (177 genes), amino acids and derivatives (258 genes), cofactor vitamins, prosthetic groups, pigments (128 genes), carbohydrates (165 genes), and membrane transport (136 genes) (Figure 6.3). The phylogeny analysis of the strain BOVIS40 is presented in Figure 6.4.

6.3.3 Functional genes annotation information of *S. indicatrix* BOVIS40

Various metabolic genes were detected in the bacterial genome (Tables 6.3-6.21). Notable secretion systems genes found in strain BOVIS40 are presented in Table 6.3. The expression of genes involved in carbohydrates such as trehalose, glucose, hexose, xylose, galactose, mannose, and fructose degradation pathways is presented in Table 6.4. The carbohydrate metabolism pathways include the pentose phosphate pathway, glycolysis – Embden-Meyerhof pathway, and Leloir pathway. Other genes responsible for organic phosphate solubilization, sulfur metabolism, and nitrogen fixation and transport were detected in the genome (Tables 6.5-6.7).

Table 6.2: Genome characteristics and annotation information

Genome annotation features	***S. indicatrix* BOVIS40**
Domain	Bacteria
Taxonomy	Bacteria, Proteobacteria, Gammaproteobacteria, Lysobacterales, Lysobacteraceae, *Stenotrophomonas*
Size (bp)	4,427,090
G + C content (%)	66.4
N_{50} (bp)	29,344
L_{50}	44
Number of contigs	525
Number of subsystems	304
Number of coding sequences	4,160
Number of RNAs	62

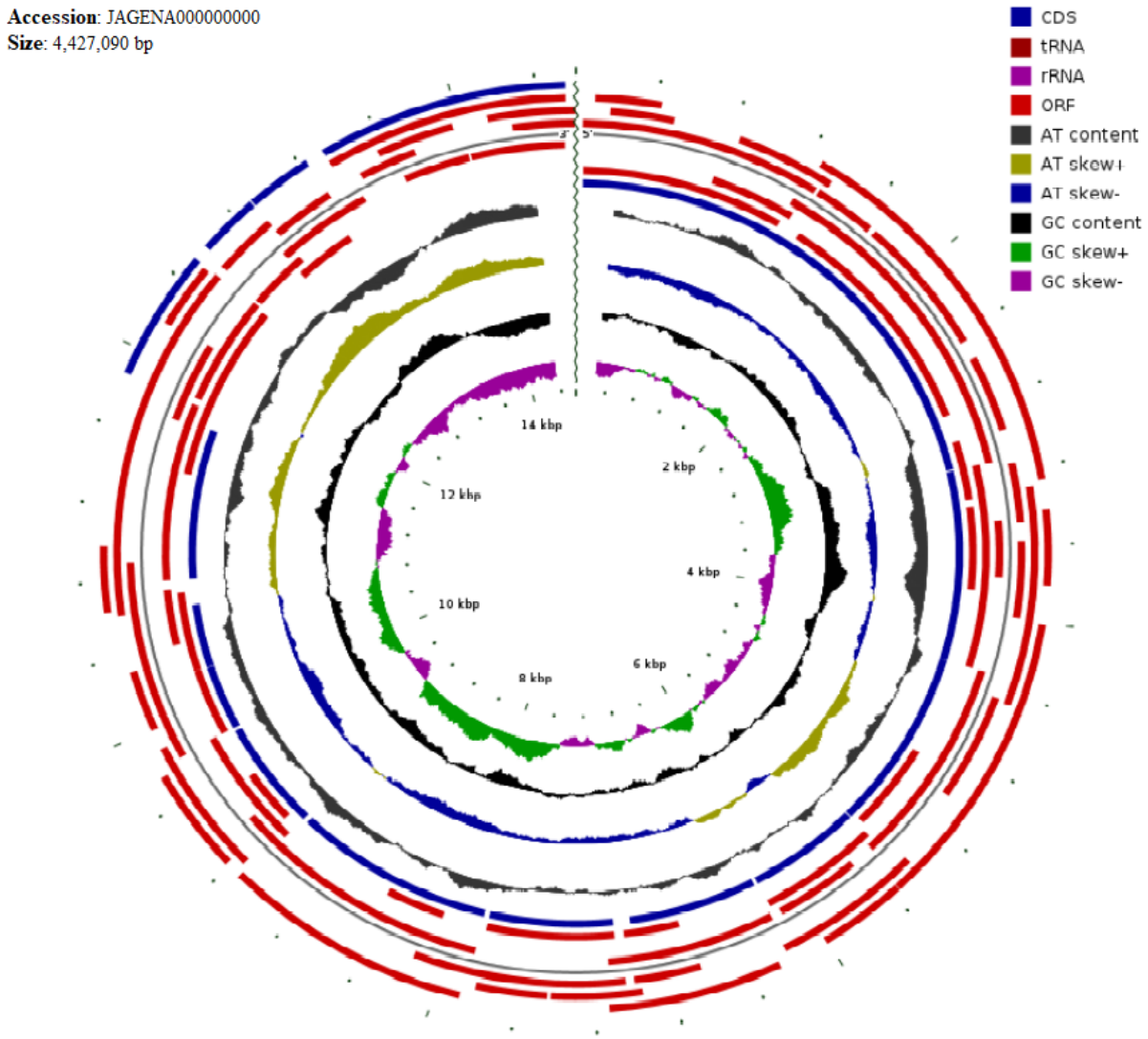


Figure 6.2: Circular genome visualization of whole-genome *S. indicatrix* BOVIS40. Each color from the external to internal circle depicts, green (ORF), red (stop codon), and blue (start codon). The ring black coloration (GC content) at the peak indicated higher or lower values than average GC content. The GC Skew (-/+) in purple/green peaks in/outside the circle indicated values greater or smaller than 1

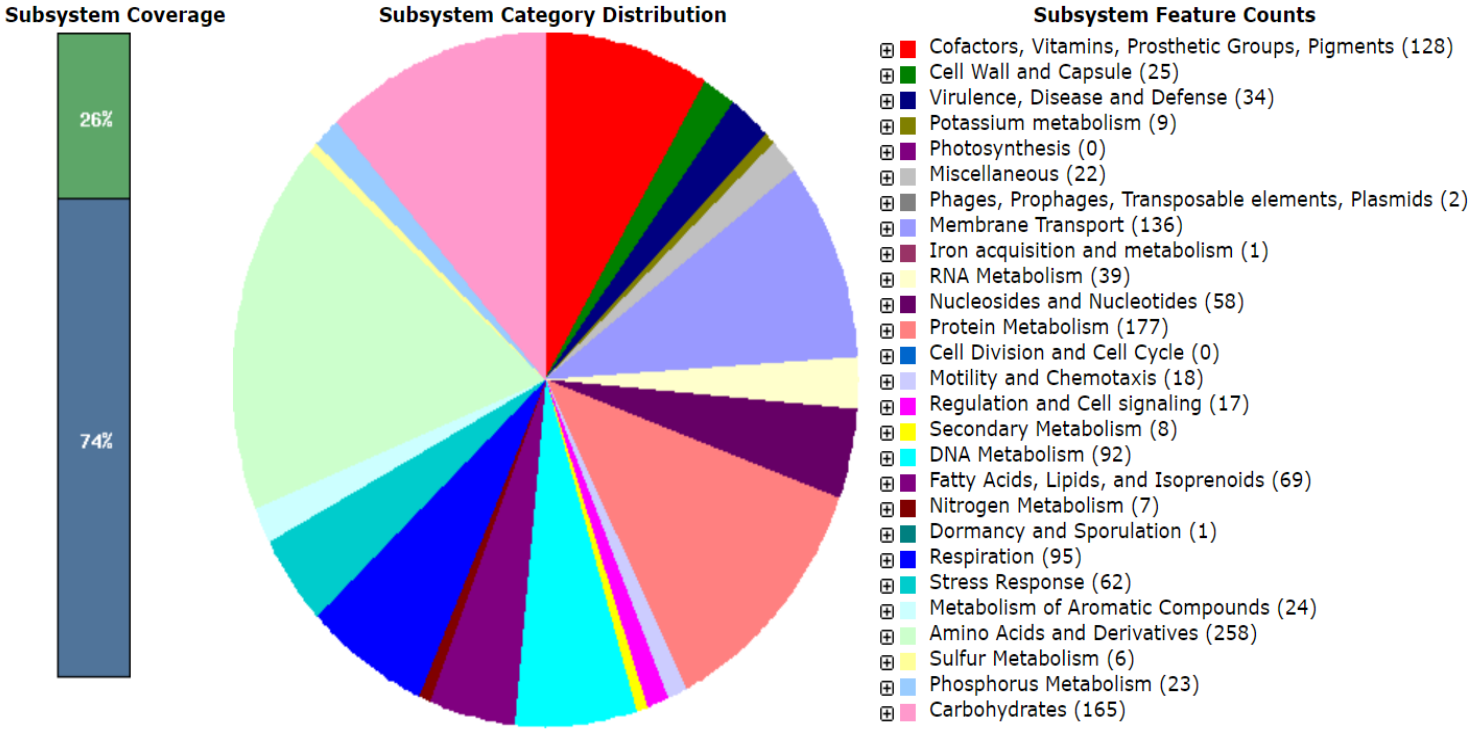


Figure 6.3: Subsystem category distribution of key PCG of *S. indicatrix* BOVIS40 annotated in the RAST SEED Viewer annotation online server. The green/blue bar shows the subsystem coverage in percentage. Blue bar correlates to the percentage (%) of proteins present

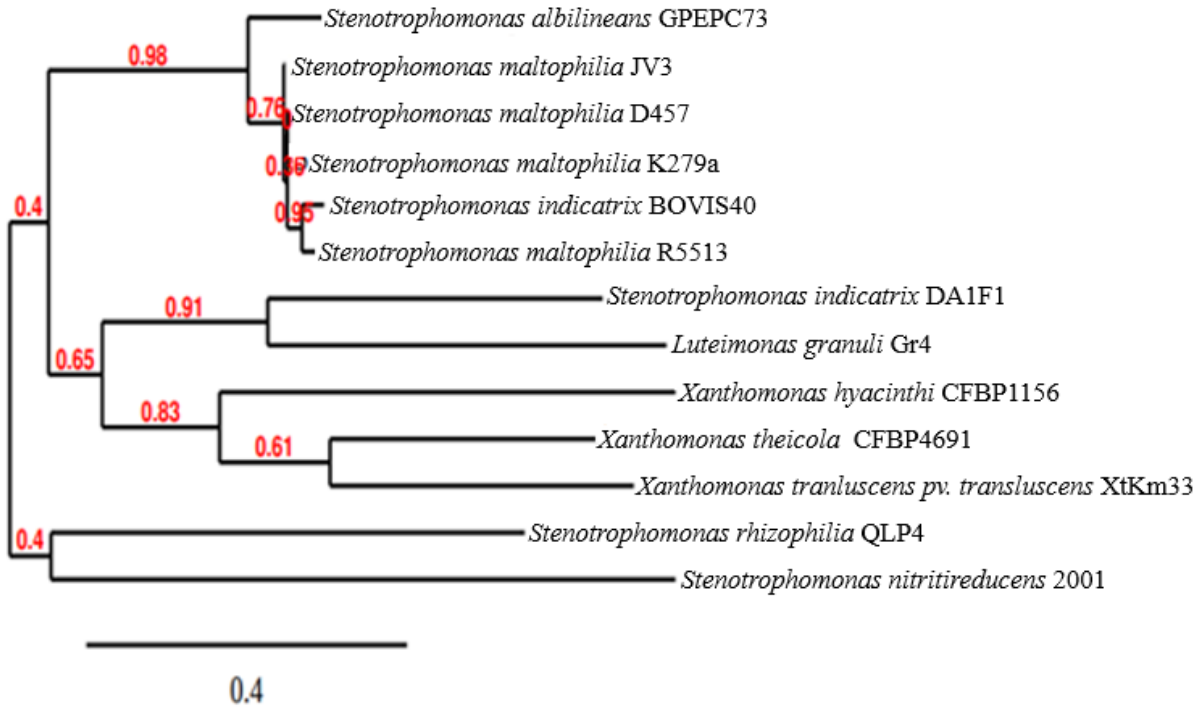


Figure 6.4: Phylogeny analysis of *S. indicatrix* BOVIS40

Table 6.3: Genes involved in secretion systems

Gene	Locus tag	Product	Pathway
*sec*A	J0657_16415	preprotein translocase subunit *Sec*A	Sec (secretion) system
*tat*A	J0657_08405	Sec-independent protein translocase subunit *Tat*A	Twin-arginine translocation (*Tat*)
*tat*B	J0657_08400	twin-arginine translocase subunit *Tat*BC	system
*tat*C	J0657_08395	twin-arginine translocase subunit *Tat*C	
*lap*B	J0657_05850	lipopolysaccharide assembly protein *Lap*B	Adhesin protein transporter
*tol*C	J0657_19275	outer membrane protein	RaxAB-RaxC type I secretion
*lol*A	J0657_03745	outer membrane lipoprotein chaperone *Lol*A	system protein
*tss*L	J0657_02390	secretion system tip protein *Vgr*G	secretion system type VI

Table 6.4: Genes involved in carbohydrate metabolism and transport

Gene	Locus tag	Product	Pathway
zwf	J0657_08080	glucose-6-phosphate 1-dehydrogenase	Pentose phosphate
pgl	J0657_08070	6-phosphogluconolactonase	
glk	J0657_19810	glucokinase	EMP glycolysis
pgk	J0657_19335	phosphoglycerate kinase	
eno	J0657_03350	enolase	
*yid*A	J0657_09895	sugar-phosphatase	Glucose and glucose-1-phosphate
*ygg*S	J0657_10680	pyridoxal phosphate phosphatase	degradation
fdh	J0657_14715	6-phosphofructokinase	Fructose and other sugars degradation
*fdh*D	J0657_19420	formate dehydrogenase	
*xyl*A	J0657_15415	xylose isomerase	Xylose degradation
*xyl*B	J0657_15420	Xylulokinase	
*gal*U	J0657_05865	glucose-1-phosphate	D-Galactose degradation
*man*A	J0657_01190	mannose-6-phosphate isomerase	Mannose degradation
*tre*A	J0657_14205	alpha, alpha-trehalase	Trehalose degradation
*ots*B	J0657_09435	trehalose-phosphatase	
*tre*A	J0657_14205	alpha-trehalase	

Table 6.5: Genes involved in nitrogen fixation

Gene	Locus tag	Product	Pathway
*ntr*C	J0657_03480	nitrogen regulation protein NR(I)	Nitrogen fixation
nifS	J0657_16100	cysteine desulfurase	
*nif*F	J0657_01315	flavodoxin	
amt	J0657_18850	ammonium transporter	Ammonia transport

Table 6.6: Genes involved in phosphate solubilization and transport

Gene	Locus tag	Product	Pathway
pgl	J0657_08070	6-phosphogluconolactonase	D-gluconate production
ppx	J0657_02585	exopolyphosphatase	Degradation of inorganic
ppa	J0657_14755	inorganic pyrophosphatase	polyphosphates
ppx	J0657_02585	Exopolyphosphatase	Organic phosphate solubilization
*pho*U	J0657_09385	phosphate signaling complex protein *Pho*U	
*pho*A	J0657_04540	alkaline phosphatase	
*pst*A	J0657_09395	phosphate ABC transporter (P(ABC)T) permease	Phosphate transport
*pst*C	J0657_09400	P(ABC)T permease subunit *Pst*C	
*pst*S	J0657_08775	P(ABC)T substrate-binding protein *Pst*S	
*pst*B	J0657_09390	P(ABC)T ATP-binding protein *Pst*B	

Table 6.7: Genes involved in sulfur metabolism

Gene	Locus tag	Product	Pathway
*cys*J	J0657_05140	sulfite reductase (NADPH) flavoprotein alpha-	Sulfate reduction
	J0657_20210	component	
*cys*Q	J0657_11815	bisphosphate nucleotidase *Cys*Q	
*cys*K	J0657_19305	cysteine synthase A	Sulfate transport

Similarly, functional genes involved in iron transport, enterobactin, and siderophore production were also found (Table 6.8). Bacterial colonization in the root endosphere mediated by several genes involved in chemotaxis, flagella, pilus, and fimbriae biosynthesis is presented in Table 6.9. The signaling molecules that aid bacterial biofilm production and genes responsible for their attachment were also detected (Table 6.10). Genes, such as *cyc*A, *gcv*A, *pst*J, *lld*R, and *bet*L coding for quorum-sensing in the N-acyl-homoserine-lactone pathway were detected in the genome of strain BOVIS40 and presented in Table 6.11. Plant protection against oxidative stress based on gene expression ability in strain BOVIS40 is presented in Table 6.12. Genes involved in organic acids metabolism, GABA, amino acid metabolism and transport, and phenolic gene, *phe*A coding for catechol metabolism were found in the strain BOVIS40 (Tables 6.13-6.16). The detection of genes involved in lignin degradation and degradation against toxic peroxides and plant growth hormones was profound in strain BOVIS40 (Tables 6.17-6.18). Notable biocontrol, stress tolerance, and temperature resistance genes that stimulate an internal response in plants were presented in Tables 6.19-6.21.

6.3.4 Predicted secondary metabolite cluster genes by antiSMASH

The predicted secondary metabolite gene clusters from antiSMASH analysis of strain BOVIS40 are presented in Table 6.22. The biosynthetic gene clusters identified include bacillibactin type non-ribosomal peptide siderophore and APE Vf type arylpolyene. Three (3) notable secondary metabolite gene clusters detected include transport-related genes, resistance, regulatory genes, additional biosynthetic genes, core biosynthetic genes, other genes, and TTA codons (Figures 6.5a and 6.5b). The genes cluster exhibiting 35% and 60% similarity for gene type arylpolyene and NRPS with the most similar known cluster for other and NRP: siderophore at node region 11.1 and 15.1 were further analyzed (Table 6.22).

Table 6.8: Genes involved in iron transport and siderophore production

Gene	Locus tag	Product	Pathway
*feo*B	J0657_02445	ferrous iron transport (FIT) protein B	Iron(II) transport
*feo*A	J0657_02450	FIT protein A *feo*A	
*fhu*E	J0657_10270	ferric-rhodotorulic acid	Iron complex transport
*ent*H	J0657_11000	thioesterase	Enterobactin production
*ent*E	J0657_01105	2,3-dihydroxybenzoate-AMP ligase	
*ent*C	J0657_01130	isochorismate synthase	
fiu	J0657_11665	catecholate siderophore receptor *Fiu*	Siderophore receptor

Table 6.9: Genes involved in motility and chemotaxis

Gene	Locus tag	Product	Pathway
*che*A	J0657_18770	chemotaxis protein *Che*A	Chemotaxis
*che*B	J0657_13650	chemotaxis protein *Che*B	
*che*Y	J0657_18780	chemotaxis response regulator *Che*Y	
*mot*B	J0657_08750	flagellar motor stator protein *Mot*A	
*mot*A	J0657_08745	flagellar motor protein *Mot*B	
*flh*B	J0657_18805	flagellar biosynthesis protein *Flh*B	Flagella biosynthesis
*flg*I	J0657_00020	flagellar basal body P-ring protein *Flg*I	
*flg*K	J0657_00010	flagellar hook-associated protein *Flg*K	
*fli*K	J0657_04855	flagellar hook-length control protein *Fli*K	
*pil*X	J0657_07000	protein *Pil*X	Pilus and fimbriae biosynthesis
*pil*C	J0657_06995	pilus assembly protein *Pil*C	
*pil*M	J0657_12600	pilus assembly protein *Pil*M	
*fim*C	J0657_12625	fimbria/pilus periplasmic chaperone	
*tad*D	J0657_18950	*Flp* pilus assembly (PA) protein *Tad*D	Type IV pilus homolog protein
-	J0657_18985	PA protein	
*cpa*B	J0657_18980	*Flp* PA protein *Cpa*B	

Table 6.10: Genes involved in attachment to plant surfaces

Gene	Locus tag	Product	Pathway
*bcs*F	J0657_13495	cellulose biosynthesis (CB) protein *Bcs*F	Cellulose biosynthesis
*bcs*G	J0657_13500	cellulose biosynthesis protein *Bcs*G	
*yhj*Q	J0657_13510	cellulose synthase operon protein *Yhj*Q	
*bcs*A	J0657_13515	UDP-forming cellulose synthase catalytic subunit	
*bcs*B	J0657_13520	CB cyclic di-GMP-binding regulatory protein *Bcs*B	
*bcs*C	J0657_13525	cellulose biosynthesis protein *Bcs*C	
*bcs*Z	J0657_13530	Cellulose	
*lpx*K	J0657_06895	tetraacyldisaccharide 4'-kinase	LPS, Lipid A biosynthesis
*lpx*L	J0657_19260	Kdo2-lipid IVA lauroyltransferase	and modifications

Table 6.11: Genes involved in quorum-sensing mechanisms

Gene	Locus tag	Product	Pathway
*cyc*A	J0657_07815	D-serine/D-alanine/glycine transporter	N-Acyl-homoserine-lactone production
*gcv*A	J0657_09785	transcriptional regulator *gcv*A	
*pst*J	J0657_10685	transcriptional regulator *Pts*J	
*lld*R	J0657_12195	transcriptional regulator *Lld*R	
*bet*L	J0657_19625	transcriptional regulator *Bet*I	

Table 6.12: Genes involved in the nitrosative stress and protection against oxidative

Gene	Locus tag	Product	Pathway/function
*kat*G	J0657_06470	catalase-peroxidase	Degradation of hydrogen peroxide and organic hydroperoxides
-	J0657_08595	glutathione peroxidase	Detoxification of peroxidized compounds
-	J0657_13015	glutathione S-transferase	
*grx*C	J0657_04095	glutaredoxin 3	
*grx*D	J0657_19130	*Grx*4 family monothiol glutaredoxin	
*gor*A	J0657_04475	glutathione-disulfide reductase	

Table 6.13: Genes responsible for organic acid metabolism

Gene	Locus tag	Product	Pathway
*acn*A	J0657_19640	aconitate hydratase	TCA cycle
*acn*D	J0657_00110	Fe/S-dependent 2-methylisocitrate dehydratase	
*ace*A	J0657_07520	isocitrate lyase	Glyoxylate bypass TCA
*acn*A	J0657_19640	aconitate hydratase	
acs	J0657_08335	Acetyl-coenzyme A synthetase	Acetate degradation
*fab*D	J0657_12360	[acyl-carrier protein] S-malonyltransferase	Malonate degradation I (biotin-
*mdc*A	J0657_09220	malonate decarboxylase subunit alpha	independent)
*mdc*H	J0657_06210	malonate decarboxylase subunit epsilon	
*mdc*G	J0657_06200	malonate decarboxylase holo	

Table 6.14: Genes involved in amino acid metabolism and transport

Gene	Locus tag	Product	Pathway
*met*K	J0657_14560	methionine adenosyltransferase	Methionine degradation
alr	J0657_08200	alanine racemase	D-alanine degradation
*spe*A	J0657_01890	arginine decarboxylase	L-arginine degradation
*spe*A	J0657_01890	arginine decarboxylase	
*cys*K	J0657_19305	cysteine synthase A	L-cysteine degradation
*glt*B	J0657_18515	glutamate synthase large subunit	L-glutamate degradation
*hut*H	J0657_10960	histidine ammonia-lyase	L-histidine degradation
*hut*C	J0657_10945	histidine utilization repressor	
epmB	J0657_05370	L-lysine 2,3-aminomutase	L-lysine degradation
*put*A	J0657_09160	bifunctional proline dehydrogenase	L-proline degradation
tdh	J0657_03685	Tdh L-threonine 3-dehydrogenase	L-tyrosine/threonine degradation
*hpp*D	J0657_01565	4-hydroxyphenylpyruvate dioxygenase	
*mms*B	J0657_13430	3-hydroxyisobutyrate dehydrogenase	L-valine degradation
gcvP	J0657_17820	aminomethyl-transferring glycine dehydrogenase	Glycine cleavage
*lpd*A	J0657_04555	dihydrolipoyl dehydrogenase	
-	J0657_08205	D-amino-acid dehydrogenase	D-amino acid degradation
*gln*A	J0657_18860	type I glutamate--ammonia ligase	Glutamine transport
*gln*E	J0657_02190	Bifunctional	

Table 6.15: Genes involved in opine and GABA transport and metabolism

Gene	Locus tag	Product	Pathway
*spe*A	J0657_01890	arginine decarboxylase	Putrescine and spermidine production and transport
*spe*D	J0657_01700	adenosylmethionine decarboxylase	
*spe*E	J0657_01885	spermidine synthase/polyamine aminopropyltransferase	

Table 6.16: Genes involved in the metabolism of phenolics and flavonoids

Gene	Locus tag	Product	Pathway
-	J0657_04780	oxidoreductase	Gallate catabolism
*phe*A	J0657_10905	prephenate dehydratase	Catechol metabolism

Table 6.17: Genes involved in lignin degradation and degradation against toxic peroxides

Gene	Locus tag	Product	Pathway
*kat*E	J0657_07885	catalase	Degradation of toxic peroxides
*ahp*F	J0657_18435	alkyl hydroperoxide reductase subunit F	
*ahp*C	J0657_18440	peroxiredoxin	
*adh*P	J0657_07410	alcohol dehydrogenase *Adh*P	Metabolism of alcohols and aldehydes
*fdh*D	J0657_19420	formate dehydrogenase accessory sulfurtransferase *Fdh*D	
-	J0657_16685	glutathione-independent formaldehyde dehydrogenase	

Table 6.18: Genes involved in the modulation of plant hormones

Gene	Locus tag	Product	Pathway
*trp*D	J0657_01675	anthranilate phosphoribosyltransferase	L-tryptophan production; IAA production
*trp*B	J0657_13265	tryptophan synthase (TS) subunit beta	
*trp*A	J0657_13275	TS subunit alpha	
*trp*C	J0657_01680	indole-3-glycerol phosphate synthase *Trp*C	
*ald*H	J0657_14110	aldehyde dehydrogenase	IAA production, IPA pathway
*mia*B	J0657_07115	tRNA (N6-isopentenyl adenosine(37)-C2)-*Mia*B methylthiotransferase	CK biosynthesis and transformation
*mia*A	J0657_16145	*Mia*A tRNA (adenosine(37)-N6)-dimethylallyltransferase	

Table 6.19: Genes involved in biological control

Gene	Locus tag	Product	Pathway
*ilv*D	J0657_02710	dihydroxy-acid dehydratase	2,3-Butanediol
*ilv*C	J0657_19445	ketol-acid reductoisomerase	
*ilv*N	J0657_19450	acetolactate synthase	
*min*D	J0657_00620	septum site-determining protein (SSP) *Min*D	Colonization
*min*E	J0657_00625	cell division topological specificity factor *Min*E	
*glp*K	J0657_04580	glycerol kinase *Glp*K	Stress resistance
*gro*ES	J0657_08665	co-chaperone *Gro*ES	Photosynthetic
*gro*EL	J0657_08660	chaperonin *Gro*EL	
*tru*D	J0657_16575	tRNA pseudouridine(13) synthase *Tru*D	
*tru*B	J0657_00165	tRNA pseudouridine(55) synthase *Tru*B	
*cyo*B	J0657_00995	cytochrome o ubiquinol oxidase (CUO) subunit I	Cytochrome
*cyd*X	J0657_11190	cytochrome bd-I oxidase subunit *Cyd*X	
*cyo*D	J0657_01005	CUO subunit IV	
*dip*Z	J0657_12710	cytochrome c biogenesis protein *Dip*Z	
*cyd*B	J0657_11195	CUO subunit II	

Table 6.20: Genes involved in osmotic stress tolerance

Gene	Locus tag	Product	Pathway
*bet*A	J0657_19615	choline dehydrogenase	Glycine betaine biosynthesis
*bet*B	J0657_19620	betaine-aldehyde dehydrogenase	
*pro*B	J0657_11110	glutamate 5-kinase	
*glt*B	J0657_18515	glutamate synthase	Glutamate biosynthesis
*glt*X	J0657_13135	glutamate--tRNA ligase	
*gln*A	J0657_18860	glutamine synthetase	Glutamine biosynthesis
*pro*P	J0657_19380	L-proline transporter/glycine betaine	Glycine betaine/proline transport system
cls	J0657_07495	cardiolipin synthase	Cardiolipin biosynthesis

Table 6.21: Genes involved in temperature stress resistance

Gene	Locus tag	Product	Pathway
*dna*J	J0657_03540	molecular chaperone *Dna*J	
*clp*A	J0657_02275	enzyme ATP-dependent-binding subunit *Clp*A	
*dna*K	J0657_03535	molecular chaperone *Dna*K	
*dna*K	J0657_03535	molecular chaperone *Dna*K	
*clp*B	J0657_02070	ATP-dependent chaperone *Clp*B	
*gro*ES	J0657_08665	chaperonin *Gro*ES	
*gro*EL	J0657_08660	chaperonin *Gro*EL	
*clp*X	J0657_19575	enzyme ATP-dependent-binding subunit *Clp*X	
*clp*S	J0657_02265	ATP-dependent *Clp* protease adapter *Clp*S	
*hsl*U	J0657_16380	ATP-dependent protease ATPase subunit *Hsl*U	
*clp*P	J0657_19570	ATP-dependent *Clp* endopeptidase proteolytic subunit *Clp*P	
*htp*X	J0657_00405	heat shock protein	
*htp*G	J0657_08165	molecular chaperone *Htp*G	
*hsp*33	J0657_15710	molecular chaperone *Hsp*33	
*hsp*70	J0657_02640	HSP20 family protein	

Table 6.22: Estimate of secondary metabolite gene clusters in the genomes of *S. indicatrix* strain BOVIS40

Node Rg	Type	From	To	MSKC		Similarity
Rg 11.1	Arypolyene	1	38,874	APE Vf	Other	35%
Rg 15.1	NRPS	1	13,311	Bacillibactin	NRP:NRP siderophore	60%
Rg 87.1	RiPP-like	2,929	13,774			
Rg 156.1	RiPP-like	30,429	40,343			

Key: MSKC - Most similar known cluster, Rg - region

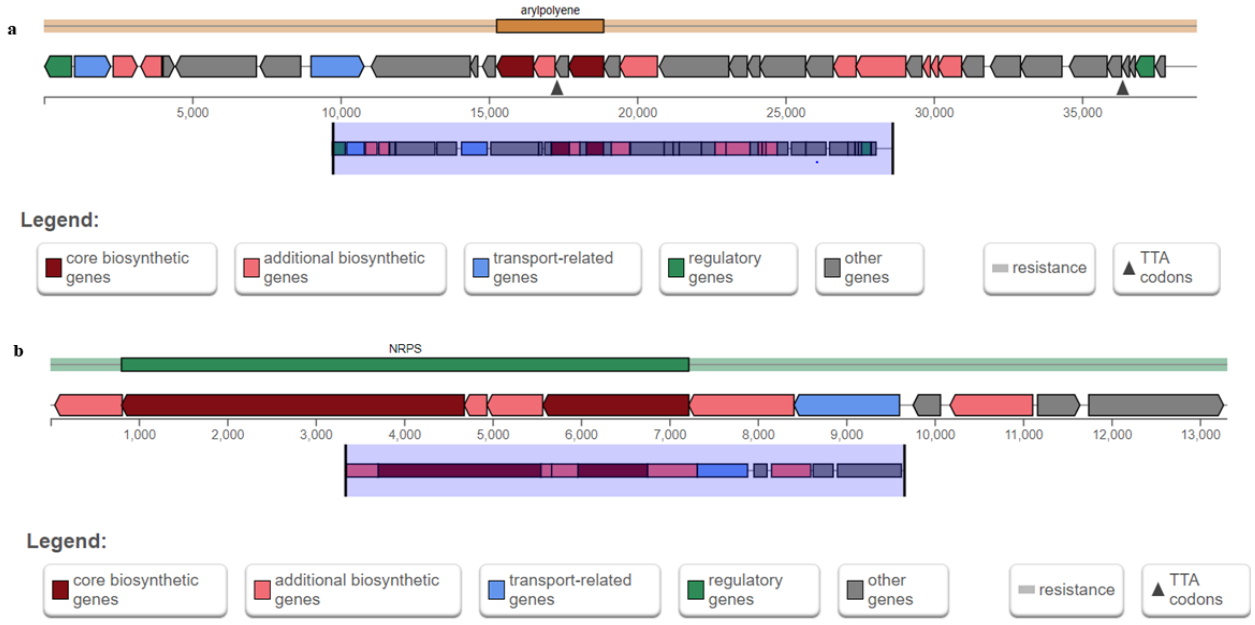


Figure 6.5a and 6.5b: Arylpolyene-and-NRPS-encoding genes

6.3.5 Growth parameters

The yield parameters of inoculated and non-inoculated sunflower from the greenhouse experiment are presented in Table 6.23. Except for taproot width, the aboveground parameters of inoculated plants compared to non-inoculated were statistically different. In terms of seed wet and dry weight, there was no significant difference compared to other belowground parameters. Inoculated plant yielded 348.04 g plant wet weight compared to un-inoculated with 332.04 g wet weight. Similar results were obtained on the shoot dry weight 57.28 g (un-inoculated) and 76.88 g (inoculated), un-inoculated head fresh and dry weight of 153.76 g and 41.55 g, and inoculated head fresh and dry weight of 182.83 g and 50.48 g respectively. The soil variables tested recorded high magnesium content of 642 mg/kg, potassium content of 399 mg/kg, nitrogen content of 0.10% and pH 7.4 (Table 6.24). The percentages of clay, sand, and silt were 22.20, 68.30, and 7.92, respectively.

Table 6.23: Sunflower yield parameters

Growth parameter	Non-inoculated	Inoculated with *S. indicatrix* BOVIS40
Belowground		
Tap root length (cm)	146.67±58.33[a]	177.33±28.31[b]
Tap root width (cm)	5.33±0.58[a]	5.33±1.15[a]
Root length (cm)	217±81.22[a]	257.33±48.44[b]
Lateral root number	25.67±1.53[a]	26.00±4.58[b]
Root wet weight (g)	44.09±16.12[a]	49.50±3.97[b]
Root dry weight (g)	8.41±1.55[a]	12.49±2.80[b]
Number of roots	935±11.30[a]	1236.00±616.83[b]
Aboveground		
Seed wet weight (g)	0.05±0.01[a]	0.06±0.00[a]
Seed dry weight (g)	0.03±0.01[a]	0.03±0.01[a]
Head fresh weight (g)	153.76±13.94[a]	182.83±23.76[b]
Head dry weight (g)	41.55±4.27[a]	50.48±10.04[b]
Plant wet weight (g)	331.04±20.16[a]	348.04±30.28[b]
Shoot wet weight (g)	165.82±5.16[a]	216.44±57.21[b]
Shoot dry weight (g)	57.28±7.04[a]	76.88±29.91[b]

Values are represented as means ± standard deviation of triplicate readings. Mean±standard deviation values with different superscripts (small letters) across the row represent a significant difference

Table 6.24: Physical and chemical analysis of the soil sample

Parameter	**Result output**
Manganese (mg/kg)	43.57
Phosphorus (mg/kg)	74.93
Organic carbon (%)	1.19
Total nitrogen (%)	0.10
pH (H_2O)	7.40
Magnesium (mg/kg)	642.00
Organic matter (%)	3.20
Potassium (mg/kg)	399.00
Iron (mg/kg)	2.00
Clay (%)	22.20
Sand (%)	68.30
Silt (%)	7.92

6.4 Discussion

Many studies have isolated and characterized bacterial strains from the endosphere of economic plants, and their presence within plant tissue has revealed mutual or antagonistic interactions with the host plants (Shastry et al., 2020, Singh et al., 2021, Annapurna et al., 2018). Diverse endophytic microbial domains associated with sunflower have been reported in many countries (Forchetti et al., 2007, Majeed et al., 2018), nevertheless, insights into sunflower-associated bacterial endophytes from Southern Africa have not been studied, and thus, the exploration of sunflower root-associated bacteria for improved productivity. Several agriculturally important bacterial endophytes contribute to plant growth through secretion of phytohormone, the bioavailability of soil nutrients, and antibiosis action against phytopathogens (Audipudi et al., 2017). The plant growth-promoting potential of the selected bacterial strain can help understand plant-bacterial mechanisms and the discovery of new bioproducts with a range of applications in agricultural biotechnology.

Endosphere represents the entire internal tissue of plants colonized by endophytic microbes with utmost beneficial effects on plant growth (Asaf et al., 2017). The beneficial endosphere inhabitants with PGP traits, as demonstrated by *in vivo* assay in this study, confirm their agricultural importance. The coevolution of endophytic microbes with the hosts has prompted complementary dynamics in their functions, such that, the host plant houses, protect, and supply nutrients to microbes. In return, these microorganisms enhance plant growth and survival (Omomowo and Babalola, 2019).

The design of the study was to isolate and characterize endophytic bacteria strains from the sunflower root with PGP potentiality. Generally, many strains of *Stenotrophomonas* species from environmental sources formerly identified as opportunistic pathogens are assigned to

closely related species without pathogenic potential. In recent times, their potential in plant growth promotion has significantly widened the research scope of this species, thus providing knowledge based on their environmental and agricultural importance (Alexander et al., 2019). So far, whole-genome analysis for detecting diverse genes involved in cellular metabolism and phytohormone production from *S. indicatrix* has not been studied. Hence, we present the first report on the genomic information on *S. indicatrix* isolated from sunflower roots.

Stenotrophomonas strain BOVIS40 was isolated on Luria Bertani medium, screened on growth-promoting media, and then sequenced. From the annotation results, it was revealed that the type of genes detected in strain BOVIS40 corroborate the *in vitro* screening. Nevertheless, other genes found aside from those screen *in vitro* were also detected. The results from plant growth-promoting tests showed that strain BOVIS40 could modulate plant growth in diverse ways. For instance, in phosphate solubilization, exopolysaccharide production, IAA, and siderophore biosynthesis. The results obtained corroborate earlier documentation on plant growth-promoting attributes of *Stenotrophomonas* strains and other root-associated endophytic bacteria (Ngoma et al., 2013, Perez-Perez et al., 2020, Mukherjee and Roy, 2016).

The ability of microbes to fix atmospheric nitrogen for plant use depends on certain genes and enzymes produced by them (Valentine et al., 2018). The protein nature of enzyme coding genes such as *nif*H and *nif*DK involved in nitrogenase production in a bacterial cell has contributed to their functions in the biotransformation of bound nitrogen in the soil into absorbable form (Guerrieri et al., 2021). The enzyme cysteine desulfurase coding for gene *nif*S involved in the nitrogen fixation pathway was identified in strain BOVIS40. Other genes involved in nitrogen regulatory protein NR (I) (*ntr*C) and flavodoxin production (*nif*F), together with the ammonia transport gene *amt* identified, could contribute to an increase in nitrogen level in the soil. The

presence of nitrogen fixation (NF) genes such as *nif*ABDEFHJKLMNSTUVWXYZ in the genome of *Klebsiella* species function in the formation of complex membrane structure and transport of molecules in the presence of nitrogenase have been employed as a model in the NF ability of *Rhodobacter capsulatus* (Jeong and Jouanneau, 2000). The detection of *nif*F in the genome of rhizobacterium *Azospirillum brasilense* Sp7 and *K. variicola* UC4115, contributing to their nitrogen fixation ability for yield improvement in tomato, has been identified with success in recent findings (Guerrieri et al., 2021). A similar gene identified in this study could contribute to bacterial functions in improving sunflower yield. Also, the expression of *fix* genes such as *fix*ABCX in the genome of *Azorhizobium caulinodans* and *Bradyrhizobium japonicum* with alternate *nif*JF and their functions in the electron transport chain system have contributed to the transfer of proteins in rhizobia (Tsoy et al., 2016). Also, identification of flavodoxin and ammonia transport gene (*amt*B) in the genome of strain MSR2 has been reported (Nascimento et al., 2020a), with similar results documented in this study.

The number of *nif* genes produced by the bacterial cell may be due to their physiological nature and source of isolation. However, unidentified genes as reported in other literature might be responsible for the replacement of these genes with other *nif* products (Shen et al., 2013). Interestingly, information relating to the nitrogen fixation potential of *S. indicatrix* isolated from the root of oilseed sunflower based on their genome studies has not been documented.

Phosphate solubilizing potential of strain BOVIS40 under *in vitro* assay on tricalcium medium was validated by identification of copious genes involved in phosphate solubilization and transport. Gluconic acid (GA) is known as a precursor that facilitates phosphate solubilization in diverse bacterial phyla. The synthesis of GA due to the catalytic actions of enzyme glucose-1-dehydrogenase and its non-protein chemical compound pyrroloquinolone quinine (*pqq*) have

contributed to microbial functions and applications (Ramachandran et al., 2006). Despite non-detection of *pqq* genes in strain BOVIS40, reports have shown the expression of heterologous *pqq* genes (*pqq*ABCDEF) that confer phosphate solubilization potential in diazotroph endophytic bacterium *Herbaspirillum seropedicae* Z67 associated with economic plants (Wagh et al., 2014, Baldotto et al., 2011). Similarly, the absence of *pqq* genes has been reported in the genome of *Klebsiella* sp. D5A (Guerrieri et al., 2021).

Phosphate uptake by strain BOVIS40 may be enhanced due to their high affinity and presence of phosphate transport genes, *pst*ABCS, which corroborate the findings earlier documented by Guerrieri et al. (2021) and Shariati et al. (2017) on the expression of phosphate transport genes, *pst*ABCS on the whole-genome analysis of *K. variicola* UC4115 and *Pantoea agglomerans* P5. The presence of *pst* genes may enhance phosphate bioavailability in the soil and uptake by plants. The detection of *pho*A gene coding for enzyme alkaline phosphatase involved in various phosphorus metabolism has been identified in *Burkholderia multivorans* WS-FJ9 (Liu et al., 2020a). Furthermore, the detection of *pgl, ppx,* and *ppa* in the genome of BOVIS40 that code for 6-phosphogluconolactonase, exoployphosphatase, and inorganic pyrophosphatase for the synthesis of D-gluconate and degradation of inorganic pyrophosphatase could contribute to the ability of the bacterial strain to solubilize phosphate in the soil. Interestingly, our results corroborate the findings of Nascimento et al. (2020b), who reported *pho*AD genes, coding for alkaline phosphatase in the genome of *Bacillus megaterium* STB1, that enhance phosphate solubilization ability in the bacterial strain.

In line with the previous studies on the PGP properties of endophytic bacteria colonizing plant root (Stoltzfus et al., 1997, Tariq et al., 2014, Eida et al., 2021), expression of genes involved in secretion system, siderophore, IAA synthesis, acetoin, and 2,3-butanediol was detected in

endophytic bacterial strain BOVIS40. The synthesis of IAA in plants and microorganisms has been postulated through multidimensional pathways which include, indole-3-acetonitrile (IAN), tryptamine, indole-3-pyruvate (IPA), and indole-3-acetamide (Spaepen and Vanderleyden, 2011). IAA, a major occurring auxin in plants can signally mediate gene expression and biosynthesis in microorganisms. Thus, plant stimuli against natural pathogenic microbes may be linked to the synthesis of auxin signal molecules by the microorganisms (Spaepen and Vanderleyden, 2011). To this premise, two major pathways, namely IPA and IAN were identified in the genome of BOVIS40 as previously reported in the genome of *K. variicola* UC4115 (Guerrieri et al., 2021). The biotransformation of IPyA - tryptophan to indole pyruvic acid in the presence of aminotransferases that convert IPyA to IAAld - indole acetaldehyde and gene *ipd*C encoding indole pyruvate decarboxylase in *Pantoea* spp has been documented (Nascimento et al., 2020a). Also, the presence of *ipd*C gene in *Enterobacter cloacae* with similar functions has been identified (Koga et al., 1991). Furthermore, the presence of aldehyde dehydrogenase in the genome of strain BOVIS40 has been identified to facilitate the conversion of intermediate IAAid to IAA (Koga et al., 1991).

Interestingly, strain BOVIS40 harbors tryptophan synthase that reversibly catalyzes indole-3-glycerol phosphate to form glyceraldehyde-3-phosphate and irreversibly involved in pyridoxal phosphate pathway to form tryptophan through the condensation of serine and indole (Ireland et al., 2008). The presence of amidase enzymes could contribute to bacterial functions in the production of IAA along the IAN pathway. Additionally, the involvement of strain BOVIS40 in two different IAA metabolic pathways significantly revealed their IAA production ability to play a vital role in plant root formation and development.

Subsequently, *mia*AB genes involving cytokinin biosynthesis and transformation were detected in the genome of BOVIS40. Enzyme coding for tRNA dimethylallyltransferase genes in the bacterial genome could enhance iPR - N_6-(dimethylallyl) adenosine production. The expression of other cytokinin genes such as *mia*BE that convert iPR to 2-methylthio-N_6-(dimethylallyl) adenosine and further to 2-methylthio-cis-ribozeatin has been identified in the genome of *P. phytobeneficialis* MSR2 (Nascimento et al., 2020a). The direct effects of these genes in plant growth enhancement suggest that cytokinin synthesis can play a vital role in plant growth promotion and plant health (Wani et al., 2016). Nevertheless, further comparative research into agriculturally important *S. indicatrix* strains will help provide information on their novel genes, as reports on cytokinin genes from this endophytic bacterium strain BOVIS40 have not been documented.

The detection of siderophore genes in *Stenotrophomonas* strain BOVIS40 can enhance plant accessibility to soil mineral nutrients and contribute to their growth. Genes coding for iron transport found in strain BOVIS40 could play a vital role in the mineralization of insoluble iron and bioavailability for plant use. Furthermore, the synthesis of 2,3-butanediol by endophytic bacteria has been linked to their biocontrol ability, induction of systemic resistance, and their ability to improve plant tolerance to drought (Samaras et al., 2020). Reports on the biocontrol activity of endophytic bacteria such as *Pseudomonas* spp against *Phytophthora capsici* that cause rot and blight disease in pepper (Aravind et al., 2009), *Bacillus* spp against wilt disease in chili (Dowarah et al., 2021), *Burkholderia cenocepacia* against *Fusarium* wilt in banana (Ho et al., 2015), and *Stenotrophomonas* spp against *Sclerotium rolfsii* that cause collar rot in tomato (Sahu et al., 2019) have been studied.

Endophytic microbes have employed indirect mechanisms in the production of metabolic compounds in the control of phytopathogens, and their pathogen suppressive actions rely on their ability to produce biocontrol agents (Santoyo et al., 2016). The biocontrol ability of strain BOVIS40 may be due to siderophore synthesis that enhances their antibiosis activity against plant pathogens (Maheshwari et al., 2019b). Notably, strain BOVIS40 harbors the siderophore gene *fiu.* Siderophore catecholate found in the bacterial genome has been recognized to play an important role in bacterial adherence to the receptor surfaces, iron chelation, and transport (Pedraza et al., 2010). Conversely, other related genes such as *ent*ABCDEFGHS and *fep*ABCDG are involved in siderophore production and transport that function in the conversion process of chorismate into enterobactin that was not detected in this study have been documented (Hubrich et al., 2021, Tortora et al., 2011).

Additionally, based on the beneficial effects on plant growth promotion, strain BOVIS40 harboring biocontrol genes could contribute to disease suppression promote plant growth. Also, detection of genes modulating the biological activity of strain BOVIS40 can stimulate the secretion of antimicrobial compounds such as g-aminobutyric acid (GABA) and 4-hydroxybenzoate, thus revealing their genome response in plant health and exploration as bioinoculants for the control of microbial infections.

The survival of endophytic microbes in the root endosphere is affected by the number of exudates released from the plant roots. Root exudate is mainly composed of amino acids, sugars, phenolics, organic acids, etc. (Baudoin et al., 2003). Various organic compounds such as sugars serve as an energy source for microbial metabolism. The release of carbon-containing compounds from the rhizo-compartment as exudate could supply endo-rhizobiome with the required energy for various cellular activities (Zhalnina et al., 2018). The sugar assimilation

potential of strain BOVIS40, as reported in Table 6.1, was validated based on the detection of various genes involved in carbohydrate metabolism and transport. Carbohydrate utilization response of strain BOVIS40 can enhance their biofilm production and colonization in the host plants. Several genes involved in the degradative pathway of organic compounds such as carbohydrates may contribute to the biotransformation of various organic substrates (Nascimento et al., 2020a). Aside from carbohydrates, the expression of genes participating in organic acid metabolism may contribute to bacterial affinity with the host plant. Also, various genes involved in the degradation and transport of amino acids and their derivatives contributing to amino acid metabolism and bacterial colonization with the host plants have been documented (Mavrodi et al., 2021).

Prephenate hydratase gene (*phe*A) involved in catechol metabolism was found in strain BOVIS40. Genes involved in gallate catabolism were also detected in strain BOVIS40. The presence of these genes could suggest their ability to modulate phenolic degradation, which plays a major role in endophytic lifestyle. Phenolics are classified as vital compounds that regulate plant growth and stimulate resistance stimuli in plants (Nascimento et al., 2020a). Furthermore, phenolic metabolism can enhance the colonization efficiency of strain BOVIS40 in plants. The ability of bacteria to degrade lignin due to their inherent lignin-degrading enzymes is known to functionally contribute to bacterial infiltration and colonization in the host endosphere compartment (Shi et al., 2015).

Bacterial colonization within the plant endosphere to form biofilm can be engineered based on certain bacterial traits and the synthesis of biofilm production genes. Genes involved in bacterial attachment to plant surfaces such as *bcs*ABCFGZ and *yhj*Q as expressed in the genome of strain BOVIS40 have been identified in the genome of Ghats1 (Shastry et al., 2020).

The expression of colonization genes in the genome of endophytic bacteria may enhance their colonization potential, host selection, and the establishment of plant-bacterial interactions in a symbiotic manner (Eida et al., 2021). Furthermore, the presence of cellulose biosynthetic genes in strain BOVIS40 may help configure endophytic lifestyle for host-bacterium mutual dependence.

The presence of exopolysaccharide genes could enhance bacterial colonization to form a biofilm, mucilaginous active substances, and boost stress tolerance in plants (Meneses et al., 2011, Santaella et al., 2008). This suggests that bacterial strain BOVIS40 could form synergistic cooperation by colonizing the plant endosphere, thus benefiting the host plants. Endophytes can infiltrate the host plant either by tissue damage, natural opening or through enzyme action (Omomowo and Babalola, 2019, Yadav et al., 2020). Hence, the ability of bacterial strain BOVIS40 to produce enzymes, and genes coding for exopolysaccharides and cellulose biosynthesis may mediate bacterial colonization and attachment for biofilm production in the root endosphere (Meneses et al., 2011).

Cellular organelles such as fimbriae, flagella, and pili play a vital role in bacterial attachment, movement, and response to chemicals (Zheng et al., 2015). The detection of chemotaxis and motility genes can mediate bacterial ability to infiltrate plant roots and establish a bacterial community for plant benefits. The results obtained from this study corroborate the findings of Shastry et al. (2020), who reported chemotaxis functional genes (*che*AVY) and other genes involved in flagella biosynthesis in *E. cloacae* Ghats1. Similarly, identification of *mot*AB and other genes responsible for chemotaxis actions such as *che*ABYWR in *P. phytobeneficialis* MSR2 have been reported (Nascimento et al., 2020a).

Plant-microbe interactions stimulating a lot of chemical reactions in the production of antioxidative and antimicrobial compounds such as phytoalexins, nitric oxide, and reactive oxygen species (ROS) have boosted plant defense against osmotic stress (Gaber et al., 2006, Samaras et al., 2020). The expression of oxidative and osmotic stress genes in the bacterial genome could reveal their strong resistance tendencies to ROS, a chemical compound produced by bacteria in a stress environment (Scott et al., 2007, Nascimento et al., 2020b). Also, the expression of osmotic genes is known to boost host immune responses. The colonization and survival of endophytic bacteria in plants growing in an environment exposed to oxidative stress can be modulated by the synthesis of antioxidant lytic enzymes such as glutathione reductase, monodehydroascorbate reductase, superoxide dismutase, ascorbate peroxidase, and catalase. The function of these enzymes is paramount in scavenging free radicals in the plant endosphere and other regulators such as reduced glutathione and ascorbic acid (Das et al., 2015). Similar functional genes coding for catalase, superoxide dismutase, peroxiredoxin, glutathione peroxidase, alkyl hydroperoxide reductase, glutaredoxin 3, glutathione monothiol glutaredoxin, and glutathione-disulfide reductase were detected in strain BOVIS40. The detection of a catalase-peroxidase gene (*kat*E) and catalase gene (*kat*G) responsible for the degradation of toxic peroxide corroborates the findings of Nascimento et al. (2020a), who reported similar genes from *Pantoea phytobeneficialis* MSR2. Hence, their ability to stimulate defense against oxidative and osmotic stress in plants could be promising in the formulation of bioinoculants for plant benefit.

The presence of osmotic stress regulatory genes could enhance plant response to heat or cold shock. Similarly, the protein-coding genes that modulate cold and heat stressors can function through multiple gene families and differential regulation (Wani et al., 2016). Plant response

to cold or heat stress has been attributed based on the expression of notable genes such *csp, hsp*, and a gene coding for the chaperone (Phadtare, 2004). As presented in this study, these genes could contribute to the resistance and survival of sunflower in temperate environments. The presence of *csp* in the bacterial genome contributing to RNA and DNA stabilization for improved translational and transcriptional efficiency of plants under cold or heat conditions has been documented (Nascimento et al., 2020b). However, the expression of these genes in strain BOVIS40 corroborates the findings of Nascimento et al. (2020b), who had earlier reported chaperones and other heat stress genes such as *gro*EL, *gro*ES, *dna*K, *dna*J, and *hsp* in the genome of *Bacillus megaterium* STBI.

Transcriptional regulator and amino acid transporter genes were found in strain BOVIS40. These genes can mediate mechanisms involved in quorum-sensing and dynamics in the lifestyle and ecology of endophytic microbes. Various antimicrobial compounds secreted by bacterial strains can exert beneficial effects in sustaining plant health. For example, the role of bacteriocin produced by endophytic bacteria as a source of new antibacterial compounds has synergistically been employed to upsurge antibiotic resistance (Oliveira et al., 2018, Lopes et al., 2017). Organic compounds synthesized by bacteria naturally play a vital role in the adaptation and survival of the host plant. *Stenotrophomonas* species are regarded as opportunistic pathogens that affect humans and plants, and in recent times, their biocontrol efficacy due to the ability to secrete vital secondary metabolite has been promising. However, documentation on secondary metabolite gene clusters from *S. indicatrix* is rare. Interestingly, two prominent gene clusters such as arylpolyene (APE Vf) and bacillibactin NRP: siderophore with type arylpolyene and non-ribosomal peptides (NRPS) detected in the genome of strain BOVIS40 could contribute to plant protection against oxidative stress and other biological

functions. For instance, the siderophore gene cluster detected in the genome of copious endophytic *Streptomyces kebangsaanensis* contributes to their biocontrol activity against pathogenic fungi *Fusarium oxysporum* and enhances plant survival (Siupka et al., 2020, Remali et al., 2017). The attribute of siderophore synthesis by bacterial strains can provide information on their ecological functions as biocontrol agents against plant pathogens. Hence, screening secondary metabolites in the genome of strain BOVIS40 can enable researchers to understand their application in the control of phytopathogens.

Under greenhouse experimental conditions, an increase in yield parameters of inoculated sunflower compared to un-inoculated was observed. This can be linked to the pheno-genotypic attribute of endophytic bacteria to solubilize phosphate, synthesize IAA and siderophore, and detection of possible biocontrol genes in the bacterial genome, and this may contribute to bacterial activity in the improvement of below-and-aboveground crop yield. The presence of IAA genes seemed evident in the root formation in sunflowers. Hence, results obtained as demonstrated, significantly reveal the agricultural importance of *S. indicatrix* BOVIS40 upon inoculation and also show their genetic capacity in the root ecosystem for plant growth and sustainable food production.

6.5 Conclusion

Detailed information on the notable genes with multiple functions of *S. indicatrix* BOVIS40 and evaluated sunflower yield parameters were documented in this study. The genome analysis highlighting important regulatory genes confirms some *in vitro* screening results. The plant growth-promoting genes exhibited by *S. indicatrix* BOVIS40 can be linked to their underlined promise in agriculture. The multiple genes identified strain; revealed their biotechnological significance in plant-microbe interactions, plant resistance to environmental stressors (such as

temperature, oxidative and osmotic stress), degradation of organic substrate (carbohydrate), and organic compounds could enhance plant growth, soil health, and crop improvement. Importantly, the presence of these genes has provided insights into the genomic functions of *S. indicatrix* BOVIS40, with a promising outlook in developing sustainable agriculture. Also, based on the limited study on endophytic bacterium *S. indicatrix* BOVIS40, the information provided in this study will help harness its potential as a suitable candidate in the formulation of bioinoculants towards addressing agricultural problems.

7.0 CHAPTER SEVEN

Effect of endophytic bacterium, *Stenotrophomonas maltophilia* JVB5 on sunflower

Abstract

The genus *Stenotrophomonas* are Gram-negative bacteria dominating soil and plants. Their mutual interaction with oilseed crops as biorational assets in ensuring ecological balancing for sustainable plant health is promising. Nevertheless, information on the versatility, exploration, and agricultural importance of these bacteria remains highly understudied, possibly due to the lack of genome information in the database. To further provide insights into the agricultural and biotechnological relevance of these genera, we present genome sequence and annotation of *S. maltophilia* JVB5 isolated from sunflower root endosphere from North West Province, South Africa. Draft genome analysis revealed a genome size of 4,771,305 bp, sequence read count of 8,764,890, 66% GC content, 57 tRNAs, 268 contigs, and 4,160 protein-coding genes with functions in various metabolic pathways. Pathways involved in IAA production were found in the *Stenotrophomonas maltophilia* JVB5 genome. The draft genome annotation predicted notable genes needed for bacterial colonization, antibiosis, and plant growth promotion. The predicted genes involved in sulfur metabolism, and oxidative stress may enhance plant growth promotion and boost plant resistance to stress. Upon inoculation, strain JVB5 efficiently colonized sunflower root under greenhouse with significant improvement in the wet plant weight of 437.20 g compared to un-inoculated with 331.04 g wet weight. The genomic analysis revealing specific functional genes in the bacterial genome suggests its bioprospecting in agriculture. Hence, understanding the mechanisms employed by strain JVB5 based on the predicted multifunctional genes will help harness their bioresource in sustainable plant health.

Keywords: Bacterial genome, crop improvement, putative phytohormone genes, root adherence, sunflower, sustainable agriculture

7.1 Introduction

Endosphere depicts an entire region within plant tissues colonized by endophytic microbes (Cavalcanti et al., 2020, Fadiji and Babalola, 2020b). In recent times, research into the endosphere microbial community to explore beneficial endophytes for improving crop health is a promising avenue in agriculture. Many studies have demonstrated the biotechnological application of endophytic microbes in combating future food scarcity and insecurity (Alkahtani et al., 2020). However, knowledge on the novel agriculturally important endophytic bacterial strains associated with sunflower in Southern Africa is limited, thus necessitating this study.

Bacterial endophytes can form a mutual or antagonistic association with plants, with beneficial types classified as PGPE (Adeleke and Babalola, 2020a). PGPE enhances plant growth by supplying essential soil nutrients, production of growth hormones, nitrogen fixation, and synthesis of chelating compounds (siderophore) that stimulate plant response in the control of phytopathogens (Wang et al., 2020a). Some examples of endophytic bacterial genera employed as bioinoculants include *Acinetobacter, Agrobacterium, Azospirillum, Bacillus, Brevibacillus, Burkholderia, Herbaspirillum, Mycoplasma, Pantoea,* and *Pseudomonas* (Rahman et al., 2018, Haidar et al., 2018).

Based on the ecological threats posed by the use of chemical fertilizer, the exploration, and use of bacterial inoculants as alternatives promise to be efficient in developing stable ecosystems and environmental-friendly agriculture. The bacterial genera *Stenotrophomonas* colonizing the plant endosphere has certain functional traits which enhance their metabolic activities in producing several metabolites to aid survival in different habitats (Rojas-Solís et al., 2018). Like other identified *Stenotrophomonas* species, *S. maltophilia* is Gram-negative, rod-like, spore former, facultative anaerobe, and found in plant and soil environments.

Stenotrophomonas species have been classified as opportunistic pathogens in humans, but findings on their agricultural importance have been achieved with success (Berg and Martinez, 2015). *S. maltophilia* is one of the dominant groups of bacteria with diverse ecological functions ranging from agricultural to industrial applications. Reports on *S. maltophilia* producing secondary metabolites, extracellular enzymes, and plant growth-promoting genes are less documented, and information on the biotechnological application of *S. maltophilia* isolated from sunflower is rare in literature. Insights into the genomic study of *S. maltophilia* will provide new information on novel genes involved in different biological functions. Hence, we performed draft whole-genome sequencing of strain JVB5 to identify its functional genes involved in plant growth and sustainable health.

7.2 Materials and methods

7.2.1 Isolation and screening of *S. maltophilia* JVB5

Sunflower roots were sourced from farmlands in Lichtenburg, South Africa (SA) (26°4′31.266″S, 25°58′44.442″E) in February 2020. An endophytic bacterium strain JVB5 was isolated from the sunflower root on Luria Bertani (LB) agar medium based on the modified method of Khamwan et al. (2018b). Briefly, the plant roots obtained were placed in zip-lock bags on an ice-box for transportation to the laboratory within 4-hour of collection for further analysis. For bacterial isolation, the roots were cut into small sizes and surface-sterilized by immersion in 70% (v/v) ethanol for 1 minute, followed by 2% (v/v) hypochlorite solution for 3 minutes, and finally, immersed in 70% (v/v) ethanol for 30 seconds. The samples were thoroughly rinsed 5 times with sterile distilled water to remove the surface sterilization agents. The surface-sterilized root samples were macerated manually and serially diluted. From the

serial dilution process, 0.1 ml from 10^6 dilutions were aseptically dispensed into sterile Petri dishes and poured plated with sterilized LB medium. Then incubated at 28^{o}C for 24 hours and then checked for colony growth. The pure isolate was obtained by streaking a bacterium inoculum on fresh LB agar plates. For cultural and morphological characterization, various biochemical tests were performed. The pure isolate was kept on slants at 4^{o}C for further analysis.

7.2.2 Morphological and biochemical characterization of *S. maltophilia* JVB5

The cultural attributes of *S. maltophilia* JVB5 were evaluated on LB media after incubation at 28^{o}C for 24 hours. The cultural morphology of *S. maltophilia* JVB5 was visualized under a light microscope (ECLIPSE E200; Nikon, Japan). The growing of bacterial isolates in the LB broth was maintained at optimal growth conditions required for bacterial proliferation. Biochemical tests, such as sugar fermentation tests (mannitol, glucose, sucrose, maltose, fructose, galactose, and raffinose), catalase test, oxidase test, citrate test, nitrate utilization test, starch and casein hydrolysis were performed.

7.2.3 Plant growth-promoting screening

7.2.3.1 Siderophore production

The siderophore-producing potential of strain JVB5 was investigated on chrome azurol S (CAS) Khan et al. (2020). The quantity of siderophore produced was determined by inoculating LB broth solution containing CAS with 0.1 ml of 24-hour old bacterial culture and incubated at 180 rpm on a rotary shaker for 164 hours. Centrifugation of cell suspension was achieved at 10,000 x g for 8 minutes. Zero-point five milliliters of the cell filtrate was added to 0.5 ml CAS reagent and properly mixed, then incubated for 120 seconds. The quantity of siderophore

released was measured at 630 nm using a spectrophotometer (Thermo Spectronic, Merck Chemicals, SA). The siderophore estimate was obtained from the siderophore standard curve.

7.2.3.2 Phosphate solubilization

The qualitative screening of bacteria isolates for the solubilization of phosphate was performed according to the modified method of Khan et al. (2020). For the quantitative assay, phosphate solubilization tendencies of the bacteria isolate were performed by inoculating 10 ml sterile Pikovskaya broth in 50 ml Falcon tubes with 0.1 ml (10^6 CFU/ml) freshly grown bacterial culture, incubated at 30^{o}C for 5 days at 160 rpm on a shaker. Bacterial supernatant was obtained after centrifugation of 10 ml bacterial cultures at 4^{o}C revolution per minute (10,000 rpm for 5 minutes). Four milliliters (4 ml) of the color reagent (1:1:1:2 ratio 3M H_2SO_4, 10% (w/v) ascorbic acid, 2.5% (w/v) ammonium molybdate and distilled water were added to 10% (w/v) of 5 ml trichloroacetic acid inside test tubes. The tubes were kept for 15 minutes upon incubation at normal room temperature. The quantity of phosphate content was measured according to phosphomolybdate, a blue method at an absorbance of 820 nm. The phosphate solubilization potential by the bacterium in the Pikovskaya broth was determined from the phosphate (KH_2PO_4) standard curve. Medium without bacterial inoculation served as the control.

7.2.3.3 Exopolysaccharide (EPS) production

Bacteria isolate was qualitatively screened for EPS production following the method of Igiehon et al. (2019). A square-sized sterile Whatman filter paper No. 1 was aseptically and gently placed onto sterile molten LB agar plates. Two microliters (2 µl) of freshly grown 24-hour bacterial culture were directly inoculated on the surface of the filter paper placed inside

sterilized media plates supplemented with sucrose (10%) with pH adjusted to 7. Subsequently, the plates were aseptically inoculated and incubated at 30°C for 120 hours. After that, mucoid formation around the colonies on the square-size filter paper depicted EPS production.

7.2.3.4 Indole acetic acid (IAA) production

Indole acetic acid synthesis by *S. maltophilia* JVB5 was conducted according to Gutierrez et al. (2009). Ten milliliters (10 ml) LB broth supplemented with tryptophan were aseptically inoculated with 100 µl freshly grown bacterial culture (10^6 $CFUml^{-1}$) and incubated at 28^{o}C for 7 days at 120 rpm in a rotary shaker incubator (SI-600, LAB Companion, Korea). The bacterial culture was cold centrifuged (4^{o}C) at 10,000 rpm for 300 seconds to obtain a free cell supernatant layer. From the supernatant, 1 ml was measured into a clean tube, and 2 ml Salkowski reagent (1:30:50 ratio of 0.5 M $FeCl_3$ solution: 95% w/w sulfuric acid: distilled water) was added. Then, a drop of 10 mM orthophosphoric acid was also added to the mixture and incubated for 10 minutes for observable color development. The appearance of pink coloration in tubes after incubation in the dark indicated a positive result. An un-inoculated tube serves as the control. The samples were measured using UV-spectrophotometer (ThermoFisher Scientific, USA) at 530 nm from the reacting mixture and control tube. IAA concentration was evaluated from the IAA gradient standard curve (SC).

7.2.4 Whole-genome sequencing of *S. maltophilia* JVB5

The standard Illumina sequencing method was employed for the WGS at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. Genomic DNA was fragmented using an enzymatic approach (NEB Ultra II FS kit). The resulting DNA fragments were selected by size (200 – 700 bp), using AMPure XP beads. Each DNA fragment was end-repaired and ligated to

Illumina-specific adapter sequences. DNA samples were singularly indexed, and a second size selection step was achieved. A Fluorometric method was used to quantify each sample at dilution standard concentration (4 nM). Sequencing was performed on Illumina's NextSeq platform, using a NextSeq mid-out kit (300 cycles), according to the manufacturer's guide. The resulting 400 Mb of 2 x 150 bp long paired-end reads dataset was obtained from *S. maltophilia* JVB5.

Genome analysis of sequences was performed on KBase (https://kbase.us/) (Arkin et al., 2018). A quality check of the sequence was performed by FastQC (version 0.11.5) (Bioinformatics, 2011), and Trimmomatic (version 0.36) (Bolger et al., 2014) was used for the trimming and removal of low sequence reads and further assembled by SPAdes (version 3.13.0) (Nurk et al., 2013). Gene annotation was performed on RASTtk (version 1.073). The bioinformatics analysis was performed using default settings. The draft genome at GenBank was annotated with the NCBI PGAP pipeline during submission as a fully automated process. Predicted functional genes were obtained from NCBI. Metabolic pathways of biomolecules were obtained from KEGG on RAST (https://rast.nmpdr.org/rast.cgi/). Biosynthetic gene clusters present in the genome of the organism were detected using antiSMASH (version 6.0.0) (https://antismash.secondarymetabolites.org) (Weber et al., 2015). The circular genome visualization was obtained from PATRIC (https://www.patricbrc.org/) (version 3.6.9), while the phylogeny analysis of the draft genome sequence was performed using MrBayes (http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes) (version 3.2.6) (Arkin et al., 2018, Huelsenbeck and Ronquist, 2001).

7.2.5 Availability of data and material

The data available from the NCBI database include Bioproject number, PRJNA706608, Sequence Read Archive, SRR13908543, and accession and strain numbers (https://www.ncbi.nlm.nih.gov/genome/?term=JAGEKL000000000) and JVB5 search (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/880/JVB5), respectively.

7.2.6 Greenhouse experiments

The effect of strain JVB5 on sunflower growth was tested in the greenhouse. Briefly, the experiment was carried out using sterile and un-sterile soil sourced from University field environment (25°49′26.256″S, 25°36′31.536″E). Sunflower seeds were surface-sterilized by washing, first, in 70% ethanol for 180 seconds, then in 3% hypochlorite for 60 seconds, and finally dispensed in 70% ethanol for 2 minutes. The seeds were further washed with water to remove the disinfectants. The bacterial isolate was cultured on LB medium at optimum growth conditions. Broth suspension was cold centrifuged at 4^{o}C revolution per minute (1,000 rpm for 5 minutes) using a centrifuge and then pelletized bacterial cells were washed in 0.85% (w/v) normal saline. The inoculum size was standardized to 0.5 x 10^{6} CFU/ml at OD_{600}. Seeds were inoculated in the bacterial suspension while seeds suspended in sterile distilled water served as control. Twenty-four plastic pots containing experimental soils were arranged at random in the greenhouse at a 10 cm distance apart. Ten seeds were planted in each pot containing sterilized moistened soil. After seed planting, the moisture content of the soil was maintained by adding 500 ml sterile water. The light conditions in the greenhouse were 14-hour photoperiod and 10 hours dark, and temperature ranged between 25 and 30^{o}C. At maturity, i.e., 132 days, harvesting of sunflower was performed. Sunflower roots were thoroughly washed with water and then excised from the shoots. Root and stem weight (dry and fresh) parameters were

recorded. Experiments were performed in triplicate. The experimented soil contains 68.30% sand, 7.92% silt, 22.20% clay, and 3.20% organic matter 3.20. The soil pH was 7.4. After 6 weeks of sunflower inoculation in the greenhouse, re-isolation of the bacterial strain was performed from the plant roots and the same endophytic bacterium was identified, which confirm the adherence of strain JVB5 to the sunflower roots.

7.2.7 Data analysis

Data analysis was performed using Statistical Package for the Social Sciences (SPSS) version 6 and Microsoft Excel version 13. Data obtained were presented as mean±standard deviation. A significant difference among the treatment groups was calculated using analysis of variance and treatment means was compared using Duncan's multiple range test (DMRT).

7.3 Results

7.3.1 Bacterial morphology, biochemical and plant growth-promoting features

The morphological, biochemical, and screening of *S. maltophilia* JVB5 are presented in Table 7.1. Strain JVB5 is Gram-negative, catalase-positive, rod in shape ferments sugars tested, growth conditions ranged between temperature 25 and 40^{o}C, and pH 4 and 10. The bacterial yielded approximately 80% siderophore activity, 32 µg/ml phosphate activity, and 30 µg/ml IAA activity, respectively. Varied enzyme activity on plate assay was exhibited by strain JVB5.

7.3.2 Draft genome information of *S. maltophilia* JVB5

The resulting output from the draft genome analysis is presented in Table 7.2. A total of 4,771,305 bp genome size, 8,764,890 sequence read count, 1,323,498,390 bases, 66% GC content, 151 bp mean read length and 268 contigs were obtained from strain JVB5.

Table 7.1: Morphological, biochemical and screening of *S. maltophilia* JVB5

Cultural characteristics							
GR	SHP	CT	SH	NT	OXD	CIT	CSH
-	Rod	+	+	+	+	+	++

Growth conditions		
Salt tolerance (% v/v)	Temperature (^{o}C)	pH
3.5	25-40	4-10

Isolate screening			**Enzyme assay**		
	Quantitative	Qualitative		ZC (mm)	Qualitative
Siderophore	79.90±0.15^{c}	++	Xylanase	22.00±0.01^{d}	+
IAA	30.0±0.01^{a}	+	Protease	2.00±0.01^{b}	+
Phosphate	32.23±0.21^{b}	+	Amylase	9.00±0.01^{c}	+
EPS		+++	Mannanase	0.00±0.00^{a}	+
			Cellulase	50.00±0.01^{e}	+

Sugar fermentation							
Glucose	Xylose	Galactose	Arabinose	Sucrose	Raffinose	Mannitol	Maltose
Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive

Key: EPS = exopolysaccharide, - = negative reaction, GR = Gram-reaction, + = positive reaction, SHP = shape, CT = catalase, ZC - zone of clearance measurement, SH = starch hydrolysis, NT - –itrate, OXD = oxidase, CT = citrate, CSH = casein hydrolysis, IAA = indole acetic acid. Different letters down the column represent significant differences

Table 7.2: Draft genome characteristics and resources of *S. maltophilia* strain JVB5

Draft genome	***S. maltophilia* strain JVB5**
Size	4,771,305 bp
G + C content	66%
Number of contigs	268
Number of genes coding RNAs	58
L_{50}	49
Number of subsystem	305
N_{50}	28,858 bp
No. of coding sequences	4,446

Key: L_{50} – is the least number of contigs that are summed up to 50% genome N_{50}- is a length of a contig

The L_{50} and N_{50} values were 49 and 28,858 bp, respectively. Figure 7.1 shows the draft genome circular view of strain JVB5. Also, the phylogeny of the draft genome sequence of strain JVB5 is presented in Figure 7.2.

7.3.3 Gene prediction of *S. maltophilia* JVB5

Predicted genes responsible for bacterial functions were predicted in the draft genome of strain JVB5 (Tables 7.3-7.18). Table 7.3 shows genes involved in the secretion system. Similarly, the predicted genes involving carbohydrates such as mannose, hexose, rhamnose, erythrulose, fructose, and inositol degradation and metabolism are shown in Table 7.4. The functional gene responsible for carbohydrate, transport, amino acid, and organic acid metabolism were detected in *S. maltophilia* JVB5 (Tables 7.5-7.7). Nitrogen fixation genes, *nif*SF, *amt*, and *azt*F coding for cysteine desulfurase, flavodoxin, ammonium transport, and allophanate hydrolase are presented in Table 7.8. Other phosphate solubilization and transport genes, such as *pho*U, *ppx*, and *pst*ABCS, and sulfate transport pathways were predicted in strain JVB5 (Table 7.9). Sulfur metabolism genes were also profound in the bacterial draft genome (Table 7.10). Siderophore genes such as *fur*, *feo*AB, *fhu*E, *tes*B, *ent*ABCF responsible for enterobactin synthesis and iron transport were detected in strain JVB5 (Table 7.11). Several genes involved in chemotaxis (*che*AY, *mot*AB, *flg*F, and *flh*ABF), and flagella biosynthesis (*fli*R), pilus and fimbriae (*fim*V, *pil*BGHOVW, and *cpa*B) were also found (Table 7.12). Genes responsible for bacterial attachment to plant surfaces are presented in Table 7.13. Quorum-sensing genes were also predicted in the draft genome (Table 7.14). Genes such as *sod*1, *sod*2, *kat*EG, and *ahp*CF coding for superoxide anion radicals, hydrogen peroxide and organic hydroperoxides degradation for plant protection against oxidative and nitrosative stress was profound in the bacterial draft genome (Table 7.15).

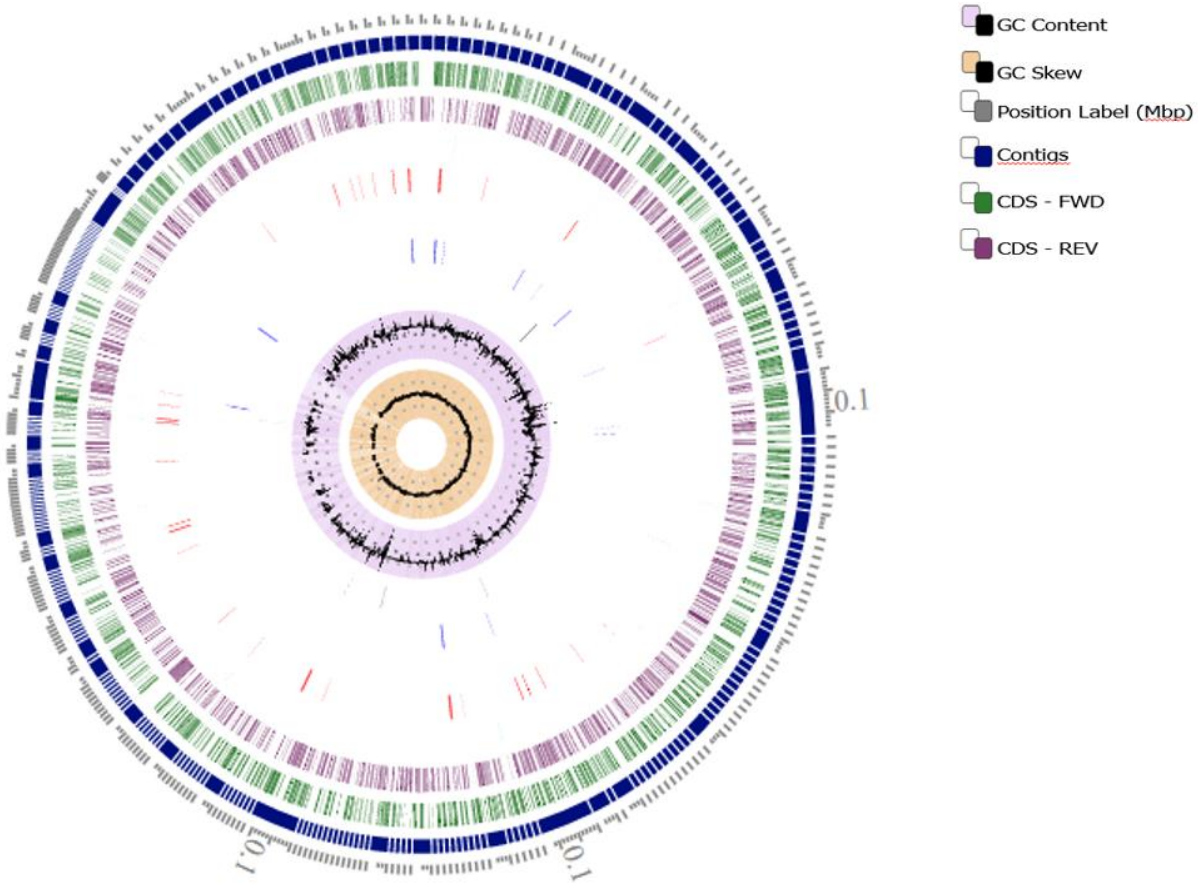


Figure 7.1: Circular visualization of the draft genome of *S. maltophilia* JVB5

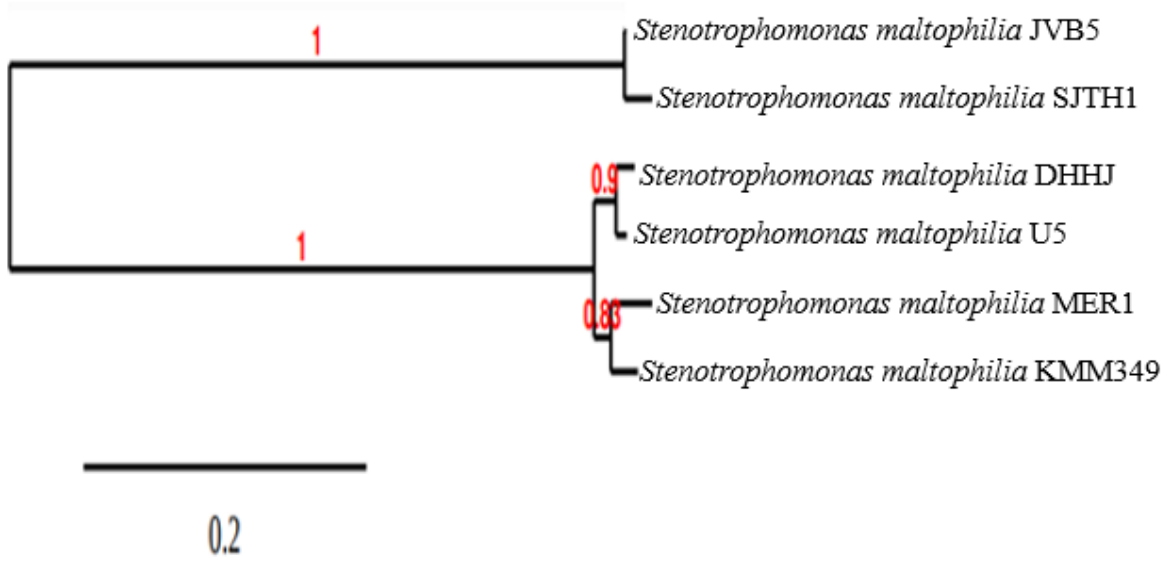


Figure 7.2: Phylogeny tree of the draft genome sequence of *S. maltophilia* JVB5. Legend: The scale bar represents branch lengths of node data in the phylogram, while numbers in red represent the number of differences between sequences (i.e. 1 means 100% difference between two bacterial sequences)

Table 7.3: Genes involved in secretion systems

Gene	Locus tag	Product	Pathway
sec	J0661_15525	preprotein translocase subunit (	Sec (secretion) system
ffh	J0661_02300	signal recognition particle subunit SRP54	
*fts*B	J0661_07960	cell division protein FtsB	
*fts*W	J0661_15475	putative lipid II flippase *Fts*W	
*fts*H	J0661_15240	ATP-dependent zinc metalloprotease *Fts*H	
*fts*Y	J0661_14735	signal recognition particle-docking protein *Fts*Y	
*tat*C	J0661_08030	twin-arginine translocase subunit *Tat*C	Twin-arginine translocation (*Tat*)
*tat*A	J0661_08040	*Sec*-independent protein translocase subunit *Tat*A	system
*tat*D	J0661_08185	*Tat*D family hydrolase	
*lap*B	J0661_13090	lipopolysaccharide assembly protein *Lap*B	Adhesin protein transporter

Table 7.4: Genes involved in carbohydrate metabolism

Gene	Locus tag	Product	Pathway
zwf	J0661_09015	glucose-6-phosphate 1-dehydrogenase	
gnd	J0661_17345	decarboxylating 6-phosphogluconate dehydrogenase	
tkt	J0661_17945	Transketolase	
gpi	J0661_15020	glucose-6-phosphate isomerase	
gap	J0661_15085	type I glyceraldehyde-3-phosphate dehydrogenase	Pentose phosphate
*gpm*A	J0661_03795	phosphoglycerate mutase	
agp	J0661_11895	bifunctional glucose-1-phosphatase	
*gal*E	J0661_12795	UDP-glucose 4-epimerase *Gal*E	
*yid*A	J0661_09635	sugar-phosphatase	
fruK	J0661_04475	1-phosphofructokinase	Fructose degradation
*rfb*D	J0661_01590	dTDP-4-dehydrorhamnose reductase	Ribose degradation
rbsK	J0661_08800	ribokinase	
*rfb*C	J0661_01595	dTDP-4-dehydrorhamnose 3,5-epimerase	Mannose degradation
*rfb*A	J0661_01600	glucose-1-phosphate thymidylyltransferase *Rfb*A	
*mms*B	J0661_09035	bifunctional 4-hydroxy-2-oxoglutarate	Myo-inositol degradation
*pdx*Y	J0661_18395	pyridoxal kinase	
*lld*P	J0661_21315	L-lactate permease	L-lactate degradation
*lld*R	J0661_21320	transcriptional regulator *Lld*R	
dld	J0661_21330	D-lactate dehydrogenase	D-lactate degradation
*glg*A	J0661_21535	glycogen synthase *Glg*A	Glycogen degradation
*glg*X	J0661_21565	glycogen debranching protein *Glg*X	

Table 7.5: Predicted genes involving in carbohydrate metabolism

Gene	Locus tag	Function/Product	Pathway
*nag*Z	J0661_13380	beta-N-acetylhexosaminidase	N-Acetylglucosamine-specific II component
*nag*A	J0661_06410	N-acetylglucosamine-6-phosphate deacetylase	
	J0661_07210		
	J0661_04480	PTS fructose transporter subunit EIIBC	
*fru*B	J0661_21150	PTS system, FS IIA	Fructose specific (FS) II component

Table 7.6: Predicted genes involving in organic acids metabolism

Gene	Locus tag	Function/Product	Pathway
*acn*A	J0661_19775	aconitate hydratase	TCA cycle
*suc*B	J0661_12230	dihydrolipoyllysine-residue succinyltransferase	
*acn*A	J0661_19775	aconitate hydratase	
*acn*B	J0661_19790	aconitate hydratase 2	
*fdh*E	J0661_00530	formate dehydrogenase accessory protein *Fdh*E	Formate degradation
Acs	J0661_04655	Acetyl-coenzyme A ligase	Acetate degradation
*mdc*H	J0661_09275	malonate decarboxylase subunit epsilon	Malonate degradation I (biotin-independent)
*mdc*E	J0661_15270	gamma biotin-independent subunit	
	J0661_15275	Beta acyl carrier protein	

Table 7.7: Predicted genes involved in amino acid metabolism and transport

Gene	Locus tag	Product	Pathway
map	J0661_00880	type I methionyl aminopeptidase	Methionine degradation
alr	J0661_20820	alanine racemase	D-alanine degradation
*spe*A	J0661_11230	biosynthetic arginine decarboxylase, partial	L-arginine degradation
*cys*K	J0661_13170	cysteine synthase A	L-cysteine degradation
*glt*B	J0661_11115	glutamate synthase (NADPH/NADH) large chain	L-glutamine degradation
*hut*C	J0661_06805	histidine utilization repressor	
*hut*G	J0661_06825	N-formylglutamate deformylase	
*hpp*D	J0661_17620	4-hydroxyphenylpyruvate dioxygenase	L-tyrosine degradation
*mms*B	J0661_13460	3-hydroxyisobutyrate dehydrogenase	L-valine degradation
*gcv*P	J0661_10905	aminomethyl-transferring glycine dehydrogenase	
*gcv*H	J0661_08310	glycine cleavage system (GCS) protein *Gcv*H	Glycine cleavage
*gcv*T	J0661_08315	GCS aminomethyltransferase *Gcv*T	
*lpd*A	J0661_11610	dihydrolipoyl dehydrogenase	

Table 7.8: Predicted genes involving in nitrogen fixation and metabolism

Gene	Locus tag	Product	Pathway
*nif*S	J0661_17475	cysteine desulfurase	Nitrogen fixation
*nif*F	J0661_20290	flavodoxin	
amt	J0661_06970	ammonium transporter	Ammonia transport
*atz*F	J0661_03650	allophanate hydrolase	Urea degradation and transport

Table 7.9: Predicted genes involving in phosphate metabolism and transport

Gene	Locus tag	Function	Pathway
*pho*U	J0661_14530	phosphate signaling complex protein *Pho*U	Organic phosphate solubilization
ppa	J0661_00840	inorganic pyrophosphatase	Degradation of inorganic
ppx	J0661_03465	exopolyphosphatase	polyphosphates
phnD	J0661_03670	phosphonate ABC transporter substrate-binding protein (SBP)	Degradation of phosphonates
*pst*S	J0661_14510	phosphate transporter (PT) ABC	Phosphate transport
	J0661_17540	SBP	
*pst*C	J0661_14515	(PT) ABC – *Pst*C - permease enzyme	
*pst*A	J0661_14520	(PT) ABC permease *Pst*A	
*pst*B	J0661_14525	(PT) ABC - *Pst*B	

Table 7.10: Predicted genes involved in sulfur metabolism

Gene	Locus tag	Product	Pathway
cysQ	J0661_13670	bisphosphate nucleotidase *Cys*Q	Sulfate transport
cysK	J0661_13170	cysteine synthase A	

Table 7.11: Predicted genes involving in iron metabolism and siderophore synthesis

Gene	Locus tag	Function/Product	Pathway
fur	J0661_18340	ferric iron uptake transcriptional regulator	Iron (III) transport
*feo*A	J0661_01485	iron transporter protein (ITP) A	Iron(II) transport
*feo*B	J0661_01490	ITP B	
*fhu*E	J0661_10800	ferric-coprogen receptor *Fhu*E	Iron complex transport
*ent*H	J0661_02500	Thioesterase	
*tes*B	J0661_17755	acyl-CoA thioesterase II	
*ent*B	J0661_20195	Isochorismatase	Enterobactin production
*ent*C	J0661_20205	isochorismate synthase	
*ent*F	J0661_20185	enterobactin synthase component F	
*ent*A	J0661_20180	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	

Table 7.12: Predicted genes involving in motility and chemotaxis

Gene	Locus tag	Function/Product	Pathway
*che*A	J0661_14170	chemotaxis protein (kinase) *Che*A	Chemotaxis
*che*Y	J0661_14215	chemotaxis response regulator *Che*Y	
*mot*A	J0661_20850	flagellar motor stator protein *Mot*A	
*mot*B	J0661_20855	flagellar motor protein *Mot*B	
mcp	J0661_06080	chemotaxis protein (methyl-accepting)	
*flg*A	J0661_19330	flagellar basal body (FBB) protein	Flagella biosynthesis
*flh*F	J0661_14230	FB *Flh*F	
*flh*B	J0661_14240	FB protein *Flh*B	
*flg*F	J0661_10750	FBB rod protein *Flg*F	
*flh*A	J0661_14235	FB protein *Flh*A	
*fli*R	J0661_14255	FB protein *Fli*R	
*pil*G	J0661_06065	twitching motility response regulator *Pil*G	Pilus and fimbriae biosynthesis
*pil*B	J0661_03600	type IVa pilus assembly ATPase *Pil*B	
*cpa*B	J0661_03155	*Flp* pilus assembly protein *Cpa*B	
*pil*V	J0661_10670	type IV pilus modification protein *Pil*V	
*pil*H	J0661_10980	twitching motility response regulator *Pil*H	
*pil*O	J0661_18940	type 4a protein *Pil*O	
*fim*V	J0661_15685	FP *Fim*V	

Table 7.13: Genes involved in attachment to plant surfaces

Gene	Locus tag	Product	Pathway
*lpx*A	J0661_17880	*Lpx*A	
*lpx*B	J0661_18455	*Lpx*B	
*lpx*D	J0661_17890	*Lpx*D	
*lpx*H	J0661_03440	*Lpx*H	LPS, Lipid A biosynthesis and modifications
*waa*A	J0661_16130	*Waa*A	
*ept*A	J0661_08085	*Ept*A	
*kds*A	J0661_07940	*Kds*A	
*kds*B	J0661_13730	*Kds*B	
*waa*A	J0661_16130	*Waa*A	LPS, core biosynthesis
*wec*B	J0661_18800	*Wec*B	
rfbD	J0661_01590	RfbD	
rfbC	J0661_01595	RfbC	LPS, O-antigen biosynthesis
rfbA	J0661_01600	RfbA	
rfbB	J0661_01605	dTDP-glucose 4,6-dehydratase	

Table 7.14: Genes involved in quorum-sensing mechanisms

Gene	Locus tag	Product	Pathway
*cyc*A	J0661_15400	D-serine/D-alanine/glycine transporter	N-Acyl-homoserine-lactone production

Table 7.15: Genes involved in the protection against oxidative and nitrosative stress

Gene	Locus tag	Product	Pathway/function
*sod*2	J0661_11640	superoxide dismutase family protein	Degradation of superoxide anion radicals
*sod*1	J0661_11645	superoxide dismutase family protein	
*kat*E	J0661_04990 J0661_10925	Catalase	Degradation of hydrogen peroxide
*kat*G	J0661_18670	catalase-peroxidase	
*ahp*C	J0661_20970	Peroxiredoxin	Degradation of hydrogen peroxide and organic hydroperoxides
*ahp*F	J0661_20965	alkyl hydroperoxide reductase subunit F	

Furthermore, strain JVB5 harbors lignin-degrading genes (Table 7.16). Several genes, such as *trp*ABCD, *ami*E, and *mia*A involved in IAA production in the indole-3-acetamide pathway (IAM) was predicted in the bacterial draft genome (Table 7.17). Notable osmotic stress tolerance genes (*bet*ABLT) involved in glycine betaine and proline biosynthetic pathways were predicted in strain JVB5 (Table 7.18). The identifiable secondary metabolite gene clusters such as arylpolyene, non-ribosomal peptides, lanthipeptide-class II, and RiPP-like were predicted in the bacterial draft genome (Table 7.19). Four notable secondary metabolite gene clusters detected include regulatory genes, TTA codons, additional biosynthetic genes, other genes, resistance transport-related genes, and core biosynthetic genes (Figures 7.3a and 7.3b). The gene clusters with 17% and 42% non-ribosomal peptides (NRPS) and arylpolyene similar to the known cluster for arylpolyene and streptobactin were detected at node regions 1.1 and 258.1 (Table 7.19).

7.3.4 Plant yield output

The comparison between inoculated and un-inoculated sunflower yield parameters obtained from the greenhouse is presented in Table 7.20. Except for the taproot width and seed weight (wet and dry), the yield output above and below ground levels of the inoculated sunflower show a significantly different compared to the un-inoculated sunflower plants. Inoculated plant yielded 437.20 g plant wet weight compared to un-inoculated with 331.00 g wet weight. Similar results were obtained on the shoot dry weight 57.3 g (un-inoculated) and 76.2 g (inoculated), un-inoculated head fresh and dry weight of 153.7 g and 41.6 g, and inoculated head fresh and dry weight of 200.8 g and 55.1 g, respectively. Also, the root wet and dry weight values of sunflower inoculated (84.2 and 19.4) and un-inoculated (44.1 and 8.4) were recorded.

Table 7.16: Predicted genes involving in the degradation of lignin

Gene	Locus tag	Function/Product	Pathway
*kat*E	J0661_04990 J0661_10925	Catalase	
*kat*G	J0661_18670 J0661_18670	catalase-peroxidase	Degradation of toxic peroxides
*ahp*C	J0661_20970	Peroxiredoxin	
*ahp*F	J0661_20965	alkyl hydroperoxide reductase subunit F	
*ald*H	J0661_06320	aldehyde dehydrogenase	Metabolism of alcohols and
-	J0661_06405	zinc-type alcohol dehydrogenase-like protein	aldehydes

Table 7.17: Predicted genes involving phytohormone synthesis and modulation

Gene	Locus tag	Function/Product	Pathway
*trp*A	J0661_15745	subunit alpha (tryptophan synthase)	L-tryptophan production; IAA production
*trp*B	J0661_15735	subunit beta (tryptophan synthase)	
*trp*D	J0661_17720	anthranilate phosphoribosyltransferase	
*trp*C	J0661_17725	indole-3-glycerol phosphate synthase *Trp*C	
*ami*E	J0661_12320	Amidase	IAA production, IAM pathway
*mia*A	J0661_15225	*mia*A	CK biosynthesis and transformation
*mia*B	J0661_08550	*mia*B	
*xdh*C	J0661_04265	xanthine dehydrogenase iron-sulfur-binding subunit	

Table 7.18: Genes involved in osmotic stress tolerance

Gene	Locus tag	Product	Pathway
*bet*A	J0661_19755	choline dehydrogenase	Glycine betaine biosynthesis
*bet*T	J0661_06100	choline BCCT transporter *Bet*T	
	J0661_20740		
*bet*L	J0661_19765	transcriptional regulator *Bet*I	
*bet*B	J0661_19760	betaine-aldehyde dehydrogenase	
-	J0661_03005	gamma-glutamyl phosphate reductase	Proline biosynthesis

Table 7.19: Secondary metabolite gene composition in *S. maltophilia* JVB5

Region	**Kind/Type**	**Start/From**	**To/Stop**	**Most similar known cluster**		**% Score**
Region 1.1	ARE - arypolyene	1	27,392	Arypolyene (APE)	Other	42
Region 64.1	RiPP-like	42,315	48,197	-	-	-
Rg 208.1	RiPP-like	1	5,829	-	-	-
Rg 22.1	Lanthipeptide-classii	1	2,889	-	-	-
Rg 258.1	NRPS	1	27,229	streptobactin	NRP	17

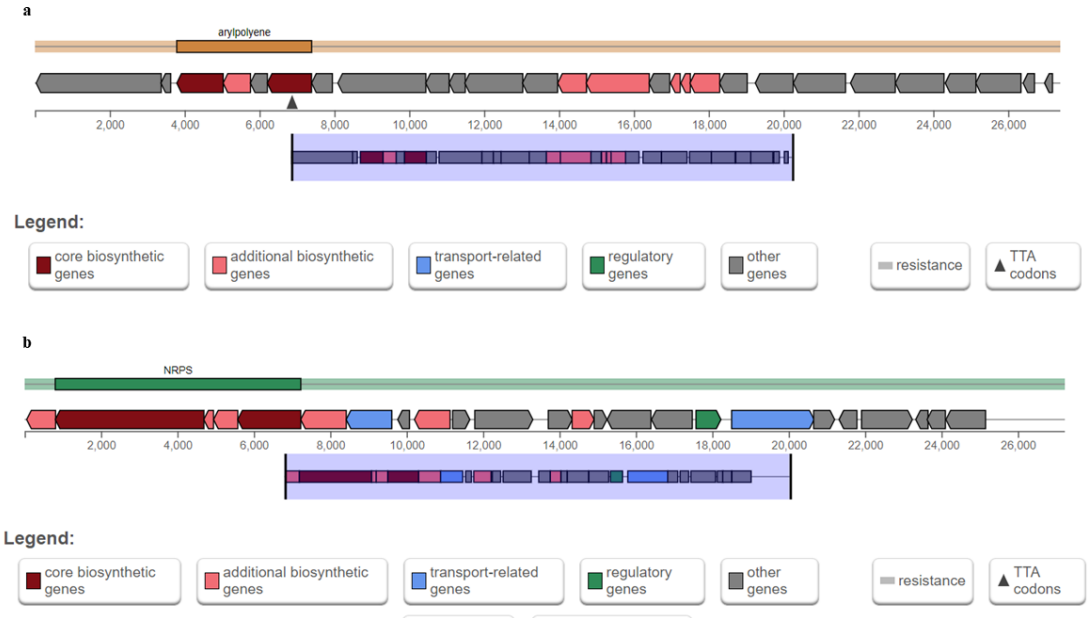


Figure 7.3a and 7.3b: Arylpolyene-and-nonribosomal peptides (NRPS)-encoding genes

Table 7.20: Sunflower yield output

Growth parameters (Belowground)							
	TRL (mm)	TRW (g)	RL (mm)	LRN	RWW (g)	RDW (g)	NR
Inoculated	191.7±10.69[b]	6.3±0.58[a]	294.3±12.50[b]	31.7±0.58[b]	84.2±13.78[b]	19.4±19.41[b]	1163.7±61.92[b]
Uninoculated	146.7±58.33[a]	5.3±0.58[a]	217.0±81.22[a]	25.7±1.53[a]	44.1±16.12[a]	8.4±1.55[a]	935.0±11.30[a]
Growth parameters (Aboveground (g))							
	SWW	SDW	HFW	HDW	PWW	SHWW	SHDW
Uninoculated	0.1±0.01[a]	0.03±0.01[a]	153.7±13.94[a]	41.6±4.27[a]	331.0±20.16[a]	165.8±5.16[a]	57.3±7.04[a]
Inoculated	0.1±0.01[a]	0.03±0.01[a]	200.8±32.58[b]	55.1±13.55[b]	437.2±21.08[b]	204.6±8.07[b]	76.2±10.09[b]

Key: SDW - seed dry weight, SWW – seed wet weight, HFW - head fresh weight, HDW - head dry weight, PWW - plant wet weight, SHWW - shoot wet weight, RDW – root dry weight, SHDW - shoot dry weight, RWW – root wet weight, TRL – taproot length, TRW – taproot width, RL – root length, LRN – lateral root number, NR – number of roots. Mean values with different superscripts (small letters) within the same column represent significant difference

7.4 Discussion

In this study, we investigated the draft genome sequence of *S. maltophilia* JVB5 and its potential for improving sunflower yield under greenhouse experimental conditions. The bacterial identification was confirmed by 16S rRNA gene sequencing before genome sequencing. The preliminary *in vitro* screening reveals the plant growth-promoting traits of strain JVB5. The ability of bacterial endophytes to produce certain phytostimulants and metabolic compounds, such as siderophore, IAA, ammonia, hydrogen cyanide, lytic enzymes, and antibiotics suggests their use in sustaining plant growth and immunity (Alkahtani et al., 2020, Fouda et al., 2021). The results obtained in this study conformed to Bashir et al. (2020), who in their findings have evaluated plant growth-promoting attributes of endophytic bacteria isolated from sunflower. Nevertheless, few reports have been documented on the genome analysis of agriculturally important *Stenotrophomonas* species (Pinski et al., 2020), as findings on endophytic bacteria *S. maltophilia* JVB5 can serve as a model in future studies. Therefore, in our view, this is the first documentation on the genome analysis of endophytic bacterium *S. maltophilia* JVB5 isolated from the sunflower root endosphere, although other *Stenotrophomonas* species might have been reported in sunflower.

The genomic assessment of *S. maltophilia* JVB5 as investigated in this study is aimed to provide insights into the PGPE genes and other metabolic genes underlining bacterial functions in enhancing sunflower yield. Interestingly, notable genes involving siderophore production, phosphate solubilization, carbon metabolism, phytohormone synthesis, cell attachment, and colonization were predicted in the strain JVB5 genome. Bacterial DNA contains an array of genes with varied genome sizes and specific functions (Westoby et al., 2021). In this study, a strong correlation exists between the genome size (4.7 Mb) of strain JVB5 compared to the

genome size (4.3 Mb) and (4.7 Mb) of endophytic bacteria *S. maltophilia* strains RR-10 and B418 isolated from rice and barley (Zhu et al., 2012, Wu et al., 2015). Evidence has shown that bacteria with a bigger genome size tend to adapt to different habitats because of their additional encoding gene products involved in the metabolism of organic compounds and osmotic stress (Westoby et al., 2021). Also, a finding has argued on the ability of bacteria with small genome sizes to efficiently participate in the metabolism of organic substrates (Zeng et al., 2018).

In line with the plant growth-promoting screening, genes involved in phytohormone synthesis were predicted in strain JVB5. Insight into phytohormone genes from endophytic bacteria with important functions in plant growth promotion has been studied (Nascimento et al., 2020b, Samaras et al., 2020), but such reports are less documented in bacteria genera *Stenotrophomonas*. In this study, genes (*trp*ABCD) involved in IAA biosynthesis were predicted in the strain JVB5 genome. The detection of IAA genes in strain JVB5 can mediate their actions in enhancing root development in plants. The results obtained in this study corroborate the findings of Singh et al. (2021), who reported IAA genes, such as *trp*BE, *trp*ABCDE, and *trp*ABD from the whole genome analysis of endophytic bacterial strains from plants. From a previous study, *in vitro* screening of IAA-producing endophytic *Stenotrophomonas* isolated from sunflower has been documented (Majeed et al., 2015), but their genome assessment for detecting IAA genes is still lacking, hence limiting their full exploration in agricultural biotechnology. However, the results obtained in this study validate the report of Khamwan et al. (2018a) on the potential use of IAA-producing *S. maltophilia* isolated from *Helianthus tuberosus* in enhancing plant root development and growth promotion.

The predicted genes involved in the metabolism of energy-yielding compounds in the draft genome of strain JVB5 show their preference in metabolizing complex organic substrates, which enhance their colonization efficiency in the plant endosphere. Plant adaptation and survival under stress conditions have been linked to the microbial actions in utilizing accumulated trehalose in the soil environment (Pinski et al., 2020). The detection of enzymes malto-oligosyltrehalose synthase and trehalose-phosphatase coding genes (*tre*YZ) involved in trehalose degradation and uptake of exogenous trehalose in strain JVB5 can be an indication in boosting sunflower tolerance to high osmotic or salinity stress (Pinski et al., 2020).

The secretion system genes detected in strain JVB5 corroborate with the findings of Liu et al. (2016a), who reported secretion system and type I-VI were found in endophytic *Klebsiella* sp., *Variovorax paradoxus,* and *Pseudomonas fluorescens* genome. The presence of these genes in strain JVB5 may contribute to plant growth promotion and bacterial attachment/colonization in the root endosphere. Other types of secretion system genes, such as *tat*, *sec,* and type I-VI have also been reported in *Hartmannibacter diazotrophicus* E19 (Suarez et al., 2019). Interestingly, similar genes in the genome of *Bradyrhizobium diazoefficiens* and *Mesorhizobium loti* have been identified with multiple functions in biofilm production and the establishment of plant-bacterial interactions (Li et al., 2020b).

Nitrogen, phosphorus, and potassium are essential nutrients required by plants, and the role of endophytic bacteria in sustainable plant growth through nitrogen fixation and soil mineral solubilization has been reported in several studies (Passari et al., 2016, Hashem et al., 2019, Adeleke and Babalola, 2021c). Based on the gene functions, the potential of endophytic bacteria, such as *Bacillus, Pseudomonas,* and *Enterobacter* to fix nitrogen to the soil is important in improving soil profiling and plant growth (Guo et al., 2020). The effects of *S.*

maltophilia on sunflower yield upon inoculation as reported in this study can be due to the expression of nitrogen fixation genes in the bacterial genome and this agrees with the report of Singh et al. (2020). The predicted multiple nitrogen fixation genes, *nif*ABDEFHJKLMNQSTUVWZYZ in the genome of *Klebsiella* have been employed as a stereotype for studying nitrogen fixation in diverse bacterial strains (Samaras et al., 2020). In this study, the detection of nitrogen fixation genes, such as *nif*SF, ammonia transport gene (*amt*), and urea degradation and metabolism gene in the bacterial genome may suggest their use in formulating bioinoculants as an alternative to nitrogen fertilizer in the organic farming system. Similar genes involved in nitrogen metabolisms, such as *nif*HU, *aat*JMPQ, and *glt*BDPS have been detected in the genome of *P. aeruginosa* B18 (Singh et al., 2021).

The ability of bacterial endophytes to fix phosphorus in the soil has played important role in plant nutrition (Passari et al., 2016). Endophytic *S. maltophilia* strains, namely, SEN_1 (Etesami and Alikhani, 2016c), SY-2 (Liaquat et al., 2020), B11 (Kasim et al., 2021), have been identified as phosphate solubilizers with the potential in enhancing plant growth. The phosphorus-solubilizing potential of strain JVB5 in tricalcium medium was confirmed in the genome analysis, as diverse genes involving phosphate transport and metabolism were predicted in the genome. The detection of phosphate transport genes (*pst*ABCS) and other regulatory genes, such as *pho*U, *phn*D, and *agp* as revealed in this study suggest the role of strain JVB5 in the degradation and assimilation of phosphate compounds, thus making phosphorus available in the soil for plant use. Besides phosphates, studies have argued on phosphonates as another source of phosphorus in the soil (Oliverio et al., 2020), with a similar gene identified in this study. The results obtained in this study corroborate with Singh et al.

(2021) and Nascimento et al. (2020a), whose findings reported *pst*ABCS and *pho*U in the genome of *P. aureginosa* B18 and *Pantoea phytobeneficialis* MSR2, respectively.

The genome analysis revealed genes (*cys*KQ) involved in sulfur transport in strain JVB5, thus classifying this bacteria as a sulfur producer. The predicted genes in the bacterial genome can mediate the mineralization of sulfur-containing compounds, recycling of organic sulfate, root colonization, and plant tolerance to stress (Battu and Ulaganathan, 2020). The enzyme-coding genes bisphosphate nucleotidase and cysteine synthase A detected in strain JVB5 might be a unique feature of this bacteria compared to *cys*A, *cys*W, and *cys*P genes identified in the genome of *Enterobacter* and *Pseudomonas* in transporting thiosulfate or sulfate molecules in the living cell (Guo et al., 2020, Singh et al., 2021). Exploration of strain JVB5 in agriculture, based on their ability to degrade phosphate and phosphonate, can improve soil mineral levels and supply to the plants.

The siderophore is another key plant growth-promoting trait exhibited by strain JVB5. Identification of siderophore genes, such as siderophore enterobactin genes, *fes*, *ent*D, and *fep*A has been reported to reduce the iron level and capturing of fixed iron siderophore in the soil (Singh et al., 2021, Nascimento et al., 2020a). Several siderophore genes mediating plant response against osmotic stress and pathogens have been identified in *Pseudomonas* species (Kang et al., 2020). The detection of siderophore genes, *ent*ABCFH in strain JVB5 agrees with Guerrieri et al. (2021) and Hubrich et al. (2021), who have reported similar genes in the genome of endophytic bacterium *Klebsiella variicola* UC4115, with functions in deriving enterobactin from chorismate.

Various chemotaxis genes identified in this study may underline bacterial activity to adhere, penetrate and colonize plant endosphere in overcoming surface repulse, cell displacement, and

biofilm formation due to the production of extra polymeric substances (cellulose and exopolysaccharide) and attachment organelles (flagella, fimbriae, and pili) (Zheng et al., 2015). The adherence of endophytic bacterial on surfaces can help build up signal molecules for gene expression and regulation, cell-to-cell communication, and bacterial adaptation under stress conditions (Adeleke et al., 2021). Also, the detection of flagella protein in the bacterial genome may stimulate host defensive mechanisms against plant pathogens, enhance bacterial colonization and plant tolerance to unfavorable conditions, thus increasing endosphere competence (Shastry et al., 2020). The predicted genes mediating chemotaxis, flagellar biosynthesis, fimbriae, and pilus production/biosynthesis in strain JVB5 conform to the findings of Singh et al. (2021), who reported similar genes in *P. aeruginosa* B18. Several genes associated with biofilm and exopolysaccharide production that aid bacterial biofilm formation genes have been predicted from the *E. roggenkampii* genome (Gupta et al., 2014). However, draft genome sequencing of agriculturally important *S. maltophilia* associated with a sunflower that reveals motility, chemotaxis, and biofilm production genes has not been documented.

A gene (*cyc*A) mediating quorum-sensing (QS) signal molecule, N-acyl-Lhomoserine lactones (NHL) was found in the bacterial genome. The detection of NHL hydrolase in strain JVB5 suggests their ability in the metabolism and modulation of AHLs, which may directly influence their lifestyle and ecological functions. Bacteria respond to oxidative stressors based on their ability to produce certain enzyme regulatory proteins and metabolic compounds. The predicted enzyme-coding genes, such as catalase, catalase-peroxidase, peroxiredoxin, superoxide dismutase, and alkyl hydroperoxide reductase in strain JVB5, and their function in degradation free radicals, hydrogen peroxide, organic hydroperoxides, and superoxide anion can mirror their ability in mitigating oxidative stressors in plants. The detection of *sod* genes in the genome

of *P. putida* has been reported to stimulate plant resistance and defense against pathogens (Gupta et al., 2014). In addition, the detection of oxidative stress genes in strain JVB5 can inform their potential in mitigating oxidative stress in plants. Nevertheless, the exact mechanisms employed by endophytic bacteria in mitigating oxidative stress need further study. Different secondary metabolite gene clusters such as arylpolyene (APE), NRPS (streptobactin), lanthipeptide-class II, and RiPP-like were identified in strain JVB5. Interestingly, six biosynthetic genes coding for secondary metabolites were predicted in strain JVB5. These products and their biocontrol activities against plant pathogens could help to ensure sustainable plant health. The detection of aylpolyene gene clusters in strain JVB5 may stimulate bacterial functions against oxidative stressors. Similar genes with similar functions have been reported in stress-tolerant *B. megaterium* STB1 and other prokaryotes (Cimermancic et al., 2014).

Sunflower inoculation with *S. maltophilia* JVB5 under greenhouse experiments showed an improvement in the sunflower yield compared to un-inoculated. We can say that root development and early flowering in sunflower may be linked to phytohormone synthesis and detection of phytohormone genes in the bacterial genome. The effect of co-inoculation of endophytic bacteria genera, such as *Stenotrophomonas, Bradyrhizobium, Pseudomonas, Kosakonia,* and *Azospirillum* on growth parameters and yield of sunflower and other crops under greenhouse experiments have been performed with success (Egamberdieva et al., 2016, Youseif, 2018, Singh et al., 2020, Adeleke et al., 2021, Kasim et al., 2021). Furthermore, the sunflower yield enhancement by *S. maltophilia* JVB5 upon inoculation in the greenhouse corroborates with Adeleke et al. (2021) who reported a significant difference in the yield of sunflower inoculated with endophytic *S. indicatrix* strain BOVIS40.

7.5 Conclusion

In conclusion, *S. maltophilia* JVB5 exhibited positive reactions to *in vivo* plant growth-promoting assay. The genomic elucidation of strain JVB5 has provided novel insights into the functional genes responsible for plant-bacteria interactions with profound plant growth-promoting activities rather than being an opportunistic pathogen. Sunflower yield improvement was observed based on bacterial colonization and the presence of diverse functional genes involved in phytohormone synthesis, carbohydrate metabolism, quorum-sensing, secretion system, lignin degradation, and oxidative and nitrosative stress. Strain JVB5 harboring oxidative and putative secondary metabolite genes can be employed as biocontrol agents against phytopathogens for improved crop production. Therefore, harnessing *S. maltophilia* JVB5 can serve as a model in formulating bioinoculants with bioprospecting in developing eco-friendly agriculture sustainably.

8.0 CHAPTER EIGHT

Genomic analysis of endophytic *Bacillus cereus* T4S and its plant growth-promoting traits

Abstract

This study presents whole-genome sequencing of plant growth-promoting *B. cereus* strain T4S isolated from sunflower roots. Phenotypically, strain T4S is rod-like, Gram-positive, motile, and produces acid during sugar fermentation. The NextSeq Illumina sequencing yielded 7,255,762 sequence reads, 151 bp average read length, 5,945,881 bp genome size, 198 contigs, 341 subsystems, 56 tRNA, 216 pseudogene, 6,086 gene, 6,277 coding sequences, 63 RNAs, and G+C content of 34.8%. Secondary metabolite type of most similar known clusters such as petrobactin, bacillibactin, bacitracin, molybdenum factor, zwittermicin, and fengycin with biocontrol efficacy was found in the T4S genome. Based on the established cut-off value for genome-to genome distance calculator (> 70%) and average nucleotide value (> 95%), the phylogenies output of strain T4S was similar to *B. cereus* ATCC 14579. The protein replicon information of novel genes involved in complex metabolic pathways can provide a genetic basis for the understanding of endosphere biology and their multifunctional thereof. Similarly, seed and root inoculation with stain T4S contribute to sunflower yield under greenhouse experiments. Hence, the detection of notable genes specific for plant growth promotion as validated under *in vitro* screening possibly will make these bacteria relevant in agricultural biotechnology.

Keywords: Bacterial endophytes, greenhouse, Illumina, oilseed crop, plant growth promotion, secondary metabolite genes

8.1 Introduction

In recent times, agricultural sustainability to maximally meet the nutritional food demand of the growing population needs a boost, more importantly, to avert future food scarcity and insecurity, and hunger. Notably, one of the promising measures is through agricultural intensification and research innovations into biotechnological functions of agriculturally important microbes inhabiting the endosphere of crops and how they can be explored as bioinoculants on a commercial scale (Majeed et al., 2018, Adeleke and Babalola, 2021a).

Agricultural practices such as tillage, crop rotation, and fertilizer application contribute to food production, reduce pest re-insurgence, and plant-pathogen attack (Huang et al., 2017). This has also ensured the availability of quantity and quality of food products for human beings (Adeleke and Babalola, 2020b). However, the incessant use of agrochemicals by farmers to recover the essential nutrients lost in the soil for crop improvement has continually harmed the environment (Sun et al., 2015a, Sharma and Singhvi, 2017). It has been postulated that indiscriminate or continuous use of agrochemicals does not have linkage to the quantity of food produced per annum, instead, it leads to negative environmental stimuli and consequences, particularly water contamination and pollution problems (Emami et al., 2019).

To mitigate these threats, employing best and acceptable practices to reduce the rate of chemical fertilization to enhance crop production remains fundamental as a key to sustainable agriculture. These measures include harnessing endophytic bacteria as invaluable resources in exchange for agrochemicals to improve plant resistance against biotic and abiotic stresses (Olanrewaju et al., 2017). Conversely, price instability, scarcity, and limited supply of chemical fertilizers due to the energy crisis, political, economic, and natural disasters can influence the rate of food production. In ecologically threatened regions, the plant is faced with a lot of stress

adaptors that instigate their internal and physiological cellular responses (Akinola and Babalola, 2020). Nevertheless, the recruitment of endophytic microbes as biological adjuvants to support plant growth in harsh or drought-prone areas can stimulate adaptive immune responses in plants (Rho et al., 2018).

Furthermore, employing endophytic bacteria as bio-factory in the synthesis of certain secondary metabolites can enhance their efficacy in agricultural biotechnology and implementation (Khamwan et al., 2018b). Endophytes are microorganisms that inhabit the internal tissue of the host plants without causing disease symptoms (Adeleke and Babalola, 2020a). The mechanistic effects of these bacteria on plant growth promotion can be direct or indirect. Significantly, plant growth-promoting endophytes (PGPE) support plants in diverse ways, mainly in the induction of systemic resistance, synthesis of phytohormones, antibiosis on phytopathogens, and competition for available nutrient and water in an ecological niche (Santoyo et al., 2016).

Based on the dominance of the genera *Bacillus* in different environments, their contributions to enhancing plant growth cannot be overemphasized. Additionally, their singularly or combined inoculation has shown significant effects on crop (wheat) yield compared to the un-inoculated (Ding et al., 2016, Emami et al., 2019). The improvement in plant biomass and crop yields, such as rice, *Arabidopsis*, wheat, maize, sorghum, and soybean upon inoculation with PGPE as demonstrated, has enhanced their productivity (Emami et al., 2019, Ghorchiani et al., 2018). Igiehon et al. (2019) reported inconsistent and lower effects of a single bacterial application on experimental plants. Nevertheless, co-inoculation of non-antagonistic bacteria is found significant, contributing more to plant performance in terms of quality and quantity than single inoculation. Notwithstanding, Subramanian et al. (2015) and Ríos-Ruiz et al. (2020)

reported an improvement in the yield of soybean co-inoculated with endophytic *Bradyrhizobium japonicum* and *Bacillus megaterium* and rice yields inoculated with endophytic *Burkholderia ubonensis, Citrobacter bitternis,* and *Burkholderia vietnamiensis*, respectively.

The group of bacterial species in the genus *Bacillus* can be found in various ecosystems, based on the endospore attributes that make them persist in extreme environments (Hashem et al., 2019). The multiple functional attributes of endophytic bacteria in agriculture can underline their potential based on the expression of novel genes in the bacterial genome that is involved in various metabolic pathways. For example, sulfur metabolism, phytohormone, exopolysaccharide, siderophore synthesis, enzyme biosynthesis, tryptophan metabolism, nitrogen fixation, photosynthesis, etc (Salwan and Sharma, 2020).

The discovery of vital genes in *Bacillus* isolated from sunflower can make them suitable candidates for various biotechnological applications. Significantly, bacterial inoculation of economically relevant plants, such as sunflower, can contribute to their growth and vegetable oil production. Sunflower is classified as one of the leading oilseed crops after soybean, safflower, and rapeseed, especially in Southern Africa (Adeleke and Babalola, 2020b). Despite the economic importance of sunflower and the identification of *B. cereus* from other plants (Ding et al., 2016), little information is available on the genome analysis of *B. cereus* associated with the root of sunflower in Southern Africa. *B. cereus* is a Gram-positive, spore-forming, rod-like bacterium belonging to the *Bacillus* genus. The whole-genome sequencing of *B. cereus* isolated from the rhizosphere and endosphere of plants has revealed notable genes in their genome, more importantly, plant growth-promoting genes (Egidi et al., 2016, Zeng et al., 2018). In addition, Hong et al. (2018a) reported the complete genome sequence of endophytic

bacterium *B. cereus* strain PgBE311 isolated from *Panax ginseng*. However, reports on the *B. cereus* isolated from the sunflower are less documented, thus suggesting its exploration and application in improving crop yield. Sunflower yield can be improved depending on the efficacy of the copious associated bacterial endophytes upon inoculation under experimental conditions. Therefore, we report the isolation, culture-based screening of plant growth-promoting traits, and genomic characterization of endophytic bacterium *B. cereus* T4S isolated from sunflower root endosphere, and its effect on sunflower yield under greenhouse experiment.

8.2 Materials and methods

8.2.1 Isolation of sunflower root endosphere associated endophytic bacterium, *B. cereus* T4S

Sunflower roots were sourced from farmlands in Lichtenburg, South Africa (26°4′31.266″S, 25°58′44.442″E) in February 2020. Bacteria isolate was isolated from the sunflower root endosphere on Luria Bertani (LB) agar medium following the method described by Annapurna et al. (2018). Briefly, the plant roots obtained were placed in zip-lock bags on an ice-box for transportation to the laboratory within 4-hour of collection for further analysis. For bacterial isolation, the roots were cut into small sizes and surface-sterilized using disinfectants. From the serial dilution process, 0.1 ml from 10^6 dilutions were aseptically dispensed into sterile Petri dishes and poured plated with sterilized LB medium. Incubation at 28^{o}C for 24 hours was carried out, and colony growth was monitored. The pure isolate was obtained by streaking on fresh LB agar plates. For cultural and morphological characterization, various biochemical tests

were performed. The isolate was kept on agar slants at 4^{o}C for further analysis. Furthermore, the isolate was preserved in 30% (v/v) glycerol at -20^{o}C.

8.2.2 Morphological and biochemical characterization of bacterial isolate

The cultural attributes of the bacteria isolate were evaluated on LB media after incubation at 28^{o}C for 24 hours. The cultural morphology of bacterial isolate was visualized under a light microscope (ECLIPSE E200; Nikon, Japan). Biochemical tests, such as sugar fermentation tests (mannitol, glucose, sucrose, maltose, fructose, galactose, and raffinose), catalase test, oxidase test, citrate test, nitrate utilization test, starch, and casein hydrolysis were performed.

8.2.3 DNA extraction, polymerase chain reaction, and molecular identification of the bacterial isolate

The genomic content of the pure bacterial isolate was extracted using a commercial Quick-DNA^{TM} Miniprep Kit specific for fungi or bacteria (Zymo Research, Irvine, CA, USA; Cat. No. D6005), following the manufacturer's guide. The concentration of the extracted DNA (ng/µl) was measured using a NanoDrop ND-2000 UV-Vis spectrophotometer (ThermoFisher Scientific, USA) and stored at -80^{o}C. The determination of 16S rDNA nucleotide sequences of the identified bacterial isolate was achieved using the amplified PCR products. The amplification process was initiated using universal oligonucleotide primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). A total of 25 µl reaction volume for each bacterial isolate composed of 12.5 µl OneTaq 2X MasterMix with the Standard Buffer, 1 µl for each primer, 2 µl genomic DNA, and 9.5 µl nuclease-free water were used for PCR amplification on DNA Engine DYAD^{TM} Peltier Thermal Cycler (BIO-RAD, USA, C1000 Touch^{TM}) (Turner et al., 1999). The PCR conditions

were set to initial denaturation at 94°C for 5 minutes, 35 cycles of amplification. Also, the denaturation temperature was set at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 68°C for 1 minute, and a final extension at 68°C for 10 minutes.

After amplification, PCR products were checked in 2% agarose gel prepared in 1 x TAE buffer, heat in microwave for 4 minutes. After cooling, 10 µl ethidium bromide was added for the electrophoresis. The band size of the amplicons was determined using a 1 kb molecular marker. After that, the gel was visualized in a Chemidoc^{TM} imaging system (BIO-RAD Laboratories, California, USA). Finally, 20 µl of the PCR product of the bacterial isolate was placed in an ice-box pack and sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. 16S rDNA sequences for the bacterial isolate were submitted to GenBank on the NCBI online server and were assigned an accession number. The strain code and accession number of the 16S rRNA gene sequence data of the identified bacterial endophytes deposited in the GenBank are T4S and MW265423, respectively.

8.2.4 Whole-genome sequencing (WGS)

The extracted genomic DNA of strain T4S was fragmented using an enzymatic approach (NEB Ultra II FS kit). The fragmented DNA was selected according to size range (200 – 700 bp) using AMPure XP beads. Subsequently, each fragment of DNA was end-repaired and ligated on Illumina-specific adapter sequences. Furthermore, the indexing of each sample and selection based on the size in the second step was performed. The quantity of samples at dilution of standard concentration to 4 nM was determined using a fluorometric method. After that, sequencing was performed using a NextSeq mid-out kit (300 cycles) on Illumina's NextSeq platform, following a guideline as described by the manufacturer. The resulting 400 Mb of data (2x150 bp long paired-end reads) were obtained for each sample.

WGS analysis was performed by submitting each sequence (FASTQ file) to the predictive biology online server and data science platform, KBase (https://kbase.us/) (Arkin et al., 2018). First, sequences were uploaded for read processing, and read quality assessment was achieved using FastQC (version 0.11.5) (Bioinformatics, 2011). The removal of sequence adaptor and low-quality bases of the paired-end Illumina raw sequence reads were performed with trimmomatic (version 0.36) (Bolger et al., 2014) to obtain high-quality sequence reads. Furthermore, sequence reads were assembled with SPAdes (version 3.13.0) (Nurk et al., 2013). After assembly, contigs annotation was performed using RASTtk (Rapid Annotations using Subsystems Technology toolkit - version 1.073) online server to categorize the distribution and functions of the predicted genes into a subsystem (https://rast.nmpdr.org/). The bioinformatics analysis was performed using default settings. The prediction of functional protein-coding genes (PCG) was obtained from the genomic protein output after processing in NCBI. Metabolic pathways of biomolecules were obtained from KEGG (Kyoto Encyclopedia of Genes and Genomes) on RAST (https://rast.nmpdr.org/rast.cgi/). The circular genome visualization was obtained from kbase (https://kbase.us/), while the phylogeny analysis was performed using MrBayes (http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes) (version 3.2.6) (Huelsenbeck and Ronquist, 2001, Arkin et al., 2018). Secondary metabolites were determined by antiSMASH (version 6.0.0) (https://antismash.secondarymetabolites.org) (Weber et al., 2015).

8.2.5 Genome accession number of *Bacillus cereus* T4S

From the NCBI database output, the Bioproject number is PRJNA706601, and Sequence Read Archive (SRA) number is SRR13887969, and BioSample number is SAMN18138757. The genome accession number JAFNAY000000000 was assigned to the strain T4S.

8.2.6 Plant growth-promoting screening

8.2.6.1 Phosphate solubilization

The qualitative screening of *B. cereus* T4S for the solubilization of phosphate was performed according to the modified method of Khan et al. (2020). For the quantitative assay, phosphate solubilization tendencies of the bacteria isolate were performed by inoculating 10 ml sterile Pikovskaya broth in 50 ml Falcon tubes with 0.1 ml (10^6 CFU/ml) freshly grown bacterial culture, incubated at 30^{o}C for 120 hours at 180 rpm on a rotary shaker machine. Bacterial supernatant was obtained after cold centrifugation (4^{o}C) of 10 ml bacterial cultures at 10,000 rpm for 5 minutes. Four milliliters (4 ml) of the color reagent [(1:1:1:2 ratio 3M H_2SO_4, 10% (w/v) ascorbic acid, 2.5% (w/v) ammonium molybdate and distilled water], were added to 10% (w/v) of 5 ml trichloroacetic acid inside test tubes. The tubes were allowed to stand for 15 minutes upon incubation at room temperature. The quantity of phosphate content was measured according to phosphomolybdate, a blue method at an absorbance of 820 nm. The phosphate solubilization potential by the bacterium in the Pikovskaya broth was determined from the phosphate (KH_2PO_4) standard curve. Medium without bacterial inoculation served as the control.

8.2.6.2 Siderophore production

Siderophore producing ability of *B. cereus* T4S was performed on chrome azurol S (CAS) medium following the methods of Khan et al. (2020) with little modifications. The quantity of siderophore produced was determined by inoculating LB broth solution containing CAS with 0.1 ml of 24-hour old bacterial culture and incubated at 180 rpm on a rotary shaker for 164 hours. Centrifugation of bacterial culture suspension was achieved at 8000 x g for 10 minutes.

Half a milliliter of the cell filtrate was added to 0.5 ml CAS reagent and properly mixed, then incubated for 120 seconds at room temperature. The quantity of siderophore released was measured at 630 nm using a spectrophotometer (Thermo Spectronic, Merck Chemicals, SA). The siderophore estimate was obtained from the regression equation of the standard curve.

8.2.6.3 Exopolysaccharide (EPS) production

Bacillus cereus T4S was qualitatively screened for EPS production following the modified method of Igiehon et al. (2019). The square-sized sterile Whatman filter paper No. 1 was aseptically and gently placed onto sterile molten LB agar plates. Two microliters (2 µl) of freshly grown 24-hour bacterial culture were directly inoculated on the surface of the filter paper placed inside agar plates supplemented with 10% sucrose with pH adjusted to 7. Subsequently, the inoculated plates were incubated at 28°C for 5 days. After that, the formation of mucoid colonies around the square-size filter paper depicted EPS production.

8.2.6.4 Indole acetic acid production

Indole acetic acid synthesis by *B. cereus* T4S was conducted according to Gutierrez et al. (2009). Ten milliliters (10 ml) LB broth supplemented with tryptophan were aseptically inoculated with 100 µl freshly grown bacterial culture (10^6 CFUml^{-1}) and incubated at 28^{o}C for 7 days at 120 rpm in a rotary shaker incubator (SI-600, LAB Companion, Korea). Following incubation, the bacterial suspension was cold centrifuged (4^{o}C) at 10,000 rpm for 5 minutes to obtain a cell-free supernatant layer. From the crude extract, 1 ml of supernatant was measured into a clean tube, and 2 ml Salkowski reagent (1:30:50 ratio of 0.5 M $FeCl_3$ solution: 95% w/w sulfuric acid: distilled water) was added. Then, a drop of 10 mM orthophosphoric acid was also added to the mixture and incubated for 10 minutes at room temperature for color development.

The appearance of pink coloration in tubes after incubation in the dark indicated a positive result. An un-inoculated tube serves as the control. From the reacting mixture and control tube after incubation, the absorbance was determined at 530 nm using UV-spectrophotometer (ThermoFisher Scientific, USA). IAA concentration was evaluated from the IAA gradient standard curve (SC).

8.2.7 Inoculum preparation and seed treatment for the greenhouse experiment

8.2.7.1 Inoculum preparation and seed treatment

A seed inoculation assay was used to facilitate bacterial adherence to the disinfected sunflower seeds. The effectiveness of sunflower seed inoculation was performed following the methods of Ullah et al. (2017). The bacterial inoculum size in LB broth at 24-hour incubation was standardized to 0.5 (10^6 CFU/ml) at OD_{600}. Surface sterilization of the seeds was performed by washing in sterile distilled water to remove floating-unhealthy seeds and dirt, and disinfected in 70% ethanol for 3 minutes, followed by 3% hypochlorite for 1 minute, then immersed in 70% alcohol for 2 minutes with final washing with sterile distilled water. Prepared LB broth inoculated with fresh bacterial culture was incubated on shaker incubator machine at 180 rpm for 24 hours. The bacterial cells in the broth culture were harvested by cold centrifugation (4^oC) at 8000 x g for 10 minutes to obtain the pelletized cells and then washed in 0.85% normal saline solution. The centrifugation and washing of the pellets were performed under sterile conditions. The sterilized seeds were suspended in a bacterial liquid medium (suspension) containing 1% (v/w) carboxymethyl cellulose (CMC) in a 250 ml flask and agitated for 60 minutes. The CMC serves as a sticker that causes the adhesion of bacterial mass to the seeds. The seeds suspended in sterile distilled water without bacterial inoculum served as control.

8.2.7.2 Greenhouse experimental study

The soil used for the experiment was sourced from agricultural farmlands in North-West University, Mafikeng Campus. Soil debris and other plant materials were removed, air dry, and sieved with a 2 mm micro stainless steel mesh sieve, and the soil was placed inside autoclavable plastic bags and sterilized at 121^{o}C for 15 minutes. This step was repeated three times to ensure all spore formers, vegetative cells, and forms of life were eliminated. The level of soil sterility after autoclaving was tested by plating on LB agar. Sterilized soil was allowed to cool for 2 days after which 10 kg of soil was aseptically transferred into plastic pots.

The inoculated and non-inoculated pots were arranged randomly in a complete randomized design (CRD) with 8 replicates for each treatment at a 10 cm distance apart in a greenhouse under natural light. Plastic pots measuring 34 cm in diameter and 29 cm tall were washed with sterile water and sterilized with 15% sodium hypochlorite solution before filling with 15 kg dry-sterilized loamy soil. The pots containing the sterile soil were moistened with 500 ml sterilized water before sowing. Ten seeds were sown per pot at a depth of 1.5 cm. After seed emergence, i.e., at 8 days, thinning was performed, leaving one sunflower seedling in a pot. Growing sunflower seedlings were maintained on a day-night cycle of 13-14 hours natural light, a temperature of 30±2^{o}C, and relative humidity of 85%. The pots were moistened with an equal amount of water and maintained daily. Pots containing seedlings without inoculation served as control. The plants were harvested at maturity, 132 days after planting.

8.2.7.3 Sunflower morphological parameters below-and-above-ground level

The data collected based on sunflower morphological parameters after harvesting include the number of lateral roots, root number, fresh weight, root weight (dry), shoot weight (dry), taproot length, etc. These parameters were considered following the method described by

Igiehon et al. (2019). After harvesting from a greenhouse, sunflower plants were taken to the laboratory. The soil adhering to plant roots was thoroughly washed under running sterile water. The fresh weight of roots and shoots was measured using a weighing balance (Wagi Elektroniczne, Poland). Furthermore, plant roots and shoots were oven-dried at 60°C for 24 hours and re-weighed on a weighing balance for the determination of roots and shoot dry weights.

8.2.7.4 Determination of sunflower yield parameters

The sunflower yield parameters were obtained above plant level. The parameters considered include seed weight (fresh and dry), total seed weight (dry), head weight (fresh and dry). After drying, sunflower seeds were manually separated from the sunflower head containing seeds. Seeds were dried in an oven at 60°C for 24 hours, and dry weight was measured on a weighing balance. The plant samples were kept in plastic bags for further analysis. The whole experiment was repeated in triplicate for each treatment.

8.2.7.5 Soil variable analysis

Approximately 0.5 kg of sieved soil was used for soil physical and chemical parameters determination (Igiehon et al., 2019). The chemical parameters such as a percentage (%) silt, clay, and sand, and chemical parameters such as magnesium, iron, potassium, phosphorus, manganese, organic carbon, organic matter, pH, and total nitrogen were evaluated.

8.2.8 Statistical analysis

The analysis of data from this study was analyzed using SPSS version 16.0. One-way analysis of variance (ANOVA) was performed for the data, followed by the Duncan test at a 5% level

of significance to determine differences between mean. For each treatment, generated data were presented as arithmetic means ± standard deviation.

8.3 Results

8.3.1 Biochemical and cultural features

The biochemical and cultural characterization of *B. cereus* T4S is presented in Table 8.1. The results indicated that *B. cereus* T4S is rod-like, catalase-positive, and Gram-positive. The strain T4S assimilates all the tested sugars and exhibited a positive reaction to citrate, oxidase, nitrate, casein, and starch hydrolysis. Also, strain T4S grows between pH ranging from 4 to 10, temperature from 25 to 45, and 5.5% normal saline. The results of plant growth-promoting tests were high, with a siderophore value of 87.30%, phosphate content of 30.43 µg/ml, and IAA of 11.28 µg/ml, respectively (Table 8.2). Bacterial endophytes exhibited significant reactions for protease, xylanase, mannanase, and cellulase production, except amylase during enzyme plate assay. The phylogeny of the genome sequence of *B. cereus* strain T4S is presented in Figure 8.1.

8.3.2 Whole genome sequencing analysis

The WGS analysis of *B. cereus* T4S yielded a sequence read count of 7,255,762, a genome size of 5,945,881 bp, total bases of 109,562,062, and G + C content of 34.8%. The read length mean was 151 bp while L_{50} and N_{50} values were 32 and 65,078 bp, respectively. The number of contigs and subsystems was 198 and 341, respectively. The genome analysis revealed 6,277 coding sequences and 63 RNAs. The circular genome visualization of *B. cereus* strain T4S is represented in Figure 8.2.

Table 8.1: Biochemical, cultural characterization of *B. cereus* T4S

Characteristics	**Result**
Gram reaction	+
Shape	Rod
Catalase	+
Starch hydrolysis	+
Nitrate	+
Oxidase	+
Citrate	+
Casein hydrolysis	+
Salt tolerance (% v/v) (max)	5.5
Temperature range (^{o}C)	25-45
pH	4-10
Fructose	+
Galactose	+
Sucrose	+
Glucose	+
Xylose	+
Raffinose	+
Mannitol	+
Maltose	+

Key: + = positive reaction

Table 8.2: Plant growth-promoting features of *B. cereus* T4S

Test	**Plant growth-promoting traits**				
	IAA (µg/mL)	Siderophore (%)	Phosphate (µg/mL)	Exopolysaccharide	
Qualitative	+	++	+	+++	
Quantitative	11.29±0.01[a]	87.30±0.38[c]	30.43±0.18[b]	ND	
Test	**Enzyme assay**				
	Amylase	Cellulase	Xylanase	Mannanase	Protease
Qualitative	-	++	++	++	+
ZOC (mm)	0±0.00[a]	50.00±0.01[d]	180.00±0.01[e]	35.00±0.01[c]	4.00±0.01[b]

Key: - = negative reaction, + = positive reaction, ZOC - zone of clearance measurement, ND – not determined. Values are represented as mean±standard deviation of triplicate readings. The superscript (small letters) within the same row represent a significant difference

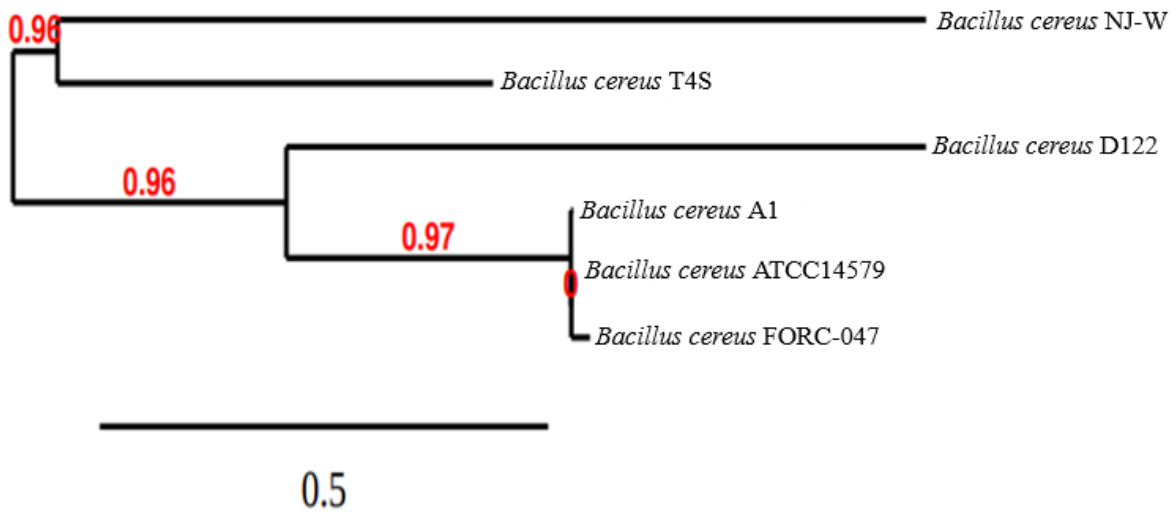


Figure 8.1: Phylogeny of the genome sequence data of *B. cereus* T4S. Legend: The scale bar represents branch lengths of node data in the phylogram, while numbers in red represents the number of differences between sequences (i.e. 0.96 means 96% difference between two bacterial sequences)

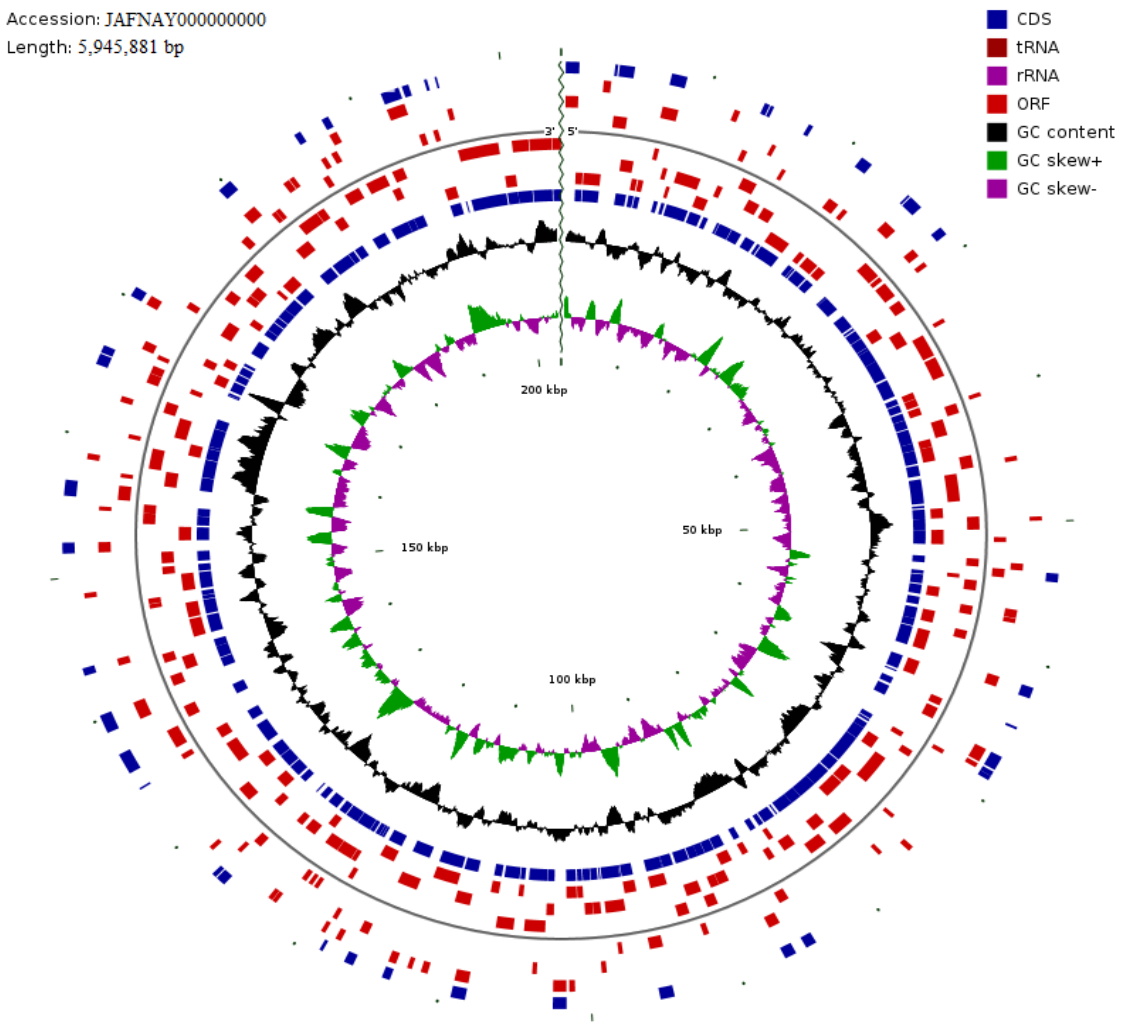


Figure 8.2: Circular genome visualization of whole-genome *Bacillus cereus* strain T4S. Each color from the external to internal circle depicts green (GC skew +) and red (ORF). The ring black coloration (GC content) at the peak indicated higher or lower values than average GC content. The GC Skew (-/+) in purple/green peaks in/outside the circle indicated values greater or smaller than 1. The GC Skew is calculated as G-C/G +C

The subsystem statistics show 27 subsystem feature counts of the coding protein into functional groups based on the annotated genome classification by SEED in RAST. The functional basis of 5,159 protein-coding genes (PCG) was assessed from the KEGG database and RAST online server. The 1,961 genes annotated by SEED viewer (version 2.0) were grouped into molecular function, cellular components, and biological processes. The topmost five groups were protein metabolism (155 genes), vitamins, prosthetic groups, pigments (161 genes), cofactor, amino acids and derivatives (377 genes), carbohydrates (271 genes), and nucleosides and nucleotides (119 genes).

8.3.4 Functional genes information of *B. cereus* T4S

The PCG from the genome of strain T4S revealed various genes involved in biocontrol, biomolecule transport and degradation, metabolic pathway, and synthesis of cellular components. Also, their locus tag, specific gene type, and gene product (Tables 8.3-8.18). In table 8.3, various secretion systems genes were highlighted. It was revealed that *B. cereus* T4S harbors nitrogen fixation genes (*nif*U and *nif*3-like) and other genes involved in ammonia and urea transport, nitrogen metabolism, nitrogen regulation, and nitrate reduction pathway (Table 8.4). The presence of PCG involved in the degradation of phosphonates such as *phn*CE and *isp*E, and phosphate transport (*pst*ABCS, *pho*UH, *glt*P, and *ugp*C) in *B. cereus* T4S is shown in Table 8.5. Similarly, the genes mediating sulfate transport were detected in the genome of *B. cereus* T4S (Table 8.6). *B. cereus* T4S possesses genes involved in iron transport and siderophore production, which include, *fbp*A, *feo*AB, and *fet*B (Table 8.7). Several other genes responsible for flagellar biosynthesis (*flg*BDEG, *flh*ABF, fliFKL, and *mot*AB), chemotaxis (*che*VW), and biofilm formation (*efp, hfq,* and *crp*) were detected in *B. cereus* T4S (Table 8.8).

Table 8.3: Genes involved in protein secretion systems genes

Gene	Locus tag	Product	Pathway
*sec*A	HWX41_RS21805	preprotein translocase subunit *Sec*A	Sec (secretion) system
*sec*E/G/Y	HWX41_RS26260	preprotein translocase subunit *Sec*E/G/Y	
*yaj*C	HWX41_RS02190	preprotein translocase subunit *Yaj*C	
*tat*E/*tat*A	HWX41_RS13235	protein translocase protein *Tat*E/*Tat*A	Twin-arginine translocation
*tat*C	HWX41_RS13230	protein translocase protein *Tat*C	(Tat) system
*tat*C	HWX41_RS13230	twin-arginine translocase subunit *Tat*C	

Table 8.4: Genes involved in nitrogen fixation and nitrogen metabolism

Gene	Locus tag	Product	Pathway
*nif*3-*like*	HWX41_RS02785	nitrogen fixation protein *Nif*3-like	Nitrogen fixation
*nif*U	HWX41_RS22890	nitrogen fixation protein *Nif*U	
*glt*P	HWX41_RS16800	glutamate/aspartate:proton symporter *Glt*P	Nitrogen metabolism
*glt*X	HWX41_RS26220	glutamate--tRNA ligase	
glnR	HWX41_RS05880	transcriptional repressor *Gln*R	
*gln*A	HWX41_RS05885	type I glutamate--ammonia ligase/glutamine ABC	
*gln*H	HWX41_RS23555	transporter substrate-binding protein *Gln*H	
*nad*R	HWX41_RS02105	transcription repressor *Nad*R	Nitrogen regulation
*nir*B/D	HWX41_RS13635	nitrite reductase (NADH) large/small subunit	Dissimilatory nitrate reduction
*nar*I	HWX41_RS13700	respiratory nitrate reductase subunit gamma	
Nark	HWX41_RS13665	nitrate transporter	
*glt*X	HWX41_RS26220	glutamate-tRNA ligase	Ammonia assimilation
*glt*P	HWX41_RS16800	glutamate/aspartate:proton symporter *Glt*P	

Table 8.5: Genes involved in phosphate solubilization and transport

Gene	Locus tag	Product	Pathway
*phn*C	HWX41_RS05935	phosphonate transport system ATP-binding	Degradation of
*phn*F	HWX41_RS04040	phosphonate metabolism transcriptional regulator *phn*F	phosphonates
*phn*E	HWX41_RS05940	phosphonate transport system permease protein	
*isp*H	HWX41_RS02790	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	
*pst*C	HWX41_RS23195	phosphate transport system permease	Phosphate transport
*pst*S	HWX41_RS02875	phosphate transport system substrate-binding protein	
*pst*B	HWX41_RS02885	phosphate transport system ATP-binding protein	
*pst*A	HWX41_RS23190	phosphate transport system permease protein	
*pho*U	HWX41_RS02890	phosphate signaling complex protein *Pho*U	
*glt*P	HWX41_RS23465	glycerol-3-phosphate transporter	
ugpC	HWX41_RS04235	sn-glycerol-3-phosphate ABC transporter ATP-binding protein *Ugp*C	
*pho*H	HWX41_RS02710	phosphate starvation-inducible protein *Pho*H and related proteins	

Table 8.6: Genes involved in sulfur metabolism

Gene	Locus tag	Product	Pathway
cysC	HWX41_RS16845	Adenylyl-sulfate kinase	Sulfate transport
Sat	HWX41_RS16850	sulfate adenylyltransferase	
Cyst	HWX41_RS18405	sulfate transport system permease protein	
*cys*W	HWX41_RS18400	sulfate ABC transporter permease subunit *Cys*W	
*cys*K	HWX41_RS15030	cysteine synthase A	
*cys*S	HWX41_RS26230	cysteine--tRNA ligase	
*sul*P	HWX41_RS25435	sulfate permease	

Table 8.7: Genes involved in iron transport and siderophore production

Gene	Locus tag	Product	Pathway
*fbp*A	HWX41_RS15335	fur-regulated basic protein *Fbp*A	Iron(III) transport
	HWX41_RS19065		
*feo*A	HWX41_RS00335	ferrous iron transport protein A	Iron(II) transport
*fet*B	HWX41_RS22925	iron export ABC transporter permease subunit *Fet*B	
*feo*B	HWX41_RS00340	ferrous iron transport protein B	
-	HWX41_RS24585	siderophore ABC transporter substrate-binding protein	Siderophore transport

Table 8.8: Genes involved in motility, biofilm formation, and chemotaxis

Gene	Locus tag	Product	Pathway
*che*V	HWX41_RS15615	chemotaxis protein *Che*V	Chemotaxis
*che*W	HWX41_RS15745	purine-binding chemotaxis protein *Che*W	
*flg*D	HWX41_RS15635	flagellar basal body rod modification protein *Flg*D	Flagella biosynthesis
*fli*K	HWX41_RS15640	flagellar hook-length control protein *Fli*K	
*flh*B	HWX41_RS15485	flagellar type III secretion system protein *Flh*B	
*flg*B	HWX41_RS15680	flagellar basal body rod protein *Flg*B	
*flh*A	HWX41_RS15480	flagellar biosynthesis protein *Flh*A	
*flg*E	HWX41_RS15630	flagellar hook protein *Flg*E	
*fli*L	HWX41_RS15650	flagellar protein export ATPase *Fli*I	
*flg*G	HWX41_RS15470	flagellar basal-body rod protein	
*flh*F	HWX41_RS15475	flagellar biosynthesis protein *Flh*F	
*fli*F	HWX41_RS15665	flagellar M-ring protein *Fli*F	
*mot*A	HWX41_RS01775	flagellar chemotaxis protein *Mot*A	
*flg*K	HWX41_RS15705	flagellar hook-associated protein *Flg*K	
*fli*M	HWX41_RS15515	flagellar motor switch protein *Fli*M	
*mot*B	HWX41_RS01780	flagellar motor protein *Mot*B	
Efp	HWX41_RS03245	elongation factor P	Biofilm formation
Hfq	HWX41_RS05840	RNA chaperone *Hfq*	
crp	HWX41_RS16660	*Crp/Fnr* family transcriptional regulator	

Other genes that mediate lipopolysaccharide biosynthesis were also detected in strain T4S (Table 8.9). Genes involved in the degradation of superoxide anion radicals and hydrogen peroxide (*sod*A and *kat*B), as well as gene *ahp*C coding for peroxiredoxin, were detected in the genome of *B. cereus* T4S (Table 8.10). Other related genes involved in carbohydrate metabolism and transport, organic acid metabolism, amino acid degradation, and transformation, opine and GABA transport and metabolism, lignin degradation and degradation against toxic peroxides, and modulation of plant hormones are represented in Tables 8.11-8.17. Subsequently, identifiable genes specific for biological control such as isoprene (*isp*G), colonization (*min*D), 2,3-butanediol (*ilv*ABCDEGN), and volatile degradation (*aco*AB) pathways were identified in the genome of *B. cereus* T4S (Table 8.18).

8.3.5 Predicted secondary metabolite cluster genes by antiSMASH

Table 8.19 shows the predicted secondary metabolite cluster genes from antiSMASH analysis of *B. cereus* T4S. Six notable secondary metabolite cluster genes detected include regulatory genes, other genes, additional biosynthetic genes, core biosynthetic genes, resistance genes, and transport-related genes (Figures 8.3a and 8.3b). *B. cereus* T4S exhibited 100% similarity for gene type siderophore and non-ribosomal peptides (NRPS), with the most similar known cluster for petrobactin and bacitracin (Table 8.19). The siderophore-and-NRPS-encoding genes in *B. cereus* T4S were found in the location between 28,853 - 42,560 nt. (total: 13,708 nt) and 1 - 57,852 nt. (total: 57,852 nt) of the entire genomic region.

Table 8.9: Genes involved in attachment to plant surfaces

Gene	Locus tag	Product	Pathway
*mur*F	HWX41_RS25735	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	LPS, Lipid A biosynthesis and modifications
*mur*A	HWX41_RS21065	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	
*mur*B	HWX41_RS04775	UDP-N-acetylmuramate dehydrogenase	
*mur*C	HWX41_RS00935	UDP-N-acetylmuramate--L-alanine ligase	
*mur*E	HWX41_RS04750	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	
*mur*D	HWX41_RS04760	UDP-N-acetylmuramoyl-L-alanine--D-glutamate ligase	
*mur*G	HWX41_RS04770	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	
*gal*E	HWX41_RS21410	UDP-glucose 4-epimerase *Gal*E	
*wec*B	HWX41_RS21750	UDP-N-acetylglucosamine 2-epimerase (non-hydrolyzing)	
*glm*U	HWX41_RS26655	Bifunctional glucosamine-1-phosphate N-acetyltransferase *Glm*U	
*upp*P	HWX41_RS17035 HWX41_RS27450	bacitracin resistance undecaprenyl-diphosphatase, undecaprenyl-diphosphate phosphatase *Upp*P	
*rfb*D	HWX41_RS17900	dTDP-4-dehydrorhamnose reductase	LPS, O-antigen biosynthesis
*rfb*B	HWX41_RS17905	dTDP-glucose 4,6-dehydratase	
*rfb*C	HWX41_RS17910	dTDP-4-dehydrorhamnose 3,5-epimerase	

Table 8.10: Genes involved in the protection against oxidative and nitrosative stress

Gene	Locus tag	Product	Pathway/function
*sod*A	HWX41_RS20530	superoxide dismutase	Degradation of superoxide anion radicals
*kat*B	HWX41_RS18175	catalase	Degradation of hydrogen peroxide
tpx	HWX41_RS01185	Thiol peroxidase	Degradation of hydrogen peroxide and organic hydroperoxides
bcp	HWX41_RS25065	thioredoxin-dependent thiol peroxidase	
*osm*C	HWX41_RS23535	*Osm*C family protein (peroxiredoxin osmc)	
*ahp*C	HWX41_RS24440	peroxiredoxin	
*ahp*F	HWX41_RS24435	alkyl hydroperoxide reductase subunit F	
*bsa*A	HWX41_RS13745	glutathione peroxidase	Detoxification of peroxidized compounds
grx	HWX41_RS22030	glutaredoxin family protein	
hcp	HWX41_RS06975	hydroxylamine reductase	Hydroxylamine reduction
-	HWX41_RS11455	PQQ-dependent sugar dehydrogenase	ROS resistance

Table 8.11: Genes involved in carbohydrate metabolism

Gene	Locus tag	Product	Pathway
tkt	HWX41_RS05950	transketolase	Pentose phosphate
*glc*K	HWX41_RS02915	glucokinase	EMP
*glc*U	HWX41_RS00785	glucose uptake protein *Glc*U	Glycolysis
*pfk*A	HWX41_RS01390	6-phosphofructokinase	
*gnt*K	HWX41_RS08285	Gluconokinase	Glucose
*glg*A	HWX41_RS00085	glycogen synthase *Glg*A	and glucose-1-phosphate degradation
*glg*P	HWX41_RS00090	glycogen phosphorylase	
*pst*G	HWX41_RS04055	PTS glucose transporter subunit IIABC	Glucose transport
*pst*H	HWX41_RS04060	phosphocarrier protein HPr	
*pfk*A	HWX41_RS01390	6-phosphofructokinase	Fructose degradation
*pfk*B	HWX41_RS05815	1-phosphofructokinase	
*rbs*K	HWX41_RS23445	ribokinase	Ribose degradation
*rbs*D	HWX41_RS23440	D-ribose pyranase	
*rbs*C	HWX41_RS23430	ribose ABC transporter permease	Ribose transport
*tre*C	HWX41_RS23595	alpha, alpha-trehalase	Trehalose degradation
*iol*E	HWX41_RS07140	myo-inosose-2 dehydratase	Myo-inositol degradation
*iol*G	HWX41_RS07120	inositol 2-dehydrogenase	
*gnt*K	HWX41_RS19365	gluconokinase	D-gluconate degradation
*gnt*P	HWX41_RS19360	gluconate permease *Gnt*P	D-gluconate transformation to 2-keto
*nag*A	HWX41_RS04030	N-acetylglucosamine-6-phosphate deacetylase	D-tagatose degradation
*cel*B	HWX41_RS21665	PTS cellobiose transporter subunit IIC	Cellobiose transport

Table 8.12: Genes involved in carbohydrate transport

Gene	Locus tag	Product	Pathway	
*mal*R	HWX41_RS04265	maltose operon transcriptional repressor	Maltose	
*tre*R	HWX41_RS23605	trehalose operon repressor	Trehalose-specific component	EIIBC
-	HWX41_RS23665	MFS transporter	Sugar transport	
bcr	HWX41_RS26020	*Bcr/Cfl*A family efflux MFS transporter		
-	HWX41_RS23330	peptide MFS transporter		
-	HWX41_RS12690	OFA family MFS transporter		
*lld*P	HWX41_RS21585	L-lactate permease	Sugar acids transport	
	HWX41_RS17765			

Table 8.13: Genes involved in the metabolism of organic acids

Gene	Locus tag	Product	Pathway
*ace*A	HWX41_RS18305	isocitrate lyase	TCA cycle
*prp*B	HWX41_RS12770	methylisocitrate lyase	
*ace*B	HWX41_RS18310	malate synthase A	
icd	HWX41_RS01420	NADP-dependent isocitrate dehydrogenase	
*pur*B	HWX41_RS24180	adenylosuccinate lyase	
*arg*G	HWX41_RS01225	argininosuccinate synthase	
*sdh*C	HWX41_RS01750	succinate dehydrogenase cytochrome B558	
*sdh*A	HWX41_RS01755	succinate dehydrogenase flavoprotein subunit	
*sdh*B	HWX41_RS01760	succinate dehydrogenase iron-sulfur subunit	
*fum*A	HWX41_RS24845	fumarate hydratase class I	
mdh	HWX41_RS01425	malate dehydrogenase	
*cit*Z	HWX41_RS01415	citrate synthase	
*mmg*D	HWX41_RS12780	citrate synthase	
*acn*A	HWX41_RS06265	aconitate hydratase	Glyoxylate bypass TCA
*ace*A	HWX41_RS18305	isocitrate lyase	
*ace*B	HWX41_RS18310	malate synthase	
*fdh*D	HWX41_RS15580	formate dehydrogenase accessory sulfurtransferase	Formate degradation
*fdh*F	HWX41_RS15595	formate dehydrogenase subunit alpha	

Table 8.14: Genes involved in amino acid metabolism and transport

Gene	Locus tag	Product	Pathway
*met*K	HWX41_RS00560	methionine adenosyltransferase	Methionine degradation
alr	HWX41_RS13960	alanine racemase	D-alanine degradation
*spe*A	HWX41_RS04510	arginine decarboxylase	L-arginine degradation
*asp*B	HWX41_RS16185	aspartate aminotransferase *Asp*B	L-aspartate degradation/conversion
*cys*K	HWX41_RS26760	cysteine synthase A	L-cysteine degradation
*suf*S	HWX41_RS01140	cysteine desulfurase	Cysteine transformation
*gud*B	HWX41_RS16465	NAD-specific glutamate dehydrogenase	L-glutamate degradation
*rac*E	HWX41_RS01860	glutamate racemase	
*hut*H	HWX41_RS06090	histidine ammonia-lyase	L-histidine degradation
*hut*U	HWX41_RS06095	urocanate hydratase	
*hpp*D	HWX41_RS25765	4-hydroxyphenylpyruvate dioxygenase	L-tyrosine degradation
*xer*S	HWX41_RS25285	tyrosine recombinase *Xer*S	
*thi*O	HWX41_RS20150	glycine oxidase *Thi*O	Glycine cleavage
*gcv*H	HWX41_RS22695	glycine cleavage system protein *Gcv*H	
asn	HWX41_RS13200	asparagine synthase	Branched-chain amino acid transport
dat	HWX41_RS13175	D-amino-acid transaminase	

Table 8.15: Genes involved in opine and GABA transport and metabolism

Gene	Locus tag	Product	Pathway
*iol*E	HWX41_RS07140	myo-inosose-2 dehydratase	Myo-inositol, rhizopine
*iol*G	HWX41_RS07120	myo-inositol 2-dehydrogenase	metabolism
*iol*D	HWX41_RS07135	3D-(3,5/4)-trihydroxycyclohexane-1,2-dione	
		acylhydrolase (decyclizing)	
*iol*C	HWX41_RS07125	5-dehydro-2-deoxygluconokinase	
-	HWX41_RS07155	5-deoxy-glucuronate isomerase	
*spe*B	HWX41_RS07085	agmatinase	Putrescine and spermidine
*spe*E	HWX41_RS20905	spermidine synthase	production and transport
*pot*D	HWX41_RS17570	spermidine ABC transporter substrate-binding protein	
*pot*C	HWX41_RS17575	spermidine ABC transporter permease	
*pot*B	HWX41_RS17580	spermidine/putrescine ABC transporter permease	
*pot*A	HWX41_RS17585	spermidine ABC transporter ATP-binding protein	
-	HWX41_RS17805	gamma-glutamyl-gamma-aminobutyrate hydrolase	GABA biosynthesis
-	HWX41_RS07225	family protein	

Table 8.16: Genes involved in lignin degradation and degradation against toxic peroxides

Gene	Locus tag	Product	Pathway
-	HWX41_RS14790	multicopper oxidase domain-containing protein	Lignin degradation
-	HWX41_RS16575	superoxide dismutase	Degradation of toxic peroxides
*sod*A	HWX41_RS20530	superoxide dismutase	
-	HWX41_RS23120	superoxide dismutase family protein	
*ahp*C	HWX41_RS24440	peroxiredoxin	
*kat*B	HWX41_RS18175	catalase	
bcp	HWX41_RS25065	thioredoxin-dependent thiol peroxidase	
tpx	HWX41_RS01185	thiol peroxidase	
*ahp*C	HWX41_RS24440	peroxiredoxin	
*adh*P	HWX41_RS13135	alcohol dehydrogenase *Adh*P	Metabolism of alcohols and
*adh*P	HWX41_RS13135	alcohol dehydrogenase *Adh*P	aldehydes

Table 8.17: Genes involved in the modulation of plant hormones

Gene	Locus tag	Product	Pathway
*acd*A	HWX41_RS21030	acyl-CoA dehydrogenase *Acd*A	ACC catabolism
*kdp*A	HWX41_RS15255	potassium-transporting ATPase subunit A	Potassium transport
-	HWX41_RS17780	tryptophan synthase subunit alpha	L-tryptophan production; IAA production
*trp*B	HWX41_RS17785	tryptophan synthase subunit beta	
trpC	HWX41_RS17795	indole-3-glycerol phosphate synthase *Trp*C	
*trp*D	HWX41_RS17800	anthranilate phosphoribosyltransferase	
*trp*E	HWX41_RS17810	anthranilate synthase component I	
*trp*S	HWX41_RS18055	tryptophan--tRNA ligase	
*dha*S	HWX41_RS06485	aldehyde dehydrogenase *Dha*S	IAA production, IPA pathway
*mia*A	HWX41_RS05835	tRNA (adenosine(37)-N6)-dimethylallyltransferase *Mia*A	CK biosynthesis and transformation
*nad*E	HWX41_RS14365	ammonia-dependent NAD(+) synthetase	Ammonia production

Table 8.18: Genes involved in biological control

Gene	Locus tag	Product	Pathway
*aco*A	HWX41_RS10755	acetoin:2,6-dichlorophenolindophenol oxidoreductase subunit alpha	Volatile degradation
*aco*B	HWX41_RS10760	acetoin:2,6-dichlorophenolindophenol oxidoreductase subunit beta	
ilvA	HWX41_RS14970	threonine ammonia-lyase *ilv*A	2,3-Butanediol
*ilv*D	HWX41_RS14975	dihydroxy-acid dehydratase	
*ilv*C	HWX41_RS16965	ketol-acid reductoisomerase	
*ilv*N	HWX41_RS16970	acetolactate synthase small subunit	
*ilv*B	HWX41_RS16975	acetolactate synthase large subunit	
*ilv*E	HWX41_RS16980	branched-chain-amino-acid transaminase	
*isp*G	HWX41_RS02835	4-hydroxy-3-methylbut-2-enyl-diphosphate synthase	Isoprene
*min*D	HWX41_RS02025	septum site-determining protein *Min*D	Colonization

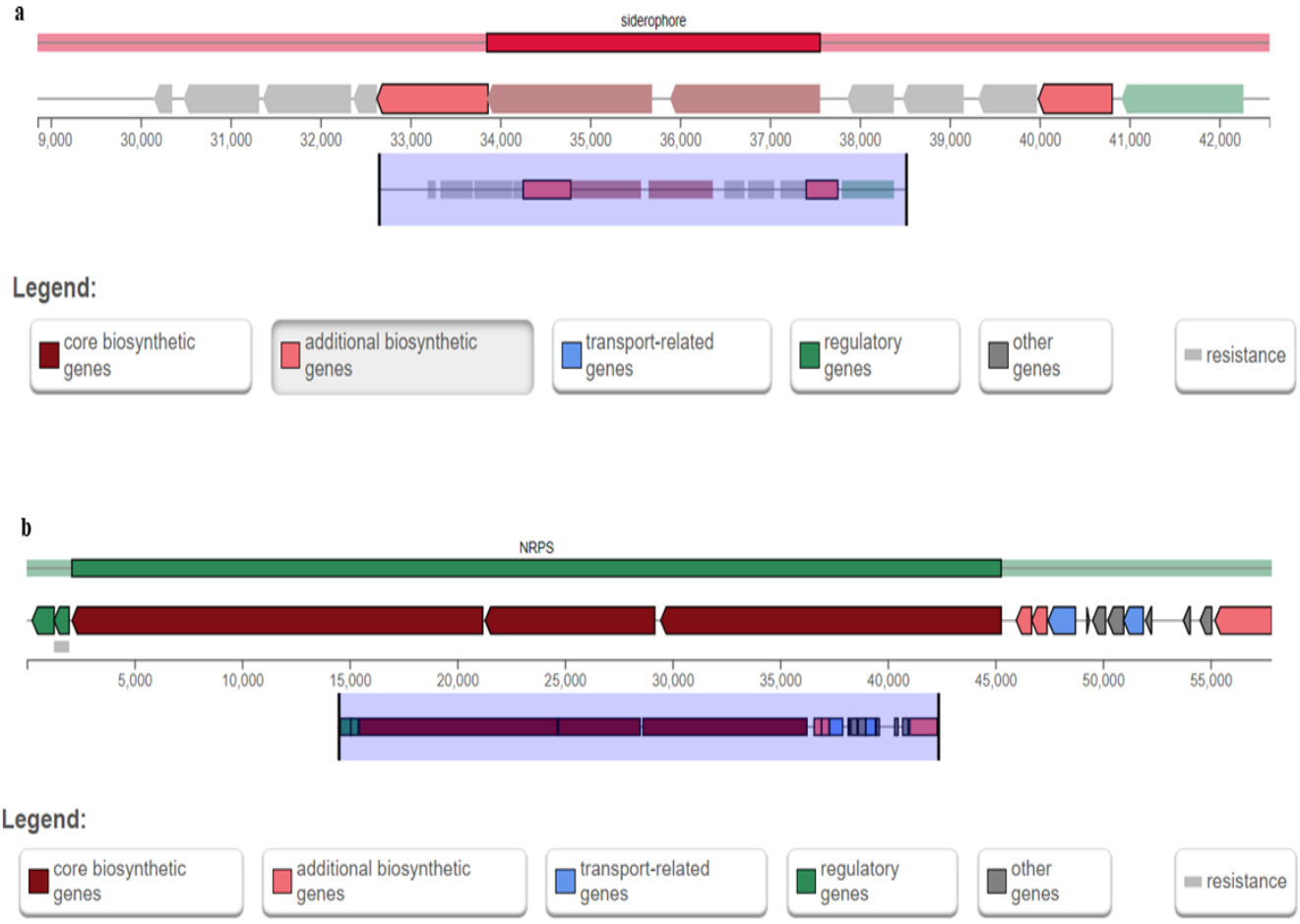


Figure 8.3a and 8.3b: Graphical representation of siderophore-and-nonribosomal peptides (NRPS)-encoding genes. These loci are predicted from the whole genome assembly of *B. cereus* T4S

Table 8.19: Estimate of secondary metabolite genes in genome of *B. cereus* T4S

Node Rg	Type	From	To	MSKC		Similarity
Rg 1.1	Siderophore	28,953	42,560	Petrobactin	Other	100%
Rg 3.1	NRPS	93,263	140,279			
Rg 3.2	RiPP-like	155,248	165,496			
Rg 21.1	NRPS	20,448	75,995			
Rg 28.1	NRPS	22,582	65,892	Bacillibactin	NRP	46%
Rg 35.1	NRPS	1	57,852	Bacitracin	NRP	100%
Rg 42.1	Terpene	16,640	38,493	Molybdenum cofactor	Other	17%
Rg 46.1	NRPS, T1PKS	1	45,939	Zwittermicin A	NRP + Polyketide	77%
Rg 62.1	NRPS-like	5,236	34,100			
Rg 71.1	RiPP-like	8,999	19,310			
Rg 86.1	NRPS	1	23,433			
Rg 87.1	LAP, RiPP-like	1	17,771			
Rg 89.1	NRPS, T1PKS	1	22,451	Zwittermicin A	NRP + Polyketide	7%
Rg 91.1	Betalactone	7,584	22,279	Fengycin	NRP	20%

Key: MSKC - Most similar known cluster, Rg – region, NRPS – nonribosomal peptides, NRP – nonribosomal peptide, T1PKS – type 1 polyketide synthases, LAP - lantipeptide, RiPP - ribosomally synthesized and post-translationally modified peptide

8.3.6 Effect of bacterial inoculation on sunflower yield

The resulting output from the greenhouse experiment is presented in Table 8.20. Inoculated sunflower with *B. cereus* T4S statistically was significantly different in the growth parameter compared to non-inoculated (Figure 8.4). Here, the results show higher yields in the parameters measured in the inoculated plant than un-inoculated, except for the seed dry weight with no significant difference. The physical and chemical analysis of the soil sample recorded high magnesium content, 642 (mg/kg), followed by potassium content, 399 (mg/kg), and the least value 0.10% of total nitrogen content (Table 8.21). The clay, sand, and silt values were 22.20%, 68.30%, and 7.92%, respectively.

Table 8.20: Sunflower yield parameters

Level	Growth parameter	Non-inoculated	Inoculated with *B. cereus* T4S
Belowground	Taproot length (cm)	146.67±58.33[a]	158.33±40.20[b]
	Tap root width (cm)	5.33±0.58[a]	7.00±1.00[b]
	Root length (cm)	217±81.22[a]	339.67±82.78[b]
	Lateral root number	25.67±1.53[a]	29.67±1.53[b]
	Root wet weight (g)	44.09±16.12[a]	65.54±18.41[b]
	Root dry weight (g)	8.41±1.55[a]	11.43±5.44[b]
	Number of roots	935±11.30[a]	1209.67±348.26[b]
Aboveground	Seed wet weight (g)	0.05±0.01[a]	0.25±0.33[b]
	Seed dry weight (g)	0.03±0.01[a]	0.035±0.00[a]
	Head fresh weight (g)	153.76±13.94[a]	165.50±7.55[b]
	Head dry weight (g)	41.55±4.27[a]	45.92±13.33[b]
	Plant wet weight dry (g)	331.04±20.16[a]	392.69±8.70[b]
	Shoot wet weight (g)	165.82±5.16[a]	183.55±13.75[b]
	Shoot dry weight (g)	57.28±7.04[a]	68.24±22.13[b]

Values are represented as means±standard deviation of triplicate readings. The superscript (small letters) within the same column represent a significant difference

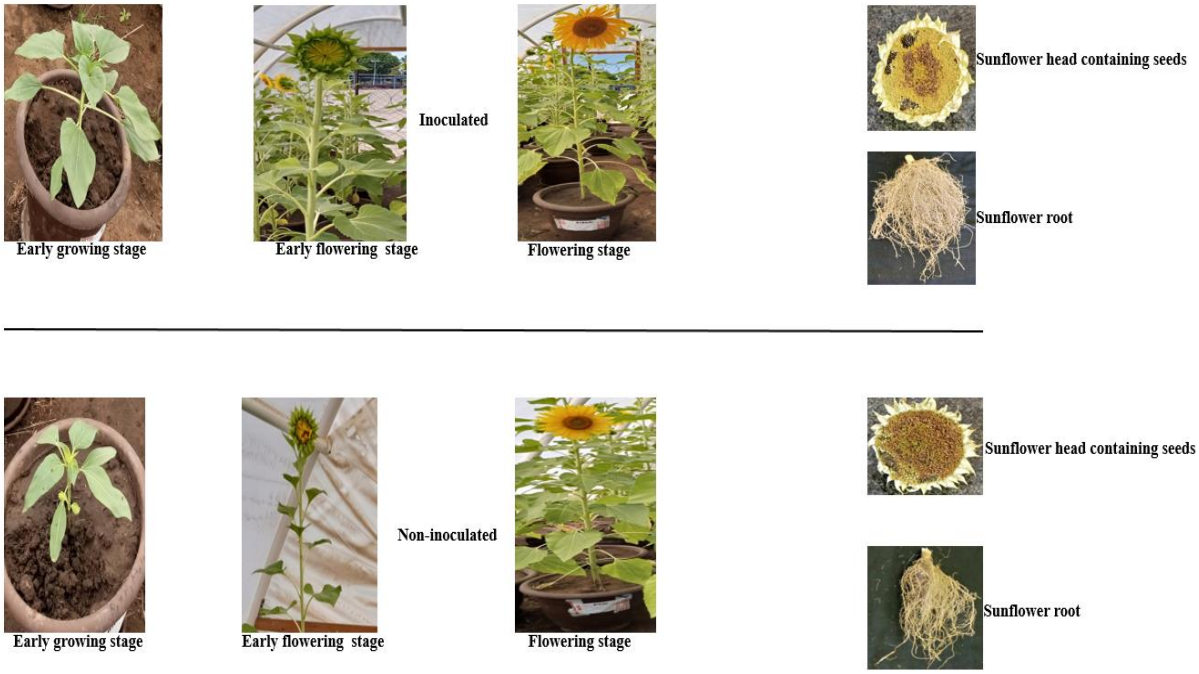


Figure 8.4: Inoculated and non-inoculated sunflower growth and yield parameters

Table 8.21: Physical and chemical analysis of the soil samples

Parameter	**Result output**
Manganese (mg/kg)	43.57
Phosphorus (mg/kg)	74.93
Organic carbon (%)	1.19
Total nitrogen (%)	0.10
pH (H_2O)	7.40
Magnesium (mg/kg)	642.00
Organic matter (%)	3.20
Potassium (mg/kg)	399.00
Iron (mg/kg)	2.00
Clay (%)	22.20
Sand (%)	68.30
Silt (%)	7.92

8.4 Discussion

In recent times, the potential of microbial endophytes ranging from plant growth promotion to control of phytopathogens to ensure agriculture sustainably remains fundamental (Bamisile et al., 2018). Significantly, the detection of multifunctional genes from the diverse bacterial community in the roots endosphere by whole genome sequence analysis with biotechnological prospecting has provided salient information on the metabolic activities and their agricultural importance (Singh et al., 2021). Nevertheless, this approach has provided researchers with a motivational framework in the exploration of environmental microbes as a key bio-factory of various compounds, importantly for both present and future use. Biotechnologically, the accumulated information on less studied endophytic microbes in the sunflower endosphere would necessitate significantly more research, focusing on plant beneficial bacterial endophytes with multifaceted metabolic potential. To this premise and based on scanty information relating to the whole genome analysis of endophytic bacteria in the sunflower root endosphere (SRE), we present this research as a promising genomic overview into SRE biology.

The genomic sequence dataset of *B. cereus* strain T4S from the SRE was analyzed. The identified strain T4S potentially produces lytic enzymes, IAA, siderophores, and also, displayed the ability to solubilize phosphate. To corroborate this, research findings into plant growth-promoting activities of endophytic bacteria colonizing the root endosphere of sunflower based on their multifunctional traits could functionally ensure better crop yields (Kumar et al., 2020).

The genomic insights into *B. cereus* T4S might perhaps unravel its diverse bioprospecting abilities, channeling appropriate specific mechanisms of action in the host plants. The

abundance of exudates in the root environment can facilitate rhizobacteria infiltrating from the external root environment into the root endosphere and becoming endophytes (Passari et al., 2016). Notably, exudate secretions from the plant roots can enhance bacterial colonization and the establishment of plant-microbes equilibrium in the root endosphere, thus significantly improving plant performance (Ellouze et al., 2015). Bacterial root colonization has been attributed to the presence of specific genes involved in biofilm formation, chemotaxis, and flagellation in their genome, thus enabling them to establish a bacterial community in the plant endosphere with multiple functions to promote plant growth under water-deficit stress. The phylogeny analysis of the genome sequence of *B. cereus* T4S revealed a close relatedness to *B. cereus* NJ-W. The positioning of taxonomic classification of bacteria can be resolved based on gene sequences.

The detection of several genes involved in protein secretion systems and TAT pathways is presented in this study. Peptidase could mediate the export of protein by cleaving the N-terminal of a signal peptide. Secretions of complex genes such as *sec*Y, *sec*E, *sec*D, *sec*G, and *sec*F in the cytoplasm of bacteria are known (Samaras et al., 2020). The presence of *Sec*A in most bacteria facilitates the binding of signal peptides as a driver in the translocation of protein through other gene channels, like *sec*YEG. Also, *sec*B in the cytoplasm could contribute to protein stability and secretion within the cell. Exhibition of oxidative gene encoding pyrroloquinoline quinone (PQQ)-dependent sugar dehydrogenase by strain T4S could be an indication of bacterial resistance or adaptation to reactive oxygen species (ROS) produced under a stress-induced environment in building strong defense mechanisms in the host plants (Nascimento et al., 2020a, Nascimento et al., 2020b).

The presence of nitrogen fixation genes, *nif*3-like and *nif*U by strain T4S revealed that they can participate effectively in biological nitrogen fixation through the conversion of dinitrogen gas into nitrate and ammonium within the plant tissues. The biological nitrogen fixation by *Bacillus* spp in chicken-pea grown in nitrogen-deficient soil environment has been reported (Singh et al., 2014). Recently, the identification of *nif*U in *P. aeruginosa* B8 isolated from the root of sugar with nitrogen fixation potential has been documented (Singh et al., 2021). Phosphorus is either fixed or immobilized in the soil, thus limiting its usage by plants. Phosphorus is made available to plants by phosphate-solubilizing microorganisms, especially the endophytic type capable of synthesizing phosphatases and organic acids (Khamwan et al., 2018b). Different *Bacillus* strains such as *B. pumilis, B. subtilis, B. amyloliquenfaciens,* and *B. methylotrophicus* are regarded as phosphate solubilizers, and their use in soil management has enhanced phosphorus levels in soil (Mehta et al., 2014, Ribeiro et al., 2018). The screened genome of strain T4S uncovered genes involved in phosphonate degradation and phosphate transport pathways. Various genes involved in phosphate solubilization and transport include *phb*CEF, *isp*H, *pst*ABCS, *pho*U, *glt*P, and *ugp*C. The presence of *pst* encoding phosphate transport system permease protein as predicted can account for an increase in phosphate uptake and bioavailability in a phosphate-limiting environment (Nascimento et al., 2020a), thus contributing to plant growth. The role of *pst* in the phosphate transport pathway by *B. subtilis* MBI 600, *B. cereus* 905, and *Paenibacillus polymyxa* HK4 has been documented (Samaras et al., 2020, Zeng et al., 2018, Soni et al., 2021). Notably, the detection of phosphate transport genes (*pst*ABCS) correlates with the finding of Zeng et al. (2018), who reported the same genes in the whole genome of plant growth-promoting *B. cereus* strain 905. Similarly, Singh et al. (2021) reported the presence of phosphate transport genes (*pst*ABCS) in the genome of *P.*

aeruginosa B18. Nevertheless, the authors had also reported *pho*BDHR, which was not detected in this study. In another study, the presence of the phosphate transporter gene, *pho*RP in the genome of *B. cereus* was documented (Zeng et al., 2018). The presence of phosphonate degradation and phosphate transport genes in stain T4S could enhance the rate of phosphate utilization by the host plants.

The presence of sulfur transport genes in the genome of an endophytic bacterium can facilitate the degradation of sulfur-containing xenobiotic compounds. It also contributes to the sulfur acquisition ability of bacterial strain and possibly in the modulation of sulfur levels in plants and soils. The identification of sulfate transport genes (*sul*P, *cys*APUW, and *yln*A) in *B. megaterium* strain STB has been documented to enhance plant growth and mitigate stress in plants (Nascimento et al., 2020b).

Plants tend to survive in iron-limiting soil due to the abundance of siderophore-producing microbes that ensure the acquisition and bioavailability of soluble iron for plants and microbial uptake (Hardoim et al., 2015). Siderophore production can exert lethal effects on targeted phytopathogens due to the strong affinity of microbes to compete for iron in the soil (Zhang et al., 2020). Siderophore activity of strain T4S was profound. The genome revealed the presence of genes, such as *fep*A, *feo*A, *feo*B, and *fet*B that code for iron transport, thus suggesting their strong linkage for siderophore production. The expression of siderophore genes namely, pyoverdine homologous genes, *pvd, fhu, mbt*H, *fpv*A, and *acr*AB from bacterial genera with potential plant growth promoting traits have been studied (Gupta et al., 2014). Douriet-Gámez et al. (2018) had identified siderophore genes *dhb*ABCF from *Bacillus* sp. strain B25. The results here corroborate the study of Nascimento et al. (2020a) who reported the presence of genes (*feo*AB) involved in iron transport in *Pantoea phytobeneficialis* strain MSR2. Also,

reports on several putative siderophore genes (*rha*ABCDEF) that somewhat mediate the synthesis of rhizobactin for the production of hydroxamate-like siderophore of *B. megaterium* STB1 and *Sinohizobium meliloti* 1021 are known (Nascimento et al., 2020b). Interestingly, active participation of *B. megaterium* STB1 in the siderophore transport pathway due to the presence of siderophore genes, *yfi*Z, *yfh*A, *yfi*Y, and *yus*V that regulate iron transport in *B. subtilis* have suggested their influence in competitive interactions with potential pathogens and enhancement of plant growth (Ollinger et al., 2006). In this study, various genes involved in biofilm formation, chemotaxis, and flagellation were detected in the genome of an endophytic bacterium strain T4S. The presence of lipopolysaccharide (LPS) genes can enhance bacterial functions to firmly stabilize their outer membrane from desiccation. Interestingly, strain T4S harbors gene *glm*U encoding glucosamine-1-phosphate N-acetyltransferase involved in LPS, lipid A biosynthesis pathways. The presence of flagellar motor protein genes (*mot*AB) was in line with Nascimento et al. (2020b), who had earlier reported the same genes in the genome of *B. megaterium* STB1. The authors further made documentation on several other genes that are involved in the synthesis of exopolysaccharides that enhance biofilm formation and adherence of bacteria to the plant root. Similarly, teichoic acid genes responsible for the biofilm formation and root attachment were documented (Nascimento et al., 2020b).

One of the key attributes of endophytic bacteria is the ability to produce exopolysaccharides (EPS). The expression of EPS genes in the bacterial genome has contributed to plant resistance under drought conditions and the formation of biofilm that aids surface colonization (Janczarek et al., 2015). EPS functions in biofilm production confer defensive mechanisms in response to ROS and plant protection against pathogens. The biofilm formation/production by endophytic microbes could enable plants to resist environmental stressors and firm colonization within the

endosphere, thus suggesting their functions in plant-microbe interactions (Nascimento et al., 2020a). Furthermore, EPS synthesis enhanced bacterial affinity within the host plants for various biological functions (Singh et al., 2021). Various genes, such as *flg*BDFGK, *flh*ABE, *fli*CEHFKLMNPQRSY, and *mot*AB are found in the genome of strain T4S involved in the flagellar biosynthesis pathway. Protein coding gene *crp* that is primarily involved in biofilm production was also identified in strain T4S. The identification of specific genes involved in biofilm production from the genome of endophytic bacterial strain associated with the root endosphere is known (Samaras et al., 2020, Singh et al., 2021). Based on a previous study by Xu et al. (2014), it can be inferred that *B. cereus* T4S might have potential as a biocontrol agent due to the presence of certain gene families which have been demonstrated to be responsible for the biocontrol efficacy in experiments performed in other hosts/microbe systems.

Carbon sources provide the required energy for bacterial metabolism. Endophytic bacterial strain T4S contains genes involved in carbohydrate metabolism and transport through dynamic pathways, which include, pentose phosphate pathway, EMP - Embden-Meyerhof-Parnas glycolysis pathway, maltose, cellobiose, glucose, and ribose transport pathways, hexose metabolism pathway, GDP-mannose, N-acetyl-D-galactosamine and D-galactosamine, trehalose, myo-inositol, D-gluconate, L-lactate, L-lyxose, beta-glucosides, 2,3-diketo-L-gulonate-and-ascorbate, chitin, D-tagatose, and fructose degradation pathways, sugar transport, sugar acids transport, and trehalose-specific EIIBC component. The ability of bacterial endophytes to utilize complex carbohydrate compounds has significantly contributed to the establishment of plant-microbe interactions. Reports have shown that the amount of metabolizable sugar released from plant roots could mediate the colonization tendencies of bacterial in the root endosphere compartments and then contribute to crop yield (Paul and Lade,

2014, Jha and Subramanian, 2018). Thus, the genomic sequencing of *B. cereus* T4S reveals the presence of gene families with previously reported beneficial roles in other crop-microbe associations (Annapurna et al., 2018), which suggests that *B. cereus* T4S may be a candidate in stimulating phytohormones in enhancing sunflower growth.

Owing to the genetic profiling of bacterial endophytes, they can participate in carbohydrate metabolic pathways and related carbon derivatives from the plant root exudates. The amount of carbohydrate content in plants can mediate sugar signal networking for cellular regulation and a shift in plant response to environmental stress adaptors (Fouda et al., 2021). Reports on the utilization of glucose, sucrose, maltose, and ribose as a carbon source by bacterial endophytes have been shown to have contributed to their metabolic activities (Joshi et al., 2019). Biotransformation of organic compounds for organic acids production could share a direct link for enhanced bacterial colonization with the host plants.

Production of notable genes involved in amino acid biodegradation and biotransformation could contribute to the functional attributes of bacteria in eco-niches. The presence of genes in the polyamine, amino acid opine, and 4-aminobutyrate (GABA) metabolism in strain T4S have suggested their ability to metabolize amino acid, thus contributing to microbial colonization for improved plant growth. Also, the presence of opines could be responsible for amino acid condensation. Opines are known as one of the chemical compounds produced by rhizobacteria that participate in plant-bacteria colonization, thus modulating root bacterial-nodulation processes. The protein-encoding myo-inositol and rhizopine metabolism (*iol*CDEG) involved in the degradation of these compounds suggested the importance of these compounds in bacterial colonization with plants. The role of GABA synthesis by bacterial species in

synergistic interactions with plants for enhanced plant performance has been reported (Bown and Shelp, 2016, Nascimento et al., 2020a).

The possession of lignin degradation encoding genes, multicopper oxidase domain-containing protein has yielded other organic compounds such as alcohols, phenolic and toxic aldehydes, several intermediates-like alcohols (gallate and protocatechuate), and aromatic compounds, vanillate (Shi et al., 2015, Nascimento et al., 2020a). The presence of lignin-degrading genes and other plant components could significantly facilitate endophytic colonization.

The role of root-associated bacterial endophytes such as *Burkholderia*, *Bacillus, Pseudomonas*, etc. in plant growth promotion has been reported in recent times with a promising outlook in agriculture sustainably (Dubey et al., 2021, Khalil et al., 2021, Singh et al., 2021). Nevertheless, information regarding the phytohormone production ability of bacterial endophytes from the sunflower root endosphere as revealed by whole-genome analysis is less studied. Plant hormone synthesis is a major feature that distinguishes agriculturally important bacteria from one and another (Daud et al., 2019). The detection of *acd*A could serve as a precursor in lowering ethylene levels in plants. Deamination of 1-aminocyclopropane-1-carboxylate (ACC) into ammonia and α-ketobutyrate can enhance bacterial metabolism in response to sustain plant health. The detection of *acd*A gene in the genome of bacteria may perhaps uncover their potential in stimulating plant internal responses against environmental stressors. The positive influence of *B. mojavensis* isolated from pea (*Pisum sativum*) on plant growth due to possession of *acd*S gene has been documented (Maheshwari et al., 2020). IAA biosynthesis in a culture medium depends on the utilization of precursory factor (tryptophan) by the endophytic bacteria. Various bacteria isolated from the root endosphere promote plant growth and soil health and the diverse endophytic bacterial community is known to produce

IAA and other plant growth-promoting traits. Genome analysis of strain T4S predicts the presence of *dha*S and *trp*BCDES genes that are involved in L-tryptophan production, IAA production, and IPA pathway. The presence of IAA-enzyme coding genes in the genome of endophytic bacteria confirms their involvement in IAA biosynthesis. Hence, identification of plant growth-promoting genes *trp*ABD, *trp*ABCDE, and *trp*BE from *Sphingomonas* sp. LK11, *Pseudomonas aeruginosa* B18, and *Enterobacter roggenkampii* ED5 from the sugarcane root endosphere have been documented (Singh et al., 2021). The putative candidate genes and gene families identified in *B. cereus* T4S have the potential for directing further experimental tests of their roles in sunflower growth similar to previous experiments examining cucumber yield in *B. subtilis* MBI 600 (Samaras et al., 2020).

Additionally, studies on the possible novel genes that involve in IAA synthesis through the tryptophan-dependent indole-3pyruvate (*IPy*A) pathway by *B. amyloliquefaciens* SQR9a have been studied (Shao et al., 2015). These genes are primarily involved in the conversion of IAA synthesis precursor (tryptophan) *IPy*A. Also, pyruvate decarboxylase, pyruvate oxidase, and phenolic acid decarboxylase participate in the conversion process of *IPy*A to indole-3-acetaldehyde (IAAld). The observed enzyme coding gene (aldehyde dehydrogenase genes, *dha*S) in strain T4S has also been implicated in the conversion process of IAAld to IAA, which further corroborates the results obtained from the genomic analysis of *B. amyloliquefaciens* SQR9a (Shao et al., 2015).

Some industrially and biotechnologically important enzymes from bacterial endophytes have been reported (Zaferanloo et al., 2013), and their vital role in plant defense mechanisms against phytopathogen resistance is known. Examples of these enzymes include chitinase, cellulase, glucanase, amylase, catalase, and peroxidase. In this study, various genes (*lon*B, *amy*S, and

*pul*A) that involved enzyme synthetic pathways were detected in strain T4S, thus suggesting the ability to stimulate plant immune response.

Various genes involved in the protection against oxidative and nitrosative stress were detected in strain T4S. The survival of plants inoculated with bacterial endophytes that thrive under high saline environments could suggest their possible use as bioinoculants to boost salt tolerance in plants' (Dubey et al., 2021). The result agrees with the earlier reports on the osmotic stress tolerance genes in *B. megaterium* STB1 (Nascimento et al., 2020a). However, the authors further stressed that the presence of cold and heat shock genes (*csp* and *hsp*), and chaperones in the genome of strain *B. megaterium* STB1 contributed to RNA and DNA stabilization for easy expression (translation and transcription) under cold or heat stress conditions. However, chaperone encoding genes were not detected in strain T4S.

The participatory genes in the biological control and synthesis of volatile compounds form an essential component of the bacterial cell. The detection of *ilv*ABCDEN gene coding for 2,3-butanediol could be linked to induction of systemic resistance in plants, and protect bacterial cells at low pH and other deleterious metabolites secreted from the root rhizosphere.

Commercialization of known strains of *Bacillus* species of the same ecotype and their activities in sustaining plant growth stand promising as a source of metabolic compounds, such as macrolactin, difficidin, and bacillaene (Venugopalan and Srivastava, 2015). The genomic information of strain T4S account for most similar known gene clusters specific for secondary metabolite biosynthesis that involves various biological functions in the host plants. Similar metabolic compounds found in the genome of *B. velezensis* have been reported to enhance plant growth and disease suppression (Sibponkrung et al., 2017). Also, it has been postulated that the genomic similarity of *Bacillus* species could contribute to their interaction mechanisms

based on inherent genes involved in biofilm formation (Teixeira et al., 2020). Information regarding the identification of antibiotic compounds in the genome of *Bacillus* and *Streptomyces* has been documented, thus suggesting their exploration as a source of biocontrol agents (Albarano et al., 2020).

Interestingly, the genome of *B. cereus* T4S contains six important genes coding for siderophore, terpene, betalactone, and non-ribosomal peptide (NRPS), such as petrobactin, molybdenum cofactor, fengycin, and bacillibactin, bacitracin, and zwittermicin A, respectively. All these compounds contributed to the biocontrol activity of strain T4S. Siderophore, terpene, betalactone, and NRPS have been implicated in the control of phytopathogens and boosting plant response to environmental stressors (Boncan et al., 2020). Fengycin production by strain T4S stands as potent antifungal compounds that can cause cell membrane lysis and eventually cell death. Here, the result obtained in this study corroborates the findings of Teixeira et al. (2020) who reported NRPS (bacillibactin) from the genome of *B. velezensis* CMRP 4490. Half of the similar known clusters in strain T4S had no similar identity in the database. Hence, the unidentified or yet-to-be described products have created opportunities for future research. Different gene clusters encoding for siderophore, NRPS, and polyketide, also with other novel gene clusters were found in strain T4S. The presence of these metabolite compounds in strain T4S can serve as a source of biocontrol agents for plant protection. However, several findings on the antimicrobial compounds from *B. velezensis* against pathogenic fungi have been documented (Teixeira et al., 2020), but less is known on endophytic bacterium *B. cereus* T4S.

In our view, the aforementioned genes involved in various metabolic pathways is the first report on the genome sequencing of sunflower-associated endophytic strain T4S with novel plant

growth-promoting attributes and biocontrol potential, which suggest their commercialization prospect in South Africa. A series of several genes identified may play an important role in root colonization to enhance plant growth and immunity. To confirm the implication of gene products from strain T4S on plant growth promotion, further research by chemical analytical methods is required. Hence, optimization of strain T4S based on their gene expression for use under greenhouse experiments may contribute to agricultural productivity. The effect of pure culture of strain T4S upon inoculation revealed better yield in inoculated sunflower plants than un-inoculated. This might be due to the viability and adherence of the bacterial to the surface of the seeds. It is important to state here that the sunflower seed bacterized with strain T4S germinated faster compared to the non-inoculated (Figure 8.4). Bacterization of sunflower seeds with strain T4S led to faster and higher growth under greenhouse experiments. Enhanced below and aboveground sunflower parameters can be explained by the suggestion that strain T4S which colonized roots interdependently enhanced root development in the uptake of nutrients from the soil for plant nutrition. In addition, the enhanced sunflower yield in terms of nutrient acquisition from soil could be a consequence of strain T4S adherence to the seeds before planting. Our findings are in agreement with the reports of Soni et al. (2021) and Prashanth and Mathivanan (2010) on cumin and groundnut seeds bacterized with *Paenibacillus polymyxa* HK4 and *B. licheniformis* MML2501, which showed a higher germination rate of bacterized seedlings in comparison to control. Also, soil physicochemical parameters and the ability of bacterial endophytes to secrete phytohormones serve as factors contributing to plant development. The CMC used as a binder help to retain water level on the seed surface as well as the source of nutrients, and their extensive use in food and pharmaceutical industries is known (Chukwuneme et al., 2020). A significant increase in growth parameters of some

inoculated plants with *Bacillus* spp. has been reported (Mayak et al., 2004). Thus, the results obtained from this study corroborate with the findings of Singh et al. (2019) who had earlier documented the increase in shoot and root length of sunflower inoculated with *Rhizobium* SF48. Furthermore, improvement in the yield of sunflower treated with plant growth-promoting bacteria, as reported by different researchers, can ensure sustainable sunflower production (Leff et al., 2017, Majeed et al., 2018). The increase in the yield of sunflower inoculated with strain T4S can be linked to their genetic composition, functionally in the synthesis of phytohormones which modulate bacteria activities in enhancing sunflower yield. Furthermore, a report by Zahra et al. (2020) revealed the effect of sunflower seed inoculation by endophytic actinobacteria and higher sunflower yield (plant length, weight, and flower diameter) than that of non-inoculated sunflower under greenhouse and field experiments. The authors recommended the possible exploration of these bacteria in producing bioinoculants for enhanced sunflower growth, which is the focus of this study.

8.5 Conclusion

From this study, it is evident that *B. cereus* T4S could be projected into agricultural and crop management based on the possession of putative genes annotated to different biological pathways. The presence of these genes can be overviewed from biotechnological perspectives into main functions such as synthesis of growth hormones, volatile organic compounds, and biocontrol agents, metabolism of organic substrates, and solubilization of essential nutrients. Primarily, the abundance of secondary metabolite gene clusters could provide more insights in the search for a new source of antibiotics. Interestingly, the results from this study have provided new information regarding the genomic competence of *B. cereus* T4S associated with sunflower. Furthermore, the previously demonstrated ability of other *B. cereus* strains with

strong affinity in the establishment of plant-bacterial interactions based on notable genes involved in motility, biofilm production, chemotaxis, and attachment to plant root surfaces could make *B. cereus* T4S a suitable candidate in the synthesis of bioinoculants with promising biotechnological application in agriculture for improved sunflower yield.

9.0 CHAPTER NINE

Unveiling the influence of different farming practices on the structural diversity of sunflower microbiome endophytes using 16S rRNA gene amplicon sequencing

Abstract

Identifiable endosphere microbial communities stand promising in formulating bioinoculants suitable as an alternative to chemical fertilizer in organic farming management for improved agricultural productivity. In this study, we investigated bacterial community structure in the sunflower at the flowering stage under different agricultural practices. Qiagen kit was used for extracting DNA from the sunflower roots and stems and sequencing was performed on the Illumina Miseq platform. Sequence analysis was achieved on Nephele, revealing a total of 10 bacterial phyla at each taxonomic level. The dominant bacterial phyla, such as Saccharibacteria, Verrucomicrobia, Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, Bacteroidetes, and Proteobacteria were identified. Acidobacteria and Actinobacteria dominated the roots and Chloroflexi dominated the stem collected from the Itsoseng site, while Bacteroidetes and Proteobacteria dominated the root and stem sourced from the Lichtenburg site, respectively. High bacterial diversity was detected in the sunflower root sourced from the Itsoseng site. An environmental variable that best explains the negative and positive influence on the bacterial diversity with correlation to sampling sites was deduced. Notably, the selection of bacterial functions at level 1 revealed their functional metabolism, organismal systems, cellular processes, and environmental information processing. From the results obtained, the use of urea as an organic amendment to boost crop production can be recommended in organic farming. Also, the bacterial genera identified can be promising in sunflower farming. Hence,

devising a biotechnological means to explore these microbes can help provide more information on their functions in ensuring sustainable plant health and improved crop yield.

Keywords: Endophyte bacteria, farming practices, *Helianthus annuus*, Illumina, sunflower microbiome, 16S rRNA gene

9.1 Introduction

Sunflower is a perennial flower-producing plant of the genus *Helianthus* from the Asteraceae family. *H. annuus* head at the maturity stage bears round flowers and ligules resembling the sun. It grows well in the temperate and tropical regions as food for both humans and livestock, and as well as for ornamental purposes (Adeleke and Babalola, 2020b). The plant is drought-resistant and can survive well during summer, spring and mid-summer. The internal tissue of plants is colonized by microbial domains called endophytic microbes. The endosphere bacterial communities associated with the roots and stems significantly play salient roles in modulating plant growth and soil health for improved crop productivity (Zhang et al., 2020). Until now, the dynamics of microbial assemblage within the host tissues remains fundamental.

Based on the nutritional value of some medicinal plants, its associated bacteria might exhibit a high degree of antimicrobial properties for treating human and plant pathogens (Manganyi et al., 2019). The antibacterial properties of bacterial endophytes colonizing the tissues of *Artemisia nilagirica* (Clarke) Pamp with multidrug resistance potential against clinical pathogens have been reported (Ashitha et al., 2019). Besides, endophytic bacteria have also been employed in agriculture, medical and environmental studies (Santoyo et al., 2016). The application of endophytic resources from *Ginkgo biloba* with multifaceted attributes in controlling phytopathogens for improved plant health is known (Islam et al., 2019).

In recent times, research into the endosphere community is gaining interest in understanding the diverse microbial world and their dynamics within the plants for sustainable crop productivity (Zhang et al., 2020). The ultraviolet radiation, temperature fluctuations, and limited access to soil nutrients are some of the factors influencing the bacterial community aboveground level (Shymanovich and Faeth, 2019). Belowground level, endophytic bacterial

association with plants enhances nutrient absorption tendencies that directly affect the growth of plants (Adeleke et al., 2021).

The dynamics of the bacteria community within the internal tissues and external plant surface during colonization are achieved via different mechanisms (Hashem et al., 2019). The migration of diverse endophytes community between or in the same plant tissues can be linked to many factors, such as plant type, genotype, growth stage, soil condition, geography and climate, anthropogenic disorders, multi-trophic interactions, and natural habitats (Adeleke and Babalola, 2020a). Similarly, long-term agricultural practices have been reported to influence bacterial diversity in organic and nonorganic amended soils (Babin et al., 2019). The high abundance of microbial biomass in sunflowers has suggested their interactions with the plant environment (Leff et al., 2017), but the mode of plant-bacteria interactions and factors determining the host organ-bacterial interactions remain largely understudied. More also, critical elucidation of endosphere biology will enable scientists to determine the endophytic bacterial community in plant organs and their possible exploration for plant growth promotion. In agricultural systems, the understanding of factors suitable for endophytic bacterial assemblage in plants can enable agriculturally important microbial endophytes to undergo various mechanisms for yield enhancement (Santoyo et al., 2016). Remarkably, the understanding of whether agricultural practices, sampling locations, and plant organs cause a shift in the plant bacterial composition and whether plant-bacteria interactions were influenced more positively at the flowering stage is still limited. Currently, research into the sunflower endosphere is less studied, and insights into bacteria interactions with plants and other microbes would ensure their applications in the agroecosystems. For instance, many studies have been conducted in determining bacterial community structure in plants growing in organic and non-

organic soils but without comparing their diversity in plant organs based on agricultural practices, locations, and growth stage (Moronta-Barrios et al., 2018, Kuźniar et al., 2019a, Fadiji et al., 2020a). The influence of plant growth stages on bacterial diversity in plants has been reported to influence the extent of bacteria diversity in the host plants (Xia et al., 2020). Subsequently, agricultural practices, fertilizer application, geographical locations, plant age can cause a shift in the bacterial communities associated with plants (Fadiji et al., 2020b).

Culture-independent techniques have been employed in studying bacterial communities in plants (Bashir et al., 2020, Yuan et al., 2021). Currently, the use of high-throughput sequencing in ecological studies has resulted in large sequence dataset generation for identifying uncultured microbes with more insights in assessing and making information available on the entire microbial communities from environmental samples and their functions (Mashiane et al., 2017, Kunda et al., 2018). Studying bacterial diversity in sunflower at the flowering stage does not limit diversity assessment alone but also promising in bioinoculants formulation with most application in developing eco-friendly agriculture and other downstream applications.

Previous studies on bacterial endophytes from various economic crops, such as rice (*Oryza sativa*) (Moronta-Barrios et al., 2018), soybean (*Glycine max*) (Tokgöz et al., 2020), maize (*Zea mays*) (Fadiji et al., 2020a), wheat (*Triticum aestivum*) (Žiarovská et al., 2020), rose (*Rosa*) (Xia et al., 2020), and oilseed rape (*Brassica napus*) (Etesami and Alikhani, 2016b) are known; nevertheless, little or no documentation is available on endophytic bacteria from sunflower at the flowering stage in Southern Africa. Furthermore, findings have revealed endophytic bacterial diversity in wheat at the early developmental stage with the most dominated genera, *Pseudomonas, Stenotrophomonas,* and *Bacillus* has been reported (Žiarovská et al., 2020).

In South Africa, commercial farming of sunflower cultivation is commonly practiced in the North West Province and the Free States compared to other provinces (Adeleke and Babalola, 2020b). Based on the economic and nutritional values of sunflower, the need to boost its production as a stringent measure in averting malnutrition and food insecurity becomes important (Leff et al., 2017). Significantly, sunflower seeds serve as an important source of income, quality vegetable oil, mineral, and vitamins, and also a key component in livestock feeds (Franco et al., 2018). Evidence has shown the pharmacological potential of medicinal plants and their associated endophytic microbes in treating human diseases (Adeleke and Babalola, 2021b, Bashir et al., 2015, Manganyi et al., 2019). Hence, devising a modern means of studying microbes associated with sunflower can suggest to farmers the alternative and sustainable approach in boosting sunflower production in Southern Africa (Adeleke and Babalola, 2020b). Furthermore, this study aims to investigate the influence of different farming practices and field history on the endophytic bacteria community structure in sunflower plants. Therefore, we present bacterial community structure and functions in the root and stem of the sunflower at the flowering stage using 16S rRNA gene amplicon sequencing.

9.2 Materials and methods

9.2.1 Plant material and sampling strategy

Samples (roots, stem, and soil) from sunflower (PAN 7160 CLP cultivar) at the flowering stage (14 weeks) were collected from Itsoseng (IT) (26°3′20.106″S, 25°56′24.234″E) and Lichtenburg (LT) (26°4′31.266″S, 25°58′44.442″E), North West Province (NWP) of South Africa. The geographical information in NWP has a record of the annual rainfall of 600 mm, low-level area, trees, grassland, and a temperature range from 17°C to 31°C during summer

and from 3°C to 21°C during winter. Based on the farm history, the sowing date was the same in both locations. The roots and stems were collected from healthy sunflower plants while soils were collected below ground level from the two sites (IT and LT). The farm size in IT is 15 hectares, while in LT, the farm size is 3,500 hectares with between 2,000 to 2,300 hectares are used for grazing and 400 to 450 hectares are used for growing sunflower. The size of sampled plot in IT is 5 hectares, while LT is 8 hectares, respectively. A total of 24 samples each for stems and roots (i.e. 48 plants per site) were randomly collected in triplicates from each site. Also, a 50 g sunflower rhizosphere soil from each plant across the sites was collected aseptically. The samples were separately put in sealable bags, placed on ice, and brought to the laboratory for further analysis. The plant roots were dug at 15-20 cm deep and carefully uprooted without any root damage. All the samples were collected in triplicates. The soil debris and other plant materials were removed, air dry, sieved with a 2 mm micro stainless steel mesh sieve, and stored at -20^{o}C. The physical and chemical analyses of sunflower rhizosphere soil were performed using standard analytical methods (Yeomans and Bremner, 1988). The background history of the farmland in Itsoseng showed that the only sunflower is the crop grown, mono-cropping system, and urea application to the soil, while farmland in Lichtenburg showed that, chemical fertilizer (NPK 15:8:4) is applied on the farmland and crops such as soybean, sugar beans, and maize were interchangeably planted on a rotational basis. In both farmlands, water supply is through rainfall and irrigation and the preliminary farming activities involve clearing, tilling, ploughing, and ridging. The same planting time for the sample collected from each site was recorded i.e. 14 weeks old plant.

9.2.2 Sample processing and DNA library preparation

The surface sterilization was performed following the modified method of Shishido et al. (1995), and Hong et al. (2015). Five grams each of the fresh root and stem samples were cut into sizes (2 cm) and labeled appropriately. The samples were washed 3 times with sterile distilled water to remove the residual or adhering soil particles and other surface contaminants and then surface sterilized by immersion in 70% (v/v) ethanol for 1 minute, followed by 2% (v/v) hypochlorite solution for 3 minutes and finally, immersed in 70% (v/v) ethanol for 30 seconds. The samples were thoroughly rinsed 5 times with sterile distilled water to remove the surface sterilization agents. The sterility of the samples was assessed by plating the final rinse of water on Luria Bertani (LB) culture media. Cultured plates were incubated at 28^{o}C, and no bacterial growth was observed on the plates after 8-10 days of incubation.

The surface-sterilized samples were immediately macerated manually in a sterile mortar and pestle to release the endophyte's DNA content in the plant tissue. DNA extraction was achieved from 0.2 g of the disrupted root and stem tissue using a commercial DNA isolation kit (Qiagen DNeasy® Plant Mini Kit; Qiagen, USA), following the quick-start protocol in the manufacturer's guide. The experiment was performed in triplicate and the extracted DNA was stored at -20^{o}C before sequencing. A two-step PCR standard protocol was employed and amplification of the V4 region of bacterial 16S rRNA gene using PCR primers, 515f/806r with a definite barcode on the forward primer was achieved according to the methods described by Caporaso et al. (2012). The PCR was performed in a 30 cycle PCR (5 cycles used on PCR products) using HotStar Taq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, and final elongation step at 72°C for 5 minutes. The success of

the PCR amplification process and band size were checked in 2% agarose gel. PCR products were purified using calibrated Ampure XP beads following the instructions of the manufacturer. The purified PCR product was used to prepare the DNA library, following the DNA library preparation kit protocol.

9.2.3 Sequence analyses and taxa identification

To adequately determine the bacterial community structure, molecular sequencing was performed at MR DNA molecular laboratory (Shallowater, Texas, USA), a next-generation sequencing and bioinformatics service provider, following the standard Illumina methods. The 16S rRNA gene variable V4 region of the amplicon sequence was sequenced on the Illumina Miseq platform (San Diego CA, USA).

Sequence read processing was done using Quantitative Insights Into Microbial Ecology (QIIME 2) 16S pipeline (version 2020.11) (Edgar et al., 2011) implemented on Nephele microbial bioinformatics platform (version 1.8) (https://nephele.niaid.nih.gov/) (Weber et al., 2018). The preprocessing steps included read pair joining using default parameters (% maximum difference of 25, and a minimum overlap of 10), removal of reads with an average Phred score of $\leq$ 20, removal of chimeras using VSEARCH (Rognes et al., 2016), and clustering, using Open Reference Method and SILVA 99 version 132 (Quast et al., 2012). Taxonomy assignments were done against SILVA version 132, with a default sequence similarity threshold of 0.99. The chimeric sequences, such as mitochondria, chloroplast, and singleton reads were removed.

The sequence data were further analyzed on CANOCO version 5 (Microcomputer Power, Ithaca, NY, USA) (ter Braak and Smilauer, 2012) and paleontological statistics software package (PAST) version 3.20 software package, based on which alpha diversity indices were

calculated (Hammer et al., 2001). The diversity indices across sites (Itsoseng and Lichtenburg) were assessed using the Kruskal-Wallis test in PAST software (Hammer et al., 2001). The taxonomic richness was expressed as the OTU number. A rarefaction plot obtained from the Nephele online server was employed to estimate the richness of bacterial species in the samples. The sequence data deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) can be assessed on Bioproject accession number PRJNA673781 and PRJNA673791.

9.2.4 Environmental variables influencing the bacterial diversity and functions

The correlations of environmental variables to the distribution of bacteria across the plant tissues and sites were performed using canonical correspondence analysis (CCA) on the CANOCO software (ter Braak and Smilauer, 2012). Also, the predictive functional annotation of the bacterial endophytes were performed on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and the resulting output was obtained from different levels i.e., 1st, 2nd, and 3rd regarding their functions.

9.2.5 Statistical analysis

The visualization of bacterial abundance at phylum and genus and the predicted relative abundance of the genes representing microbial functions were generated on Shinyheatmap (http://www.heatmapper.ca/expression) (Khomtchouk et al., 2017). For alpha diversity, the selected taxonomic indices, such as Pielou evenness, Simpson, and Shannon were employed for assessing bacterial diversity across the sites, and a comparison of these indices was achieved using the Kruskal-Wallis test on the PAST software version 3.20 (Kemp and Aller, 2004). The principal coordinate analysis (PCoA) plot for beta diversity was achieved by selecting a Bray-

Curtis-based and one-way analysis of similarities (ANOSIM). The PCoA plot based on the Bray-Curtis dissimilarity matrix was performed using CANOCO version 5.0.

9.3 Results

9.3.1 Sunflower rhizosphere soil physical and chemical analysis

The physical and chemical analysis of sunflower rhizosphere soil across the sampling sites (Itsoseng and Lichtenburg) is shown in Table 9.1. The soil pH value of 7.05±0.05 was neutral at site IT (sunflower rhizosphere soil samples from Itsoseng) and low acidity value of 6.36±0.05 at site LT (sunflower rhizosphere soil samples from Lichtenburg). Ca, Mg, and K values obtained were higher at site IT than lower values recorded at site LT with a significant difference.

9.3.2 Information on sequence data analysis

The resulting sequences uploaded on Nephele with varied sequence data information are presented in Table 9.2. The total number of input sequences (TNIS) varied across the sites. The average sequence count (bp) of 108,015 (LS-sunflower stem from Lichtenburg), 89,729 (LR-sunflower root from Lichtenburg), 73,286 (ITR-sunflower root from Itsoseng) and 60,480 (ITS-sunflower stem from Itsoseng) and the sequence read count of LR−2,743, LS−3,448, ITR−2,519 and ITS− 1,833 after quality control were recorded across the sampling sites. The rarefaction curve from Nephele showed the level of richness of endophytic bacteria in the samples.

Table 9.1: Physical and chemical soil analysis

Site	LT	IT
Organic matter (%)	1.36±0.05^{c}	2.02±0.03^{c}
Nitrate (N-NO_3) (mg/kg)	6.44±0.04^{e}	14.15±0.06^{h}
Ammonium (NH_4) (mg/kg)	5.08±0.08^{d}	6.04±0.05^{e}
pH	6.36±0.05^{e}	7.05±0.05^{g}
Resistivity conductivity (ohm)	2170.03±0.03^{m}	1045.03±0.02^{o}
Phosphorus (P) (mg/kg)	26.40±0.02^{h}	74.93±0.05^{j}
Calcium (Ca) (mg/kg)	424.02±0.17^{l}	973.02±0.02^{n}
Magnesium (Mg) (mg/kg)	122.06±0.28^{k}	187.02±0.02^{m}
Potassium (K) (mg/kg)	113.01±0.02^{j}	181.03±0.03^{l}
Sodium (Na) (mg/kg)	7.17±0.03^{f}	6.51±0.01^{f}
Total carbon (%)	0.42±0.03^{b}	0.63±0.04^{b}
Total nitrogen (%)	0.04±0.01^{a}	0.06±0.01^{a}
Sand (%)	77.02±0.02^{i}	77.03±0.03^{k}
Silt (%)	5.01±0.01^{d}	3.01±0.02^{d}
Clay (%)	18.02±0.02^{g}	20.01±0.02^{i}

Key: LT = sunflower soil samples from Lichtenburg, IT = sunflower soil samples from Itsoseng. Mean±standard deviation having different letters of triplicate determinations are significantly different at $P \leq 0.05$ according to DMRT

Table 9.2: Quality filter results of 16S rRNA gene sequencing

Sample information	TNS	SRAQC	CCEL	TNSAQC
ITR	73,286	2,519	4	70,763
ITS	60,480	1,833	2	58,654
LR	89,729	2,743	0	86,985
LS	108,015	3,488	2	104,525

Key: LR – root samples from Lichtenburg, ITR = root samples from Itsoseng, LS - stem samples from Lichtenburg, ITS - stem samples from Itsoseng, TNIS - Total number sequences, SRAQC – sequence read after quality control, CCEL – count of N characters exceeds the limit, MSL - median sequence length, TNSW - total number of sequence after quality control

The number of reads generated for each sample that describes the bacterial community structure is presented by the rarefaction curve and based on the saturation level at 97% operation taxonomic unit (OTU), and the maximum bacterial richness was found to be higher in LS as represented in purple color (Figure 9.1).

9.3.3 Endophytic bacteria distribution in the root and stem of sunflower at the phylum level

At the phylum level, a total of 10 most dominant bacteria phyla were observed and selected from the sequenced samples (Figure 9.2). The row Z-score from negative to a positive value (-1 to +1) indicates a low to a high abundance of bacteria in each sample. The roots (ITR) were predominated with Acidobacteria, Actinobacteria, Saccharobacteria, and Verrucomicrobia while only Bacteroidetes were found dominant in LR. Similarly, Chloroflexi and Firmicutes were dominant in ITS compared to LS with low abundance. The relative abundance of Proteobacteria (LR-89.20% and ITR-77.79%) in the roots across the sites were more abundant than Proteobacteria (ITS-69.19% and LS-66.37%) in the sunflower stem. At the genus level, *Flavobacterium, Pseudomonas,* and *Rheinheimera* dominated the ITS and LS, while *Acidovorax, Burkholderia, Chlorochromatium, Comamonas, Chryseobacterium, Stenotrophomonas,* and *Streptomyces* dominated ITR and LR, respectively (Figure 9.3).

Based on the significant difference ($p < 0.05$) observed in the bacterial composition within the sites, phylum was selected for the PCA plot (Table 9.3). A variance of 88.4% in Axis 1 and 9.9% in Axis 2, respectively were obtained (Figure 9.4). The most predominant bacterial phyla in each sample were directionally shown via a vector pointer with the most extended length.

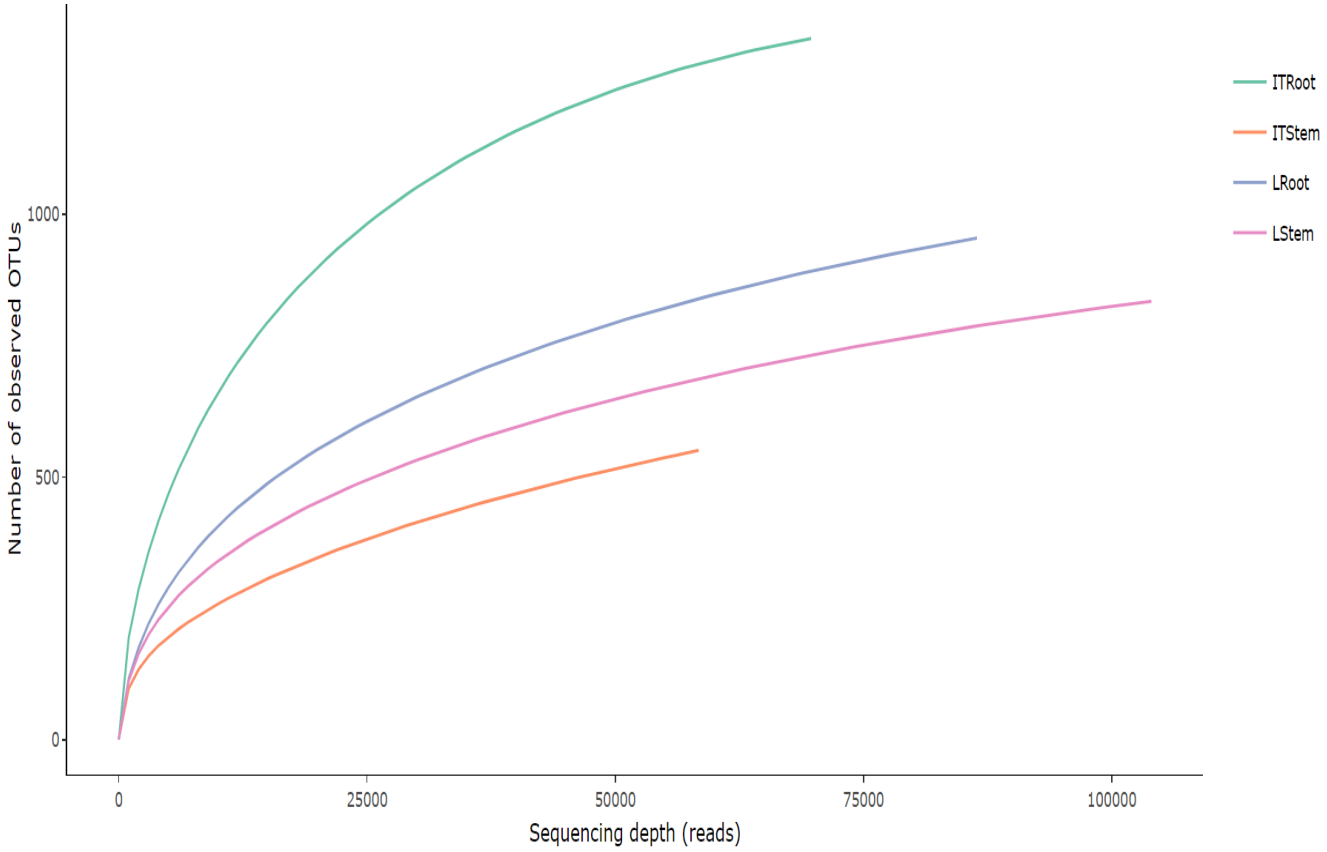


Figure 9.1: Rarefaction curves of evaluated richness in the root and stem of a sunflower. The vertical axis represents the number of OTUs estimated after quality control at 97%, while the number of sequence reads is represented on the horizontal axis

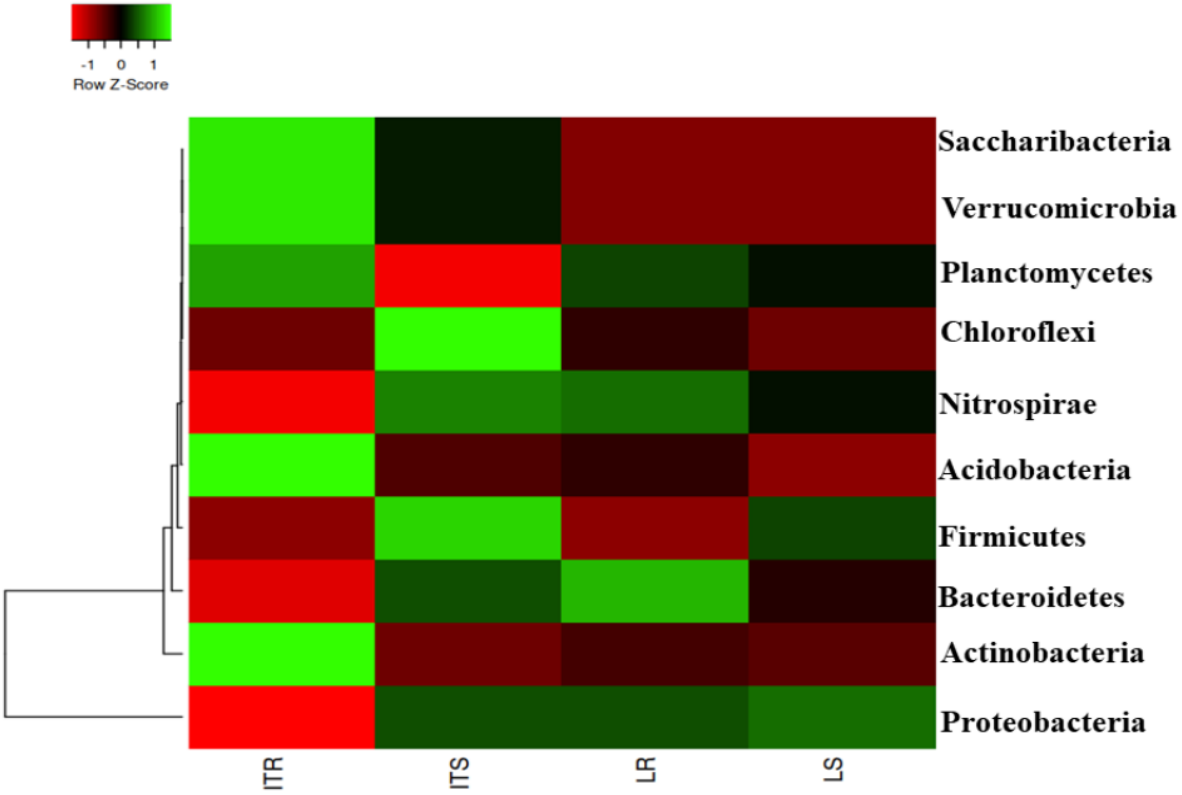


Figure 9.2: Taxonomic classification of bacterial phyla from sunflower roots and stem at flowering stage. The row Z-score from negative to a positive value (-1 to +1) indicates a low to a high abundance of bacteria in each sample. Key: LR – root samples from Lichtenburg, ITR = root samples from Itsoseng, LS - stem samples from Lichtenburg, ITS - stem samples from Itsoseng

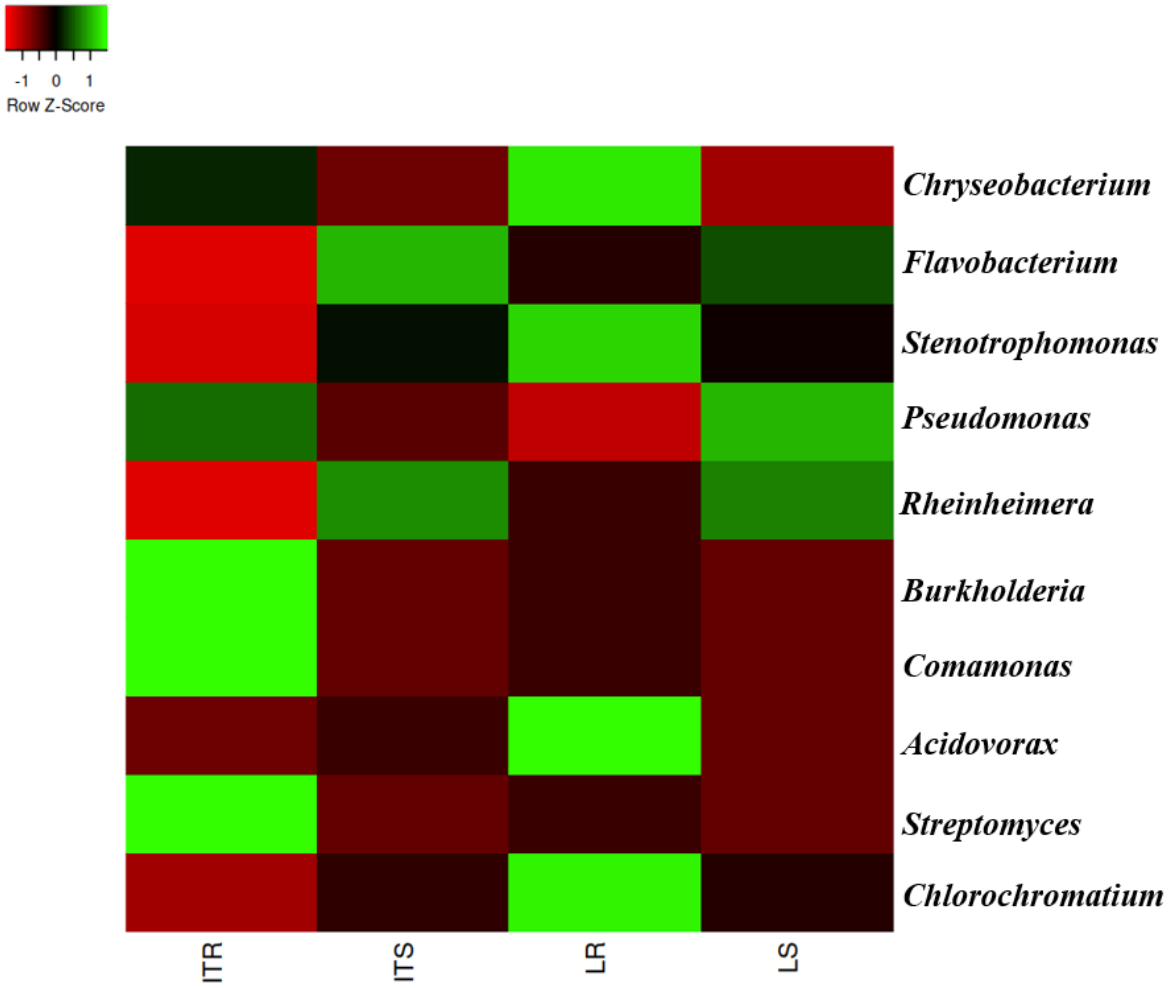


Figure 9.3: Mean relative abundance of the bacterial genus in sunflower at the flowering stage. The row Z-score from negative to a positive value (-1 to +1) indicates a low to a high abundance of bacteria in each sample. BR-sunflower root from Lichtenburg, BS-sunflower stem from Lichtenburg, NR-sunflower root from Itsoseng, and NS-sunflower stem from Itsoseng

Table 9.3: Alpha diversity of endophytic bacteria in the root and stem of sunflower at the flowering stage

Bacterial level	**Indices**	**LR**	**LS**	**ITR**	**ITS**	***p*-value**
Phylum	Evenness_e^H/S	0.20	0.22	0.25	0.18	0.0001
	Simpson_1-D	0.20	0.18	0.36	0.20	
	Shannon_H	0.44	0.42	0.68	1.47	
Genus	Evenness_e^H/S	0.45	0.24	0.52	0.23	0.1343
	Simpson_1-D	0.64	0.36	0.75	0.34	
	Shannon_H	1.51	0.86	1.65	0.85	
	OTU observed	86,391	103,980	69,726	58,391	
	Coverage	0.97	0.97	0.97	0.97	

P values were obtained through the Kruskal-Wallis test. LR – root samples from Lichtenburg, ITR = root samples from Itsoseng, LS - stem samples from Lichtenburg, ITS - stem samples from Itsoseng

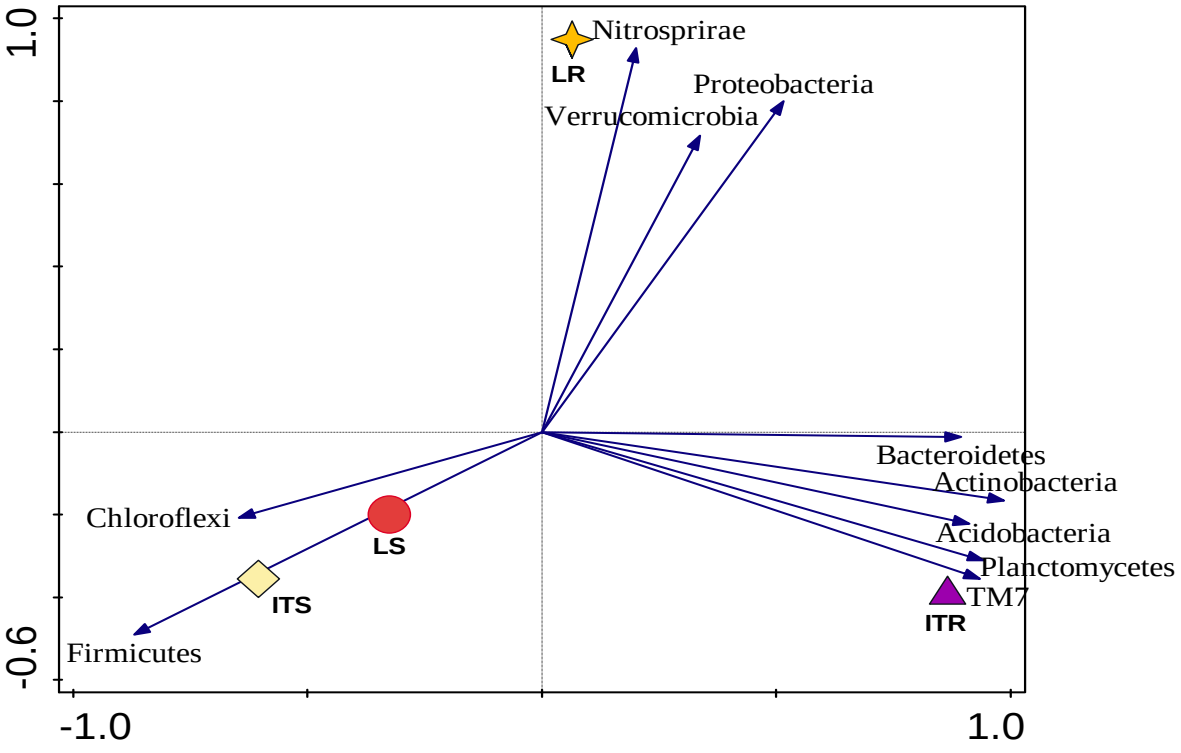


Figure 9.4: PCA plot. The strength of the bacteria taxa as indicated with the length of the vector showed a variance of 88.4% in Axis 1 and 9.9% in Axis 2. Key: LR – root samples from Lichtenburg, ITR = root samples from Itsoseng, LS - stem samples from Lichtenburg, ITS - stem samples from Itsoseng

Based on this, the predominance of Nitrospirae, Proteobacteria, and Verrucomicrobia in LR, Bacteriodetes, TM7, Acidobacteria, Actinobacteria, and Planctomycetes in ITR, Firmicutes, and Chloroflexi in LS and ITS, respectively were presented in Figure 9.4.

9.3.4 Estimate of bacterial diversity in the root and stem of sunflower at the flowering stage

The alpha diversity estimate of endosphere bacterial communities depicted by Shannon index, Simpson, and Evenness is shown in Table 9.3. Also, the Chao 1 and Shannon species diversity indices were presented in Figure 9.5. The Shannon index, Simpson, and Evenness indices at the taxonomic level were significantly different ($p < 0.05$) at the phylum level and with no significant difference ($p > 0.05$) observed at the genus level (Table 9.3). The OTU observed from each sample and the coverage levels are presented in Table 9.3. The PCoA plot for the comparison of distinct bacterial communities in each site using Bray-Curtis distance is shown in Figure 9.6. The *p*-value of 0.01 and variance (R) value 0.5853) obtained using analysis of similarities (ANOSIM) PAST version 3.2 software showed a significant difference in the samples across the sites. The distance observed in ITS to LS and LR to LS showed the uniqueness of bacterial communities in each site. However, LS and ITS were closer together compared to LR and ITR, thus indicating similarities in the bacterial communities in the samples, despite differences across the sites. Bacterial community structure based on distance measurement between ITS and ITR, and likewise LR and LS were significantly different ($p < 0.05$) (Figure 9.6). Also, compare with the PCoA plot, the weighted UniFrac dissimilarity matrix shows a significant difference ($p < 0.05$) in the bacterial diversity in the root across the site, while LS and ITS were closer compared to LR and ITR (Figure 9.7a).

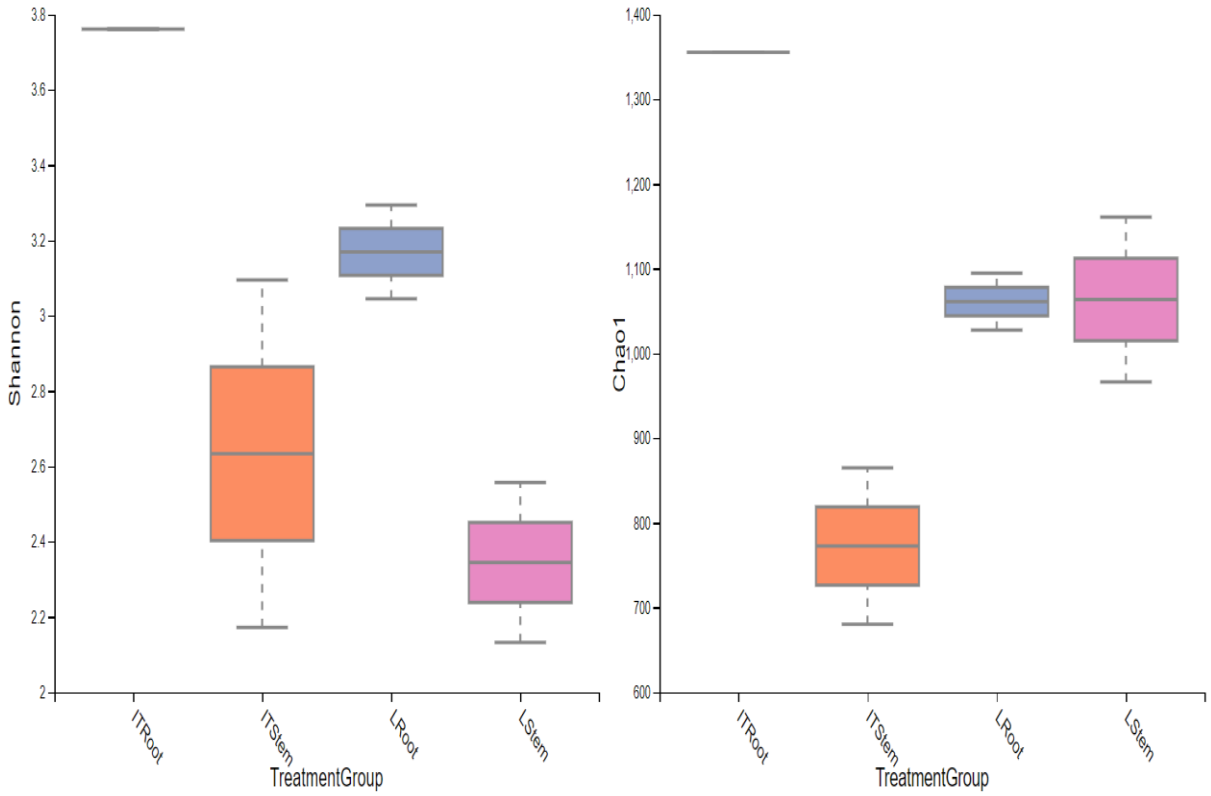


Figure 9.5: Chao 1 and Shannon species diversity

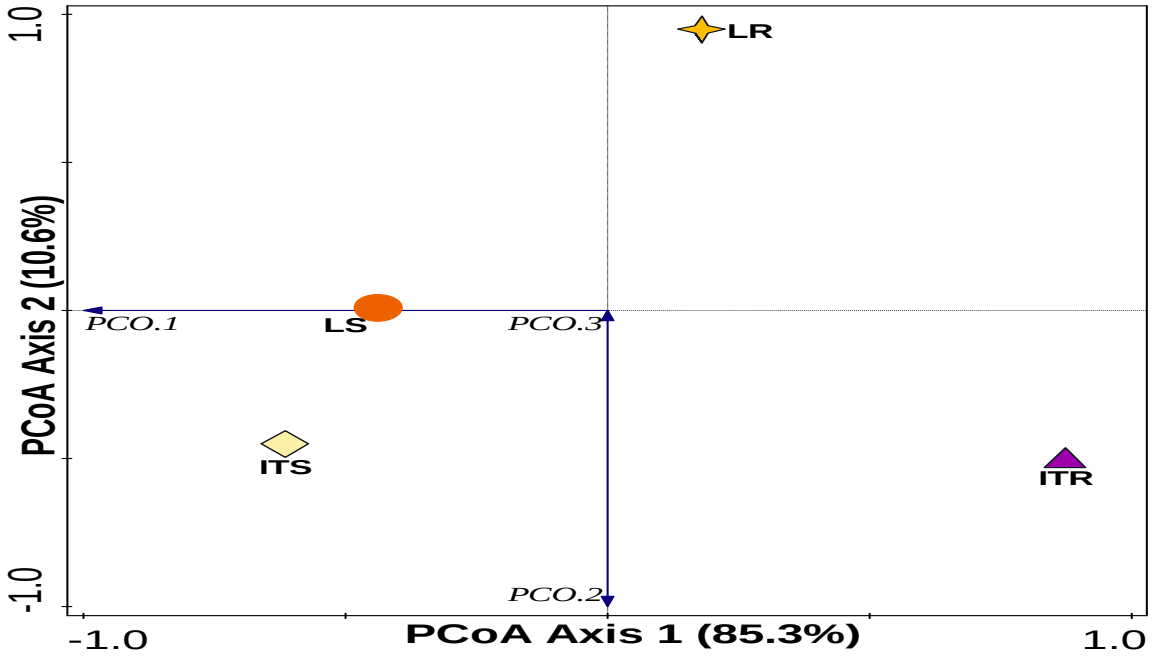


Figure 9.6: PCoA plot of bacterial phyla. Key: LR – root samples from Lichtenburg, ITR = root samples from Itsoseng, LS - stem samples from Lichtenburg, ITS - stem samples from Itsoseng

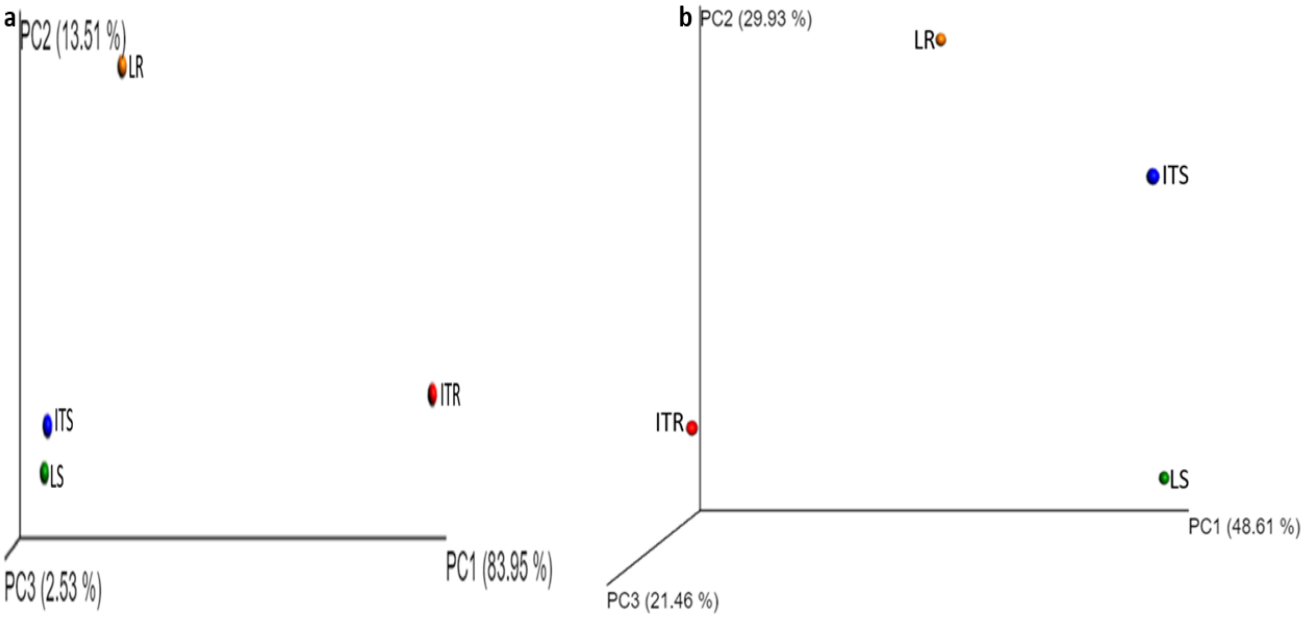


Figure 9.7: UniFrac dissimilarity matrix. Key: **a** – weighted, **b** – unweighted, LR – root samples from Lichtenburg, ITR = root samples from Itsoseng, LS - stem samples from Lichtenburg, ITS - stem samples from Itsoseng

Similarly, the unweighted UniFrac dissimilarity matrix shows a significant difference ($p < 0.05$) in the bacterial diversity across the sites with PC1 (48.61%), PC2 (29.93%), and PC3 (21.46%), respectively (Figure 9.7b).

9.3.5 Environmental variables determining bacterial diversity

The CCA plot showing the correlation based on constrained axes tests and unrestricted permutations of the soil physical and chemical properties is presented in Table 9.1 and bacterial distribution at the phylum level in Figure 9.8. The likelihood dependence of bacterial community structure on the environmental variables as shown in the CCA plot was achieved at permutation test 0.02. The influence of environmental variables tested was measured on the bacterial distribution across the sites. Acidobacteria, Actinobacteria, TM7, and Verrucomicrobia exhibited a strong positive correlation with sodium (Na). Also, Planctomycetes were observed to respond positively to pH, potassium (K), and calcium (Ca). Chloroflexi, Firmicutes, and Proteobacteria negatively correlated with sand, silt, and resistivity, respectively. Also, Nitrospirae and Bacteroidetes exhibited a positive correlation with ammonia ($N\text{-}NH_3$), magnesium (Mg), organic matter (OM), clay, potassium (P), total carbon (C), total nitrogen (N), and nitrate ($N\text{-}NO_3$). It was observed that Na, sand, K, pH, and Ca best explain the environmental factors influencing the bacterial composition across the sites with profound influence based on the vector pointer with the most extended length.

9.3.6 Predictive functional analysis on PICRUSt

The predictive functions of endophytic bacterial across the sites were analyzed using PICRUSt (Figure 9.9). The functional information at different levels was identified by plotting on the Shinnyheatmap based on the top 131 selected abundance functions.

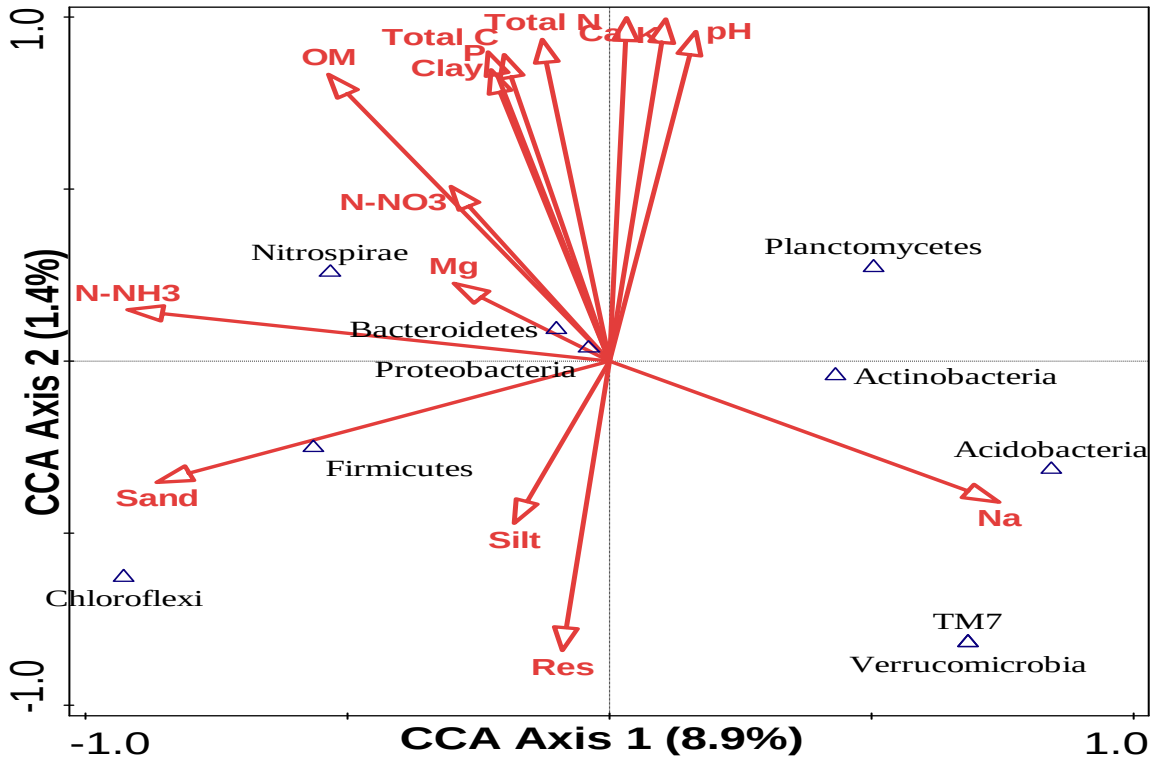


Figure 9.8: CCA plot

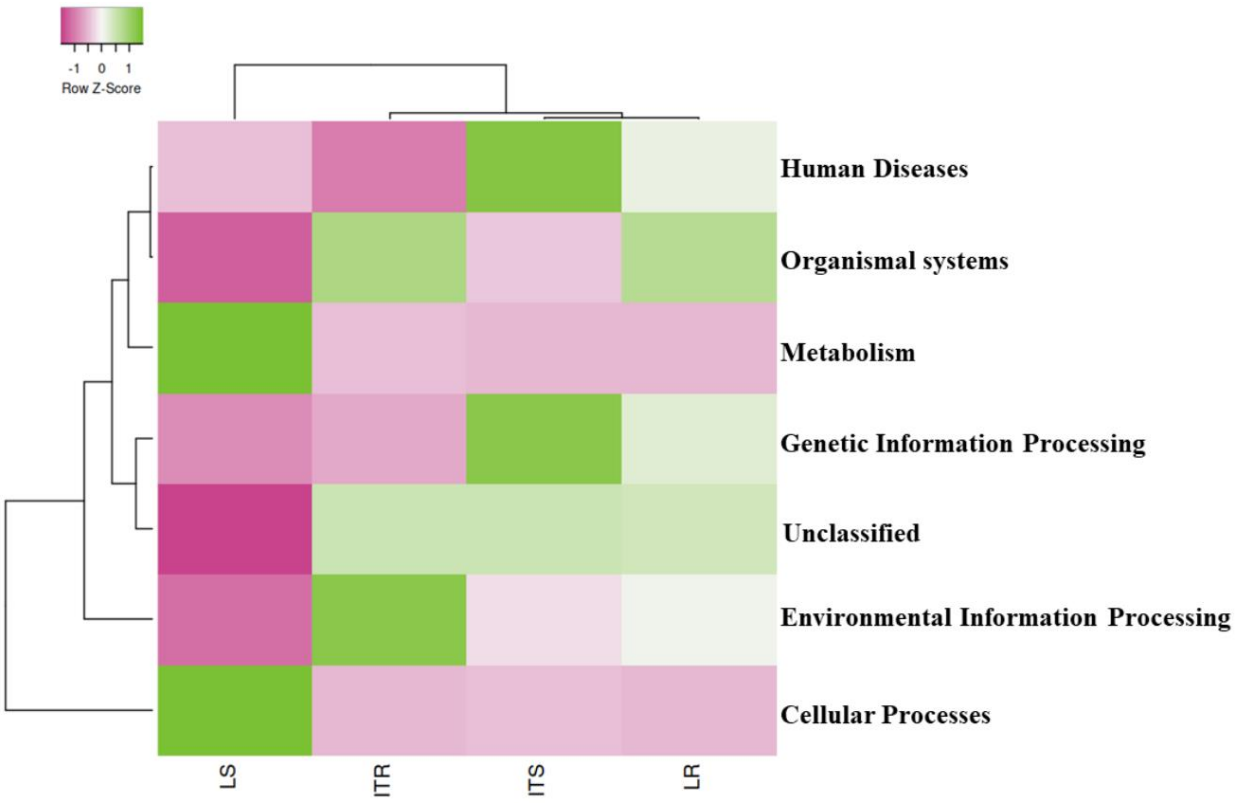


Figure 9.9: PICRUSt analysis of the bacterial endophyte in sunflower root endosphere across the site. A clustered heatmap was generated from the top 131 functional abundance information from each site

The different level of bacterial functions from each level is presented in Table 9.4. At level 1 functional selection, the bacterial in each site possesses varied cellular metabolism, organismal systems, human diseases, cellular processes, and environmental information processing. The varied abundance functions of the bacterial endophytes across the sites (Itsoseng and Lichtenburg) might suggest their impact on sunflower growth, thus modulating endosphere competence.

Table 9.4: Predicted amplicon metagenome functions

Predicted metabolism (1st level)	**Activity (2nd level)**	**Function (3rd level)**
Cellular Processes	Cell motility	Bacterial chemotaxis
		Bacterial motility protein
Environmental information processing	Membrane transport	ABC transport
		Bacterial secretion system
Genetic information processing	Sorting and degradation	Sulfur relay system
Metabolism	Amino acid	Alanine, aspertate and glutamate
		Lysine biosynthesis
		Valine, leucine, and isoleucine biosynthesis
Metabolism	Secondary metabolites	Betalain biosynthesis
		Indole alkaloid biosynthesis
Metabolism	Carbohydrate	Amino acid and sugar metabolism
		Citrate cycle
		Fructose and mannose metabolism
Metabolism	Energy metabolism	Methane metabolism
		Nitrogen metabolism
Metabolism	Metabolism of terpenoids and polyketides	Biosynthesis of siderophore group nonribosomal peptides
		Biosynthesis of polyketide products
		Zeatin (cytokinin) biosynthesis
Metabolism	Enzyme families	Peptidases
		Protein kinases

9.4 Discussion

Sunflower is one of the oilseed crops cultivated under different agricultural practices, especially in South Africa. Due to the economic importance of sunflower as a source of oil foods and medicine, employing a modern approach in their cultivation will help avert future food insecurity and in treating diseases affecting humans (Adeleke and Babalola, 2020b). Owing to the environmental threats associated with the use of chemical fertilizer in maximizing crop production, there is a need to devise affordable and biorational means to boost sunflower production and to ensure sustainable agriculture using organic fertilizer (Fadiji et al., 2020a). Agricultural practice involving the use of biochar, bioinoculants, and compost has been the major focus in recent studies, due to its less toxicity to the ecosystem in enriching the soil and for plant growth (Yu et al., 2019). Evidence has shown that growing plants on organic and non-organic amended soil influence the structural diversity, and functions of root-associated bacteria with a promising link in enhancing plant growth, and boosting plant resistance to environmental stressors (Fadiji et al., 2020a). In this study, the influence of different farming practices on the structural diversity of endophytic bacteria colonizing the root and stem of sunflower cultivated in urea and chemical fertilizer amended soil using next-generation sequencing was assessed. From a previous finding, the use of 16S rRNA in analyzing endosphere bacterial communities in sunflower has been reported (Avila et al., 2020). Nevertheless, studies on sunflower-associated bacterial endophytes using 16S rRNA amplicon-based sequencing in Southern Africa are less documented, thus necessitating this study. Based on the authors' perception, this is the first research performed on the endophytic bacterial community associated with the roots and stems of sunflower at the flowering stage in South Africa under different agricultural practices.

The analysis of the sequence datasets was performed on Nephele. The specific data for endophytic bacteria were analyzed, while a possible sequence dataset from sunflower DNA was discarded, which was achieved using a locked nucleic acid polymerase chain reaction (LNA-PCR) during the plant DNA amplification process (Puri et al., 2019). In this study, samples collected from organic amended and nonorganic amended soil revealed diverse endophytic bacterial communities in the roots and stems of sunflower at the flowering stage on farmlands. The impact of different agricultural practices was profound in determining the abundance and type of microbes present across the sites. The results obtained from this study revealed a high abundance of endophytic bacteria in the sunflower root cultivated in urea amended soil than fertilizer treated soil and this might be due to the mono-cropping system approach employed in Itsoseng farm and the less toxic effect of urea application on soil and root-associated microbial communities. Interestingly, the comparative analysis of endophytic bacterial community structure in the root and stem of sunflower at the flowering stage across different sites under different agricultural practices is rare, hence, few findings on sunflower bacterial community shall be employed for appropriate referencing in this study.

At the phylum level, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Nitrospirae, Planctomycetes, Proteobacteria, Saccharobacteria, and Verrucomicrobia, were identified in the root and stem of sunflower at the flowering stage. However, Acidobacteria, Actinobacteria, Bacteroidetes, Saccharobacteria, and Verrucomicrobia, dominated sunflower root, while Chloroflexi and Firmicutes dominated the sunflower stem across the sites (Itsoseng and Lichtenburg). Most bacteria phyla identified in this study have been reported in the sunflower cultivated under different cropping systems (Wen et al., 2016).

The high abundance of the bacterial community in the roots than in stems across the sites can be linked to the site location, agricultural practice, plant growth stage, and origin of root endophytes from the rhizosphere (Comby et al., 2016). Based on farm history across the sites on the use of chemical fertilizer (NPK 15:8:4) in Lichtenburg farm and urea in Itsoseng, this might influence the diversity of bacterial community structure in the plant organs. Plant types, soil conditions, and exudates from plant roots are known factors influencing the shape of the bacterial community in the endo-rhizosphere below ground level (Eisenhauer et al., 2017). Identification of endophytic bacteria phyla, such as Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria, Tenericutes, Acidobacteria, and Bacteroidetes have been reported in the stem and roots of many plants (Xia et al., 2020, Purushotham et al., 2020). Furthermore, the influence of cropping systems on the bacterial diversity in sunflower which accounts for 36.18% of Proteobacteria as the most dominant bacterial phyla compared to the wheat and maize plants have been reported (Wen et al., 2016). Furthermore, the results obtained in this study corroborate with the findings of Leff et al. (2017), who reported a significant difference in the microbial communities across sunflower compartments with multiple functions, hence standing promising as a key indicator to soil health and plant growth promotion.

At the genus level, *Burkholderia*, *Comamonas*, *Stenotrophomonas*, *Acidovorax*, *Chlorochromatium,* and *Streptomyces* were dominant in the roots, while *Flavobacterium*, *Pseudomonas,* and *Bacillus* were dominant in the stem across the sites. The dominance of bacterial genera in the roots and stem can suggest their biotechnological functions in plant improvement and endosphere biology (Lin et al., 2020). In our view, the use of culture-independent techniques in identifying endophytic bacteria genera, *Comamonas*, *Stenotrophomonas*, *Chlorochromatium,* and *Bacillus* from sunflower at the flowering stage is

novel, as such reports have not been documented before. The differences observed in bacterial genera in the root and stem of sunflower at the flowering stage suggest plant organs as a source of endophytic bacteria and their bioprospecting in agriculture have caused an improvement in crop yield through phytohormone synthesis, phosphate solubilization, nitrogen fixation, siderophore, and antibiotic production (Pourbabaee et al., 2018). The effect of endophytic bacteria, *Flavobacterium, Burkholderia, Streptomyces,* and *Pseudomonas* as a source of bioinoculants in improving crop productivity and as a source of biocontrol agent against phytopathogens have been reported (Liotti et al., 2018).

The observed similarity in the endophytic bacterial community structure in the roots and stems can be due to spatial distribution, organ location, and environmental factors. Müller et al. (2016) have reported differences in the bacteria identified from the plant endo-rhizosphere and their bioprospecting in agriculture. Similar to this, Maropola et al. (2015) reported agriculturally important endophytic *Bacillus, Stenotrophomonas, Erwinia, Pseudomonas, Herbaspirillum,* and *Paenibacillus* from sorghum. The predominance of endophytic bacteria communities in the root samples (ITR and LR) across the sites compared to the stem samples (ITS and LS), may suggest their multifunctional attributes in enhancing crop yield, alleviating stress in plants, and plant survival under limiting soil nutrients through their biotransformation activities, and bioavailability of bound soil nutrients for plant use (Jiang et al., 2019). The high abundance of endophytic microbes in the root endosphere of many plants has been identified using 16S rRNA amplicon gene sequencing (Unno et al., 2015, Imchen et al., 2019).

Based on the significant difference ($p < 0.05$) observed in the bacterial diversity in the plant organs across the sites at 88.4% variance (Table 9.2), endophytic bacteria at the phylum level were selected for the PCA plot (Figure 9.4). The sequence composition of each bacterial phyla

and their distribution across the sites revealed their position using vector arrows as a point indicator and this has assisted in figuring out the most abundant bacterial genera in the samples across the sites. Studying the bacterial community structures in the sunflower root and the stem can selectively reveal the difference in their diversity across the sites and recruitment dynamics in econiches (Xia et al., 2020). In this study, it was observed that bacterial phyla were found dominant in root samples sourced from Itsoseng (ITR) and Lichtenburg (LR) compared to stem samples from the two sites (Figure 9.4). The high abundance of endophytic bacteria in the plant roots from Itsoseng revealed their ability to survive and interact with plants below ground level, and this corroborates earlier findings reported by Wang et al. (2016b) on the dominance of bacteria in the plant roots. The endophytic bacteria phyla identified in this study have been reported to play an important role in plant growth promotion and soil health in organic and nonorganic amended soil for improved crop productivity (Fadiji et al., 2020b, Lin et al., 2020, Liu et al., 2020b). Based on our observations, the difference in the bacterial abundance across the sites (Itsoseng and Lichtenburg) suggest that the minimal urea application to soil can serve as an alternative to mitigate chemical fertilizer use with a promising outlook in the organic farming system to sustain plant health, reduce disease re-insurgence on farm crops, and contribute to soil health (Moronta-Barrios et al., 2018). The use of urea as an organic amendment and mono-cropping system may underline high bacterial diversity in the ITR site with promising attributes in enhancing soil quality, increasing nitrogen bioavailability in the soil, and contributing maximally for improved crop yields (Chen et al., 2020). Nevertheless, the disadvantageous effects of urea application to the soil cannot be overemphasized, hence, its long-term effects on crop yields can be controlled (Geng et al., 2015).

The low abundance of the bacterial community in the stem region of the plants from the two sites (Itsoseng and Lichtenburg) might be due to bacterial survival and movement within the plant tissue, organ location, tissue type, different environmental factors, limited nutrient supply, and competition (Maropola et al., 2015). Furthermore, bacterial endophytes aboveground level might possess unique composition, but with less diversity (Chen et al., 2016). Hence, to support our assertion in this study, reports on the higher bacterial communities in the roots of oilseed crops, such as soybean, sunflower, rapeseed, and canola than the stem have been documented in several studies (Leff et al., 2017, Glaeser et al., 2020, Cordero Elvia et al., 2021, Sohn et al., 2021).

Evenness, Simpson, and Shannon indices estimated at phylum level for endophytic bacteria showed a significant difference ($p < 0.05$) in the root and stem across the sites. Based on Chao 1 and Shannon species diversity, the bacterial diversity in the ITR site was significant compared to other samples across the sites (Figure 9.5). In this study, the results obtained showed a lower diversity of endophytic bacteria in the roots from Itsoseng at the phylum level than the genus level and this might be due to the abundance of bacterial endophytes at the genus level than the phylum level. Here, the results obtained support the hypothesis on the influence of crop rotation, soil amendment, plant developmental age, plant organs, and sampling location on the diversity of bacterial communities (Fadiji et al., 2021b). Interestingly, endophytic bacteria were found to be more dominant in the root of sunflower sourced from Itsoseng (ITR) than in other samples sourced from the two sites (Table 9.3). The results obtained, however, validate the positive influence of urea on the abundance and diversity of endophytic as shown in this study. The comparison of distinct bacterial communities in the root and stem across the sites is presented by PCoA and PC UniFrac dissimilarity matrix plots (Figures 9.6, 9.7a, and 9.7b).

The weighted UniFrac dissimilarity matrix shows a significant difference ($p < 0.05$) in the bacterial diversity in the root across the site, while LS and ITS were closer compared to LR and ITR (Figure 9.7a). Similarly, the unweighted UniFrac dissimilarity matrix shows a significant difference ($p < 0.05$) in the bacterial diversity across the sites, which further validates the abundance of endophytic bacteria in the root of sunflower compared to the stem across the sites (ltsoseng and Lichtenburg). Organic farming practices have been reported to influence the microbial community structure within the plant endosphere; and based on the farm history from the sampling sites, water use, crop rotation, and soil amendment can affect the bacterial interaction with the plant root and infiltration from the root to the stem (Fadiji et al., 2020b). Notably, water supply stands as a major factor needed for microbial growth and limited water supply can cause cell death. Also, the application of urea and fertilizer to the soil has been reported to influence bacterial diversity in the endo-rhizosphere region. In addition, tilling, ridging, ploughing, pesticide use and crop rotation have been recognized to influence microbial diversity in soil and plants (Fadiji et al., 2020a).

In this study, the physical and chemical composition of sunflower rhizosphere soil and their correlation with bacterial diversity in the root and stem of sunflower at the flowering stage across the sites were assessed. Soil parameters have been reported to influence bacterial community structure in the soil and plant roots (Shymanovich and Faeth, 2019). Furthermore, the influence of pH and other soil nutrients on the biodiversity pattern and function of bacterial in the soil environment have been reported (Amoo and Babalola, 2019). The predictive functions of bacterial communities across the sites revealed their involvement in genetic information processing, cellular biological processes, plant metabolism, and environmental processing information. The differences in the bacterial function across the sites can be linked

to their genetic makeup, source of isolation, and plant type (Hong et al., 2019). The specific genes involved in the metabolism of amino acid, terpenoids, and polyketides, carbohydrate, energy, biosynthesis of secondary, metabolites and enzyme families; cellular processes (cell motility), and genetic information processing (sorting and degradation) were more profound in stem samples (ITS and LS), while the environmental information processing (membrane transport) was expressed dominant bacteria in the ITR samples. The ability of these bacteria to degrade complex organic compounds in the soil can contribute to nutrient bioavailability in the soil for plant growth promotion (Jambon et al., 2018). Production of certain metabolites, such as terpenoids and polyketide has been attributed to the Actinobacteria and Acidobacteria inhabiting the soil based on the abundant genes involved in the metabolism of terpene and polyketide associated with the bacterial phyla (Crits-Christoph et al., 2018, van Bergeijk et al., 2020, Zhang et al., 2021). The predicted functional analysis of endophytic bacteria associated with sunflower root and stem has not been documented in any study, however, bacterial endophytes associated with flowering and nonflowering moso bamboo (*Phyllostachys edulis*) have been reported to exhibit similar functions (Yuan et al., 2021) as obtained in this study.

9.5 Conclusion

The authors presented the influence of different farming practices on the structural diversity of bacterial communities associated with the roots and stems of sunflower at the flowering stage using the next-generation sequencing approach. In our view, this is the first documentation on the use of 16S rRNA gene amplicon sequencing in assessing bacterial community structure associated with a sunflower at the flowering stage and their predicted functions across the sites under different farming systems in Southern Africa. We suggested that the application of urea as an organic amendment and crop rotational system can be adopted in growing sunflower as

the interdependence of these bacteria with the host plants may underline their function in ensuring a stable ecosystem. The identification of bacteria genera in the sunflower endosphere can provide a platform for further studies in isolating them for plant inoculation under greenhouse and field experiment trials, hence suggesting the exploration of their bioproducts in developing eco-friendly agriculture sustainably.

The biotechnological importance of endophytic bacteria has been reported, nevertheless, more insights into their mechanisms of can further reveal their potential in sustainable plant health. Also, further studies on the comparative studies of sunflower endosphere and rhizosphere bacteria will provide several opportunities in determining the potential of endo-rhizosphere microbes in enhancing plant growth and sustaining soil health. Furthermore, the predicted functions of these bacteria can suggest their biotechnological potential with a multifaceted outlook, for future exploration in formulating bioinoculants to mitigate agricultural problems arising from the use of agrochemicals.

10.0 CHAPTER TEN

Bacterial community structure of the sunflower (*Helianthus annuus*) endosphere

Abstract

Agrochemical applications on farmland aim to enhance crop yield, however, the consequence of biodiversity loss has caused a reduction in ecological functions. The positive endosphere interactions and crop rotation systems may function in restoring a stable ecosystem. Employing culture-independent techniques will help to access the total bacteria community in the sunflower endosphere. Limited information is available on the bacteria diversity in sunflowers cultivated under different agricultural practices. Hence, this study was designed to investigate the endophytic bacterial community structure of sunflower at the growing stage. Plant root and stem samples were sourced from two locations (Itsoseng and Lichtenburg), for DNA extraction and sequenced on the Illumina Miseq platform. Saccharibacteria and Acidobacteria were dominant in plant roots, while the stem is dominated with Proteobacteria, Bacteriodetes, and Gemmatimonadetes across the sites. Bacterial genera, *Acidovorax, Burkholderia-Paraburkhoderia, Flavobacterium,* and *Hydrogenophaga* were found dominant in the root while the stem is dominated by *Streptomyces*. The diverse bacterial community structure at phyla and class levels were significantly different in plant organs across the sites (Itsoseng and Lichtenburg). The influence of soil physical and chemical parameters analyzed was observed to induce bacterial distribution across the sites. This study provides novel information on the dominant bacteria community structure in sunflowers at the growing stage and their predictive functions, thus suggesting their future exploration in agricultural sustainability. Hence, devising culture-dependent techniques in the characterization of identifiable bacteria in this

study would create greater opportunities in exploring their novel attributes as bioinoculants for improved agricultural yields.

Keywords: Agricultural sustainability, bacterial diversity, culture-independent techniques, amplicon sequencing

10.1 Introduction

The understanding of the spatial distribution of bacteria communities in plant organs has provided various opportunities in harnessing their potential in agricultural systems (Afzal et al., 2019, Pathania et al., 2020). Diverse microbial communities colonize plant organs to maximally induce adaptive responses in the plant through various metabolic pathways (Kim et al., 2017; Fitzpatrick et al., 2018). Characterization of bacterial endophytes from various environmental samples is known and widely adopted in environmental studies (Puri et al., 2018), although, most microbes have not been cultured and isolated. More significantly, insights into microbial biotechnology have unveiled the use of amplicon metagenome sequencing in microbiological studies (Unno et al., 2015). Furthermore, the use of 16S rRNA gene sequencing in evaluating bacterial community structure in the sunflower endosphere is advancing compared to the culture-dependent techniques (Cai et al., 2020).

Bacteria that colonize the endosphere could be beneficial, but most do not affect plant fitness (Rustamova et al., 2020). However, limited information is available on the diversity of endophytic bacterial communities in oil food crops, such as sunflower; thus necessitating this research. Sunflower is an oilseed crop cultivated in most countries of the world (El-Hamidi and Zaher, 2018). Many countries, such as Turkey, Russia, Argentina, Ukraine, and South Africa have tapped into maximizing its potential usage for both industrial and domestic use (Adeleke and Babalola, 2020b).

On a large scale production, reports on the plant growth-promoting microbes associated with sunflower in Southern Africa for improved yield are understudied, possibly due to limited research work on sunflower using the next-generation sequencing approach (Leff et al., 2017). Promisingly, the researcher-farmer synergism in sunflower production can provide a lasting

solution to food insecurity and shortfall in the global cooking oil supply. More importantly, the use of bioinoculants from bacterial sources has been employed as a potential tool for the sustainability of agricultural production systems to meet the world food supply (Sammauria et al., 2020). Work toward devising agriculturally friendly approaches to explore below-and-above ground endophytes is promising to improve crop productivity (Brijesh et al., 2019). The plant genotype, age, soil type, locations, agricultural practices, and limiting soil nutrients can influence plants' physiological patterns and their associated bacteria community structure (Adeleke and Babalola, 2020a). Recent findings have revealed the use of culture and non-culture approaches in assessing bacteria community structure in the stem, root, leaves, and seed endosphere of most oilseed crops and medicinal plants (Kajić et al., 2020; Dubey et al., 2021; Khalil et al., 2021; Singh et al., 2021). A study by Fadiji et al. (2020a) on the metagenomics profiling of endophytic bacteria from maize has recommended further exploration of endophytic microbes in agriculture, based on their promising effects in shaping plant community, modulating plant ecology, fitness, and evolution, and exhibiting strong effects on the biological activities of the plants against pathogens. Interestingly, endophytic microbes have been studied to enable a complete understanding of the mechanism of applications and ensure a thorough assessment of the current state in the knowledge of the microbes in agricultural biotechnology (Anyasi and Atagana, 2019).

In this study, we hypothesized that environmental variables analyzed may induce varied influence on the root endosphere bacterial structure compared to stem endosphere across different locations. Presumptively, these hypotheses can be determined considering the soil physical and chemical parameters in shaping the bacterial community structure in the plant root

rhizosphere (Cherni et al., 2019). Most bacteria grow well close to neutral or slightly acidic environments, as low pH (below 4) impedes their growth (Yun et al., 2016).

The culture-based techniques in the study of bacteria associated with sunflower have been employed in identifying notable plant growth-promoting bacteria (Majeed et al., 2018). Additionally, the use of culture-dependent techniques for detecting identifiable sunflower root-associated endophytic bacteria has been reported with great promise in agriculture (Pandey et al., 2013; Adeleke et al., 2021). A study by Shahid et al. (2015) reported the use of 16S rRNA gene sequencing in the identification of phosphate-solubilizing bacteria for enhanced sunflower growth and improved yields. Evaluation of structural diversity of sunflower endophytic bacteria and comparative analysis of root and stem endosphere bacterial communities across different sites under different agricultural practices have not been documented. Therefore, this study was designed to present sunflower root and stem-associated bacteria and their predicted functions under different agricultural practices using 16S rRNA gene sequencing. Hence, the comparison of sunflower bacterial endophytes in plant organs will significantly reveal their potential traits and possible applications for improved agricultural production.

10.2 Materials and methods

10.2.1 Site location, sampling, and climatic conditions

In February 2020, samples of sunflower plants (roots and stem) of the same cultivar PAN 7160 CLP were sourced from different sites in Itsoseng (IT) (S26°3′20.106″E25°56′24.234), Thusong District, and Lichtenburg (LT) (S26°4′31.266″ E25°58′44.442), Ditsobotla District, North West Province (NWP) of South Africa. A total of 12 samples each for stem and root (i.e.

24 plants per site) were collected in triplicate from 4 points from each site. Samples were obtained, aseptically excised, and placed in sterile zip lock bags, kept on ice, and immediately transported to the Microbial Biotechnology Research Laboratory (North-West University, South Africa), and stored at -20°C before use. Also, a 50 g sunflower rhizosphere soil in triplicate from each sunflower across the sites was collected aseptically at the depth of 15-20 cm. Generally, the geographical information in NWP has a record of the annual rainfall of 600 mm, low-level area, trees, grassland, and a temperature range from 17°C to 31°C during summer and from 3°C to 21°C during winter. The historical background of the farmland in Lichtenburg revealed that, for more than four decades, sunflower had been cultivated with other crops, such as maize and soybean, on a rotational basis. Water supply is by rainfall and irrigation. The major farm activities include clearing, tilling, ridging, minimal chemical fertilizers (NPK 15:8:4) application, and herbicides (Judo 50EC, Metagon Gold) application to the soil before and after planting. Foliar insecticide spray was also applied after plant germination. The farm size is 234 hectares with 6 hectares of sunflower plantation coverage. In Itsoseng, the farm size is 3,500 hectares with 100 hectares of sunflower plantation coverage. Only sunflower is cultivated on the farmland. Urea and organic manure are employed as soil amendments.

10.2.2 Root cleaning, and surface sterilization

Samples were detached with a sterile scalpel, and washed 3 times with sterile distilled water, and allowed to drain for 10-20 minutes. The samples were cut into miniature sizes (1 cm). The surface sterilization was performed following the modified method of Shishido et al. (1995). Briefly, the washed roots and stem were immersed in 70% ethanol for 3 minutes, then in 3% hypochlorite solution for 3 minutes, and finally immersed in 70% ethanol for 30 seconds. The

sterilized samples were rinsed 5 times with sterile distilled water and allowed to dry under controlled conditions in a laminar flow cabinet (FILTA-MATIX). The level of sample sterility was validated according to the method described by Schulz et al. (1993). A few surface-sterilized samples were gently placed in 10 ml sterile distilled water and gently stirred for 1 minute. An aliquot of 0.1 ml suspension was inoculated on Luria Bertani (LB) agar plates, incubated at 28^{o}C for 8 to 10 days, then assessed for colony growth daily. No bacterial growth formation on the plates validates the removal of root surface bacterial colonizers, and sunflower roots and stem were used for DNA extraction.

10.2.3 DNA extraction process and 16S rRNA gene amplicon sequencing

The extraction of genomic DNA from the surface-sterilized samples was conducted according to the method described by Shishido et al. (1995). The surface-sterilized samples were immediately macerated manually in a sterile mortar and pestle. DNA was obtained from 1 g of the macerated sample using commercial DNA kits (DNeasy® Plant Mini kit, Qiagen, USA) following the manufacturer's protocol and then stored at -20^{o}C before sequencing. The 16S rRNA gene variable V4 region of the amplicon sequence was sequenced on the Illumina Miseq platform. The PCR primers; 515f/806r with a definite barcode on the forward primer were achieved according to the methods described by Caporaso et al. (2012). The PCR was performed in a 30 cycle (5 cycles used on PCR products) using HotStar Taq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, and final elongation step at 72°C for 5 minutes. The success of the PCR amplification process and band size were checked in 2% agarose gel. PCR products were purified using calibrated Ampure XP beads

following the instructions of the manufacturer. The purified PCR product was used to prepare the DNA library, following the DNA library preparation kit protocol.

Sequencing of the extracted DNA was done at MR DNA LP Research Laboratory (Shallowater, TX, USA). For accuracy and sensitivity, a fluorescence Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) was used to quantify the concentrated dsDNA. The accurate, easy, and quick fluorescence measurement was determined using a dual-channel and compact DQ 300 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

Sequence read processing was done using Quantitative Insights Into Microbial Ecology (QIIME 2) 16S pipeline (version 2020.11) (Edgar et al., 2011) implemented on Nephele microbial bioinformatics platform (version 1.8) (https://nephele.niaid.nih.gov/) (Weber et al., 2018). The preprocessing steps included read pair joining using default parameters (% maximum difference of 25, and a minimum overlap of 10), removal of reads with an average Phred score of $\leq$ 20, removal of chimeras using VSEARCH (Rognes et al., 2016), and clustering, using Open Reference Method and SILVA 99 version 132 (Quast et al., 2012). Taxonomy assignments were done against SILVA version 132, with a default sequence similarity threshold of 0.99. The chimeric sequences, such as mitochondria, chloroplast, and singleton reads were removed. The sequence was deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) with Bioproject accession numbers https://www.ncbi.nlm.nih.gov/bioproject/PRJNA673781 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA673791.

10.2.4 Determination of physical and chemical parameters of the sunflower rhizosphere soil

To determine soil physical and chemical parameters, soil tightly associated with the root of sunflower was collected into sterile plastic bags. Triplicates of four sunflower rhizosphere soils were collected from each site. Soil samples from each site were analyzed separately. The soil was air-dried into a fine powder and sieved through a mesh size 2 mm after removing rubble, specks of dirt, stones, and other organic debris. The soil parameters considered include soil pH, nitrate, ammonium, total soil mineral elements (carbon, calcium, phosphorus, potassium, nitrogen, sodium, magnesium), and organic matter. The soil particle size was classified following the methods described by Kettler et al. (2001). The measurement of soil pH was performed by dipping Jenway 3520 pH-meter (Cole-Parmer Instruments, Staffordshire, United Kingdom) into a soil suspension containing mixed sieved soil sample and sterile distilled water (1:2) ratio (Miller and Kissel, 2010). The moisture content (MC) was determined based on the dry mass of the soils after oven-drying at 105°C overnight using an oven (Anatech Instruments, Thermo Scientific Inc., SA). The soil total nitrogen and carbon were determined appropriately using the dry combusting technique as described by (Craft et al., 1991). Similarly, the potassium chloride (KCl) extraction method was used to determine the nitrate content (Dorich and Nelson, 1984). The organic carbon content was determined according to the methods described by Yeomans and Bremner (1988) and Shi et al. (2011). The soil mineral elements, such as calcium, potassium, sodium, calcium, and magnesium contents were determined after the extraction was performed using 1 M ammonium acetate adjusted at pH 7. Subsequently, sodium, calcium, and magnesium present in the soil were determined using an atomic absorption spectrophotometer (Anatech Instruments, Thermo Scientific Inc., SA) (David,

1960). The phosphorus and potassium contents were measured using a flame photometer spectrophotometer (Anatech Instruments, Thermo Scientific Inc., SA), respectively (David, 1960).

10.2.5 Data analysis

The graphical representation (plotting) of bacteria relative abundance was determined using Shinyheatmap (version 0.12.2) (Khomtchouk et al., 2017). The differences in the bacterial community structure across the sampling sites were assessed using a Kruskal-Wallis test in the paleontological statistics software package (PAST version 3.20) (Carrell and Frank, 2015). The alpha diversity indices, Evenness, Simpson, and Shannon derived from PAST software were used to estimate the diversity and richness of the endophytic bacteria in the samples across sites. Principal coordinate analysis (PCoA) based on a Bray-Curtis dissimilarity matrix was used to determine beta diversity. The distribution of bacteria in the samples across sites for principal component analysis (PCA) plot was performed using the Bray-Curtis distance matrix. PCoA and PCA plots were designed using CANOCO version 5 (Microcomputer Power, Ithacha, NY, USA) software. The canonical correspondence analysis (CCA) was determined on CANOCO software. CCA was employed to assess possible correlations between bacterial communities and measured environmental variables. The predictive functional annotation of the bacterial endophytes in each site was performed on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and the resulting output from different levels i.e., 1st, 2nd, and 3rd regarding their predicted functions were obtained.

10.3 Results

10.3.1 Sunflower soil analysis

The physical and chemical analysis of sunflower rhizosphere soil is shown (Table 10.1). Soil analysis showed higher nitrate values in L (sunflower rhizosphere soil samples from Lichtenburg) (14.15 mg/kg) than I (sunflower rhizosphere soil samples from Itsoseng) (6.44 mg/kg). The soil pH value was neutral at site L and had low acidity at site I. It was observed that ammonia, phosphorus, magnesium, total carbon, total nitrogen, and clay were higher in site L than in site I.

10.3.2 Root surface sterilization and sequence data analysis

Taxonomy assignments were done against the SILVA database. The differences in alpha diversity indices in the samples across sites were calculated as Shannon, Simpson, and Evenness (Table 10.2).

At phylum and class levels, a significant difference ($p < 0.05$) was observed, while at the genus level, no significant difference ($p > 0.05$) was observed in the bacterial diversity. The highest alpha diversity of endophytic bacteria in the root and stem of growing sunflower based on Shannon indices were attained in BGR (sunflower root from Lichtenburg), followed by AGR (sunflower root from Itsoseng) at class, and the least Shannon index from BGS (sunflower stem from Lichtenburg) and AGS (sunflower stem from Itsoseng) were obtained at the phylum level (Table 10.2). At the phylum, class, and genus levels, high Shannon (H) index values were obtained in the samples compared to other indices measured across the sites (Table 10.2).

Table 10.1: Physical and chemical soil analysis

Site	**L**	**I**
Organic matter (%)	2.02 ± 0.03^{c}	1.36 ± 0.05^{c}
Nitrate (N-NO_3) (mg/kg)	14.15 ± 0.06^{h}	6.44 ± 0.04^{e}
Ammonium (NH_4) (mg/kg)	6.04 ± 0.05^{e}	5.08 ± 0.08^{d}
pH	7.05 ± 0.05^{g}	6.36 ± 0.05^{e}
Resistivity conductivity (ohm)	1045.03 ± 0.02^{o}	2170.03 ± 0.03^{m}
Phosphorus (P) (mg/kg)	74.93 ± 0.05^{j}	26.40 ± 0.02^{h}
Calcium (Ca) (mg/kg)	973.02 ± 0.02^{n}	424.02 ± 0.17^{l}
Magnesium (Mg) (mg/kg)	187.02 ± 0.02^{m}	122.06 ± 0.28^{k}
Potassium (K) (mg/kg)	181.03 ± 0.03^{l}	113.01 ± 0.02^{j}
Sodium (Na) (mg/kg)	6.51 ± 0.01^{f}	7.17 ± 0.03^{f}
Total carbon (%)	0.63 ± 0.04^{b}	0.42 ± 0.03^{b}
Total nitrogen (%)	0.06 ± 0.01^{a}	0.04 ± 0.01^{a}
Sand (%)	77.03 ± 0.03^{k}	77.02 ± 0.02^{i}
Silt (%)	3.01 ± 0.02^{d}	5.01 ± 0.01^{d}
Clay (%)	20.01 ± 0.02^{i}	18.02 ± 0.02^{g}

Key: L = sunflower soil samples from Lichtenburg, I = sunflower soil samples from Itsoseng. Mean±standard deviation having different letters of triplicate determinations are significantly different at $P \leq 0.05$ according to DMRT

Table 10.2: Alpha diversity of endophytic bacteria in the root and stem of the sunflower at the growing stage

Bacterial level	**Indices**	**AGR**	**AGS**	**BGR**	**BGS**	***p*-value**
Phylum	Evenness	0.1986	0.1891	0.223	0.2089	0.000006
	Simpson	0.3517	0.1613	0.3927	0.2721	
	Shannon	0.6859	0.4142	0.8021	0.6311	
Class	Evenness	0.4231	0.3015	0.452	0.3518	0.0001
	Simpson	0.7184	0.4398	0.7185	0.5611	
	Shannon	1.442	0.9983	1.508	1.258	
Genus	Evenness	0.4069	0.2443	0.4423	0.2481	0.5435
	Simpson	0.6797	0.382	0.6787	0.3978	
	Shannon	1.404	0.8931	1.487	0.9088	

P values were obtained through the Kruskal-Wallis test. AGR – root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR = root samples from Lichtenburg, BGS - stem samples from Lichtenburg

The Chao 1 and Shannon species diversity indices at taxonomic level presented in Figure 10.1 showed the differences in the bacteria diversity in the samples across the sites (Itsoseng and Lichtenburg).

The total number of input sequences (TNIS) varied across the sites (Table 10.3). Sequence count (bp) of 74,158 (AGR-sunflower root from Itsoseng), 58,131 (AGS-sunflower stem from Itsoseng), 48,763 (BGR-sunflower root from Lichtenburg) and 56,387 (BGS-sunflower stem from Lichtenburg) and the sequence read count of AGR−2,497, AGS−1,507, BGR−1,566 and BGS−1,744 after quality control were recorded across the sampling sites. The number of reads generated for each sample that describes the bacterial community structure is presented by the rarefaction curve. Based on the saturation level, the maximum bacterial richness was found to be higher in AGR (sunflower root from Itsoseng) (Figure 10.2). The rarefaction depth for each sample across the sites, i.e. reads per species include, AGR (70,001 per 1265.5136), AGS (56,305 per 637.2572), BGR (46,781 per 784.1046), and BGS (56,305 per 637.2572), respectively.

10.3.3 Endophytic bacterial composition and community structure at the phylum level

In this study, the dominant endophytic bacteria phyla with higher richness in the samples across the sites were selected. The sunflower roots across the sites were dominated by Saccharibacteria and Acidobacteria in BGR (root samples from Lichtenburg), and unassigned bacteria in AGR (root samples from Itsoseng). Also, Proteobacteria, Gemmatimonadetes, Bacteroidetes, and unclassified bacteria were dominant in AGS (stem samples from Itsoseng) and BGS (stem samples from Lichtenburg), respectively (Figure 10.3). However, their distribution across the plant organs at the phylum level was significantly different ($p < 0.05$) from each other as shown in Table 10.2.

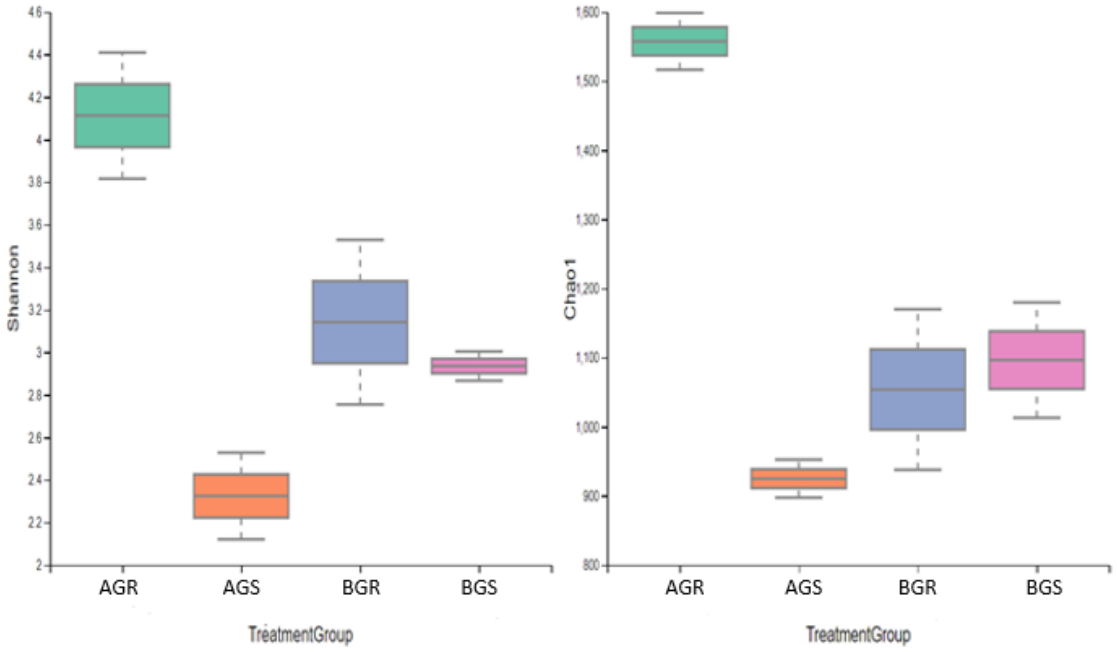


Figure 10.1: Species diversity. **Keyword**: AGR – root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR = root samples from Lichtenburg, BGS - stem samples from Lichtenburg

Table 10.3: Statistical results for 16S rDNA sequencing

Sample information	TNS	SRAQC	CCEL
AGR	74,158	2,497	0
AGS	58,131	1,507	2
BGR	48,763	1,566	0
BGS	56,387	1,744	2

Key: AGR – root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR = root samples from Lichtenburg, BGS - stem samples from Lichtenburg, TNS - total number of sequences, SRAQC – sequence read after quality control, CCEL – count of N characters exceeds the limit

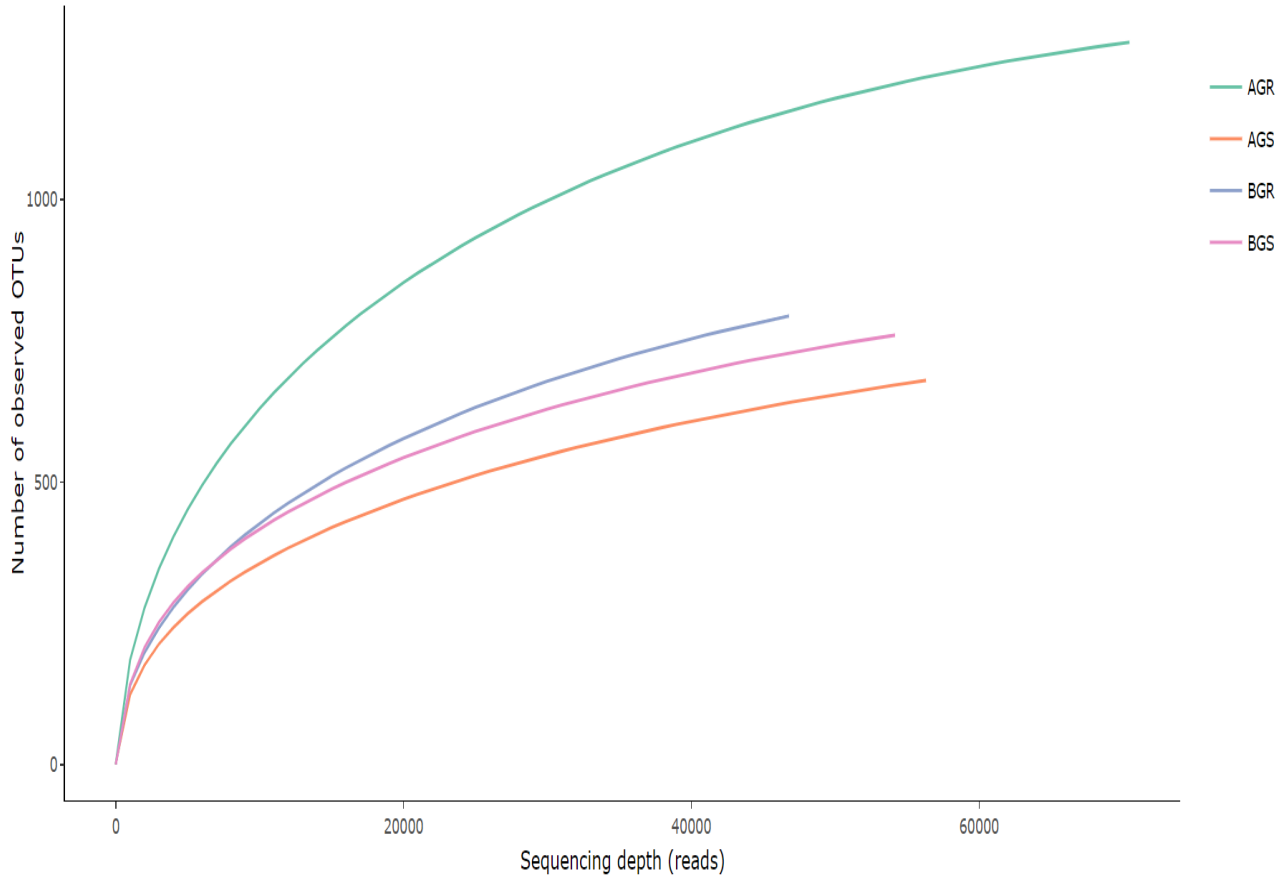


Figure 10.2: Rarefaction curves of evaluated richness in the root and stem of a sunflower. The vertical axis represents the number of OTUs estimated after sampling, while the number of sequence reads is represented on the horizontal axis

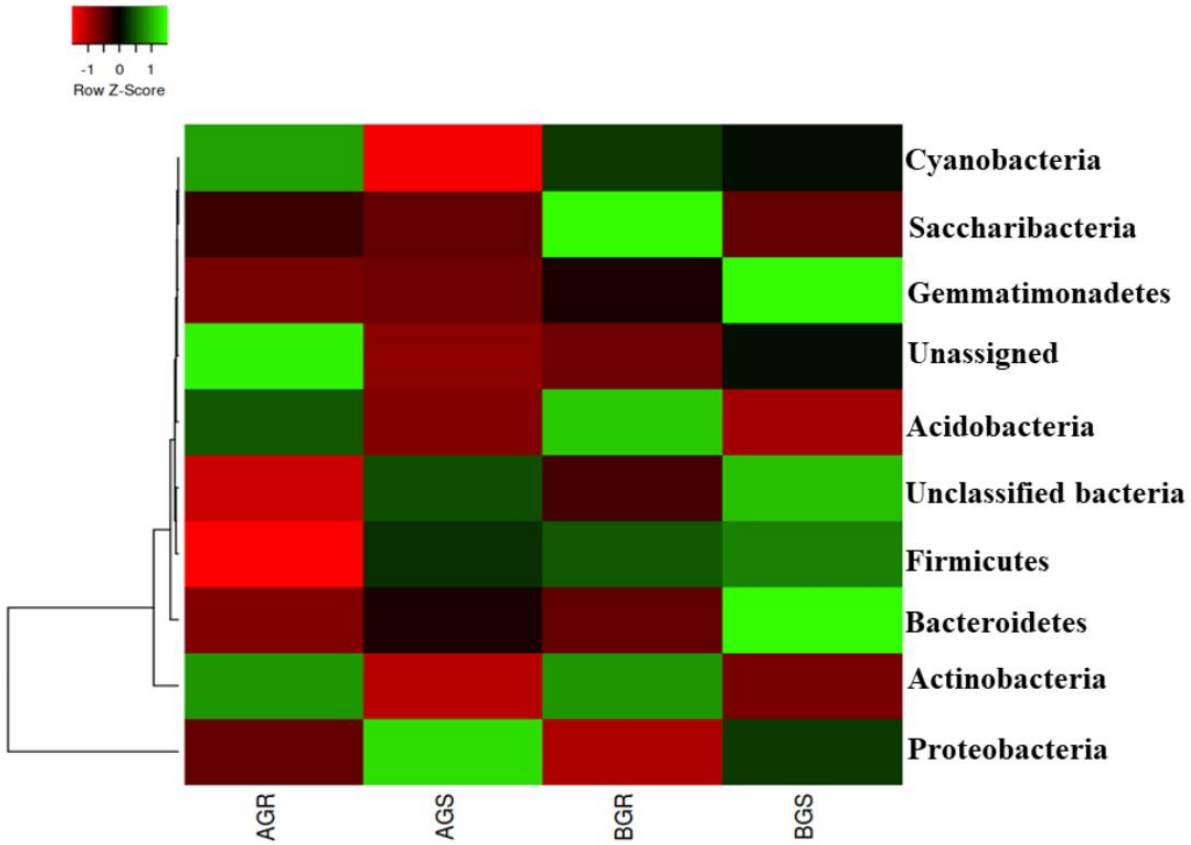


Figure 10.3: Taxonomic classification of bacterial phyla from roots and stem of growing sunflower. Key: AGR = root samples from Itsoseng, BGR – root samples from Lichtenburg, AGS - stem samples from Itsoseng, BGS - stem samples from Lichtenburg

The distribution of identifiable bacterial phyla across the two sites was more diversely distributed in root and stem samples from Lichtenburg (BGR and BGS) than the samples from Itsoseng. The distribution of bacterial genera in the root and stem across the collection sites was revealed in the PCA plot with Axis 1 explained 88.5% and Axis 2 explaining 10.1% variance, respectively (Figure 10.4). The vector distance of PCA revealed the most abundant bacterial phyla in each habitat (i.e., phyla on the longest distance length of PCA). Using the vector distance as a pointer, it is evident that Saccharibacteria, Cyanobacteria, Actinobacteria, and Acidobacteria dominated BGR, Bacteroidetes, Firmicutes, Gemmatimonadetes, and unclassified bacteria dominated BGS, while unassigned bacteria and Proteobacteria were found dominant in AGR and AGS, respectively. The selection of bacterial phyla for PCA and PCoA plots was based on the level of significance (Table 10.2).

10.3.4 Endophytic bacterial diversity indices across the sampling locations

Bacterial diversity evaluations using Evenness, Shannon, and Simpson indexes at the phylum and class levels were significantly different ($p < 0.05$), while no significant difference ($p > 0.05$) was observed in bacterial diversity at the genus level (Table 10.2). The PCoA analysis of endophytic bacteria using Bray-Curtis distance is shown in Figure 10.5. Subsequently, the PCoA graph revealed significant differences in the endophytic bacterial composition in the BGS (stem sample from Lichtenburg) compared to other samples, namely, AGR (root sample from Itsoseng), AGS (stem sample from Itsoseng), and BGR (root sample from Lichtenburg) across the sites. ANOSIM (analysis of similarities) report showed a significant difference in the root and stem endophytic bacterial communities across the sampling sites with $p = 0.01$ and $R = 0.585$.

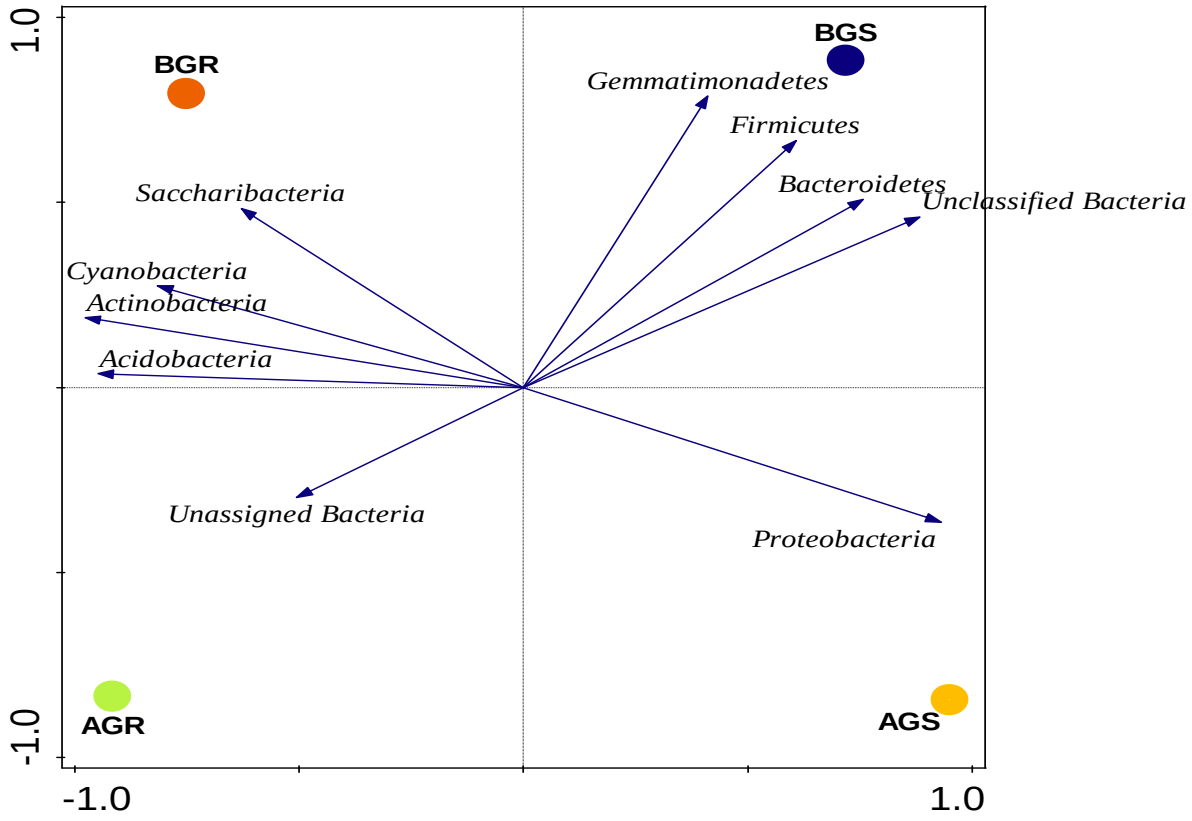


Figure 10.4: PCA plot of endophytic bacterial phyla associated with growing sunflower. The observed variance is represented on Axis 1 (88.5%) and Axis 2 (10.1%). Key: AGR = root samples from Itsoseng, BGR – root samples from AGS - stem samples from Itsoseng, Lichtenburg, BGS - stem samples from Lichtenburg

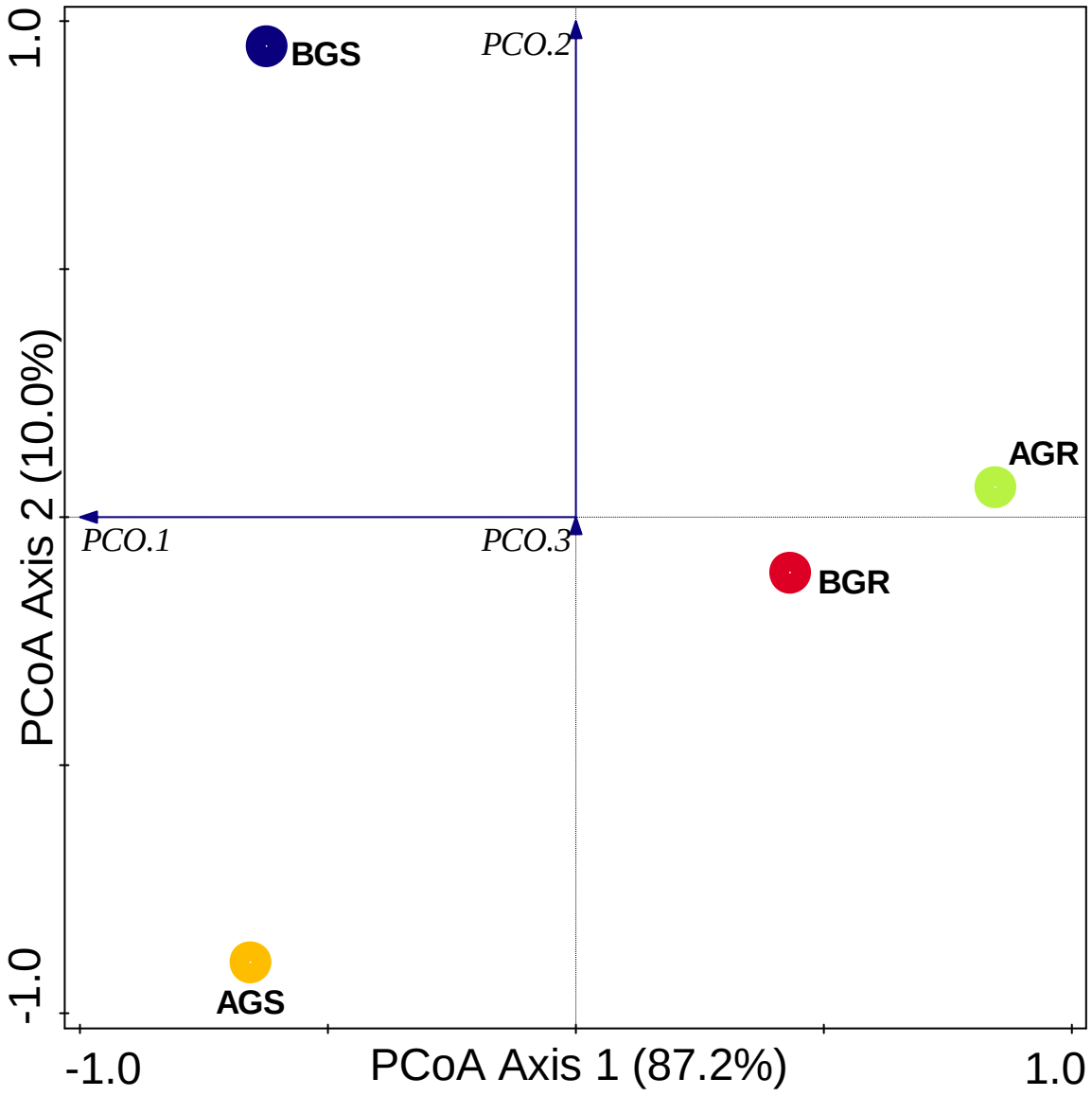


Figure 10.5: PCoA plot of bacterial phyla. Key: AGR = root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR – root samples from Lichtenburg, BGS - stem samples from Lichtenburg

10.3.5 Endophytic bacterial distribution and community structure at the class level

The representative amplicon sequences from sunflower endophytic bacteria used in this study were compared using the SILVA database for the taxonomic assignment. The taxonomic classification of bacterial at the class level from the root and stem of growing sunflower, as shown in Figure 10.6 revealed noticeable variations in the bacterial richness across the sites. Flavobacteriia was most dominant in BGR (root sample from Lichtenburg). The predominance of Alphaproteobacteria and Actinobacteria in AGR (root sample from Itsoseng) were also observed. AGS (stem sample from Itsoseng) and BGS (stem sample from Lichtenburg) were also dominated with Gammaproteobacteria, and Betaproteobacteria, Acidobacteria, Clostridia, and Actinobacteria. Furthermore, Bacilli and unclassified bacteria were also found dominant in BGR (root sample from Lichtenburg). A significant difference ($p < 0.05$) was observed in the distribution of endophytic bacterial isolates across plant organs at the class level (Table 10.2). Across the sites, Gammaproteobacteria exhibited a high relative abundance of 39.2% in AGR (root sample from Itsoseng), 73.59% in AGS (stem sample from Itsoseng), 63.94% in BGR (root sample from Lichtenburg), and 38.10% in BGS (stem sample from Lichtenburg), respectively.

10.3.6 Endophytic bacterial distribution and community structure at the genus level

At the genus level, *Acidovorax*, *Bacillus*, *Flavobacterium*, *Hydrogenophaga*, and *Pseudomonas* were the most dominant in BGR (root samples from Lichtenburg) compared to other samples. Additionally, *Streptomyces* and unclassified bacteria were predominant in BGS (stem samples from Lichtenburg), while *Rhizobium* and *Burkholderia-Paraburkholderia* were detected only in AGR (root samples from Itsoseng) (Figure 10.7).

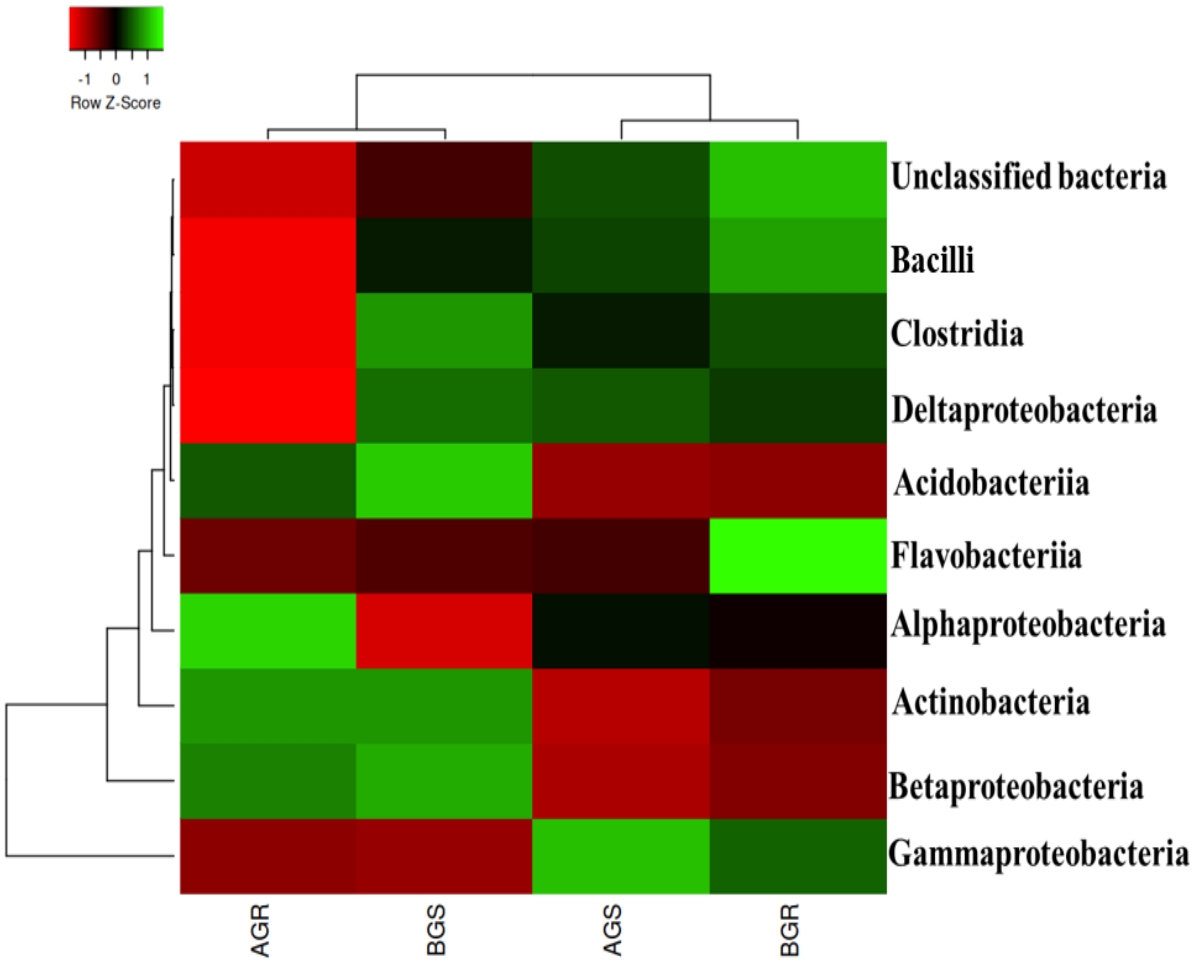


Figure 10.6: Taxonomic classification of bacterial distribution at a class level from the roots and stem of growing sunflower. Key: AGR = root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR – root samples from Lichtenburg, BGS - stem samples from Lichtenburg

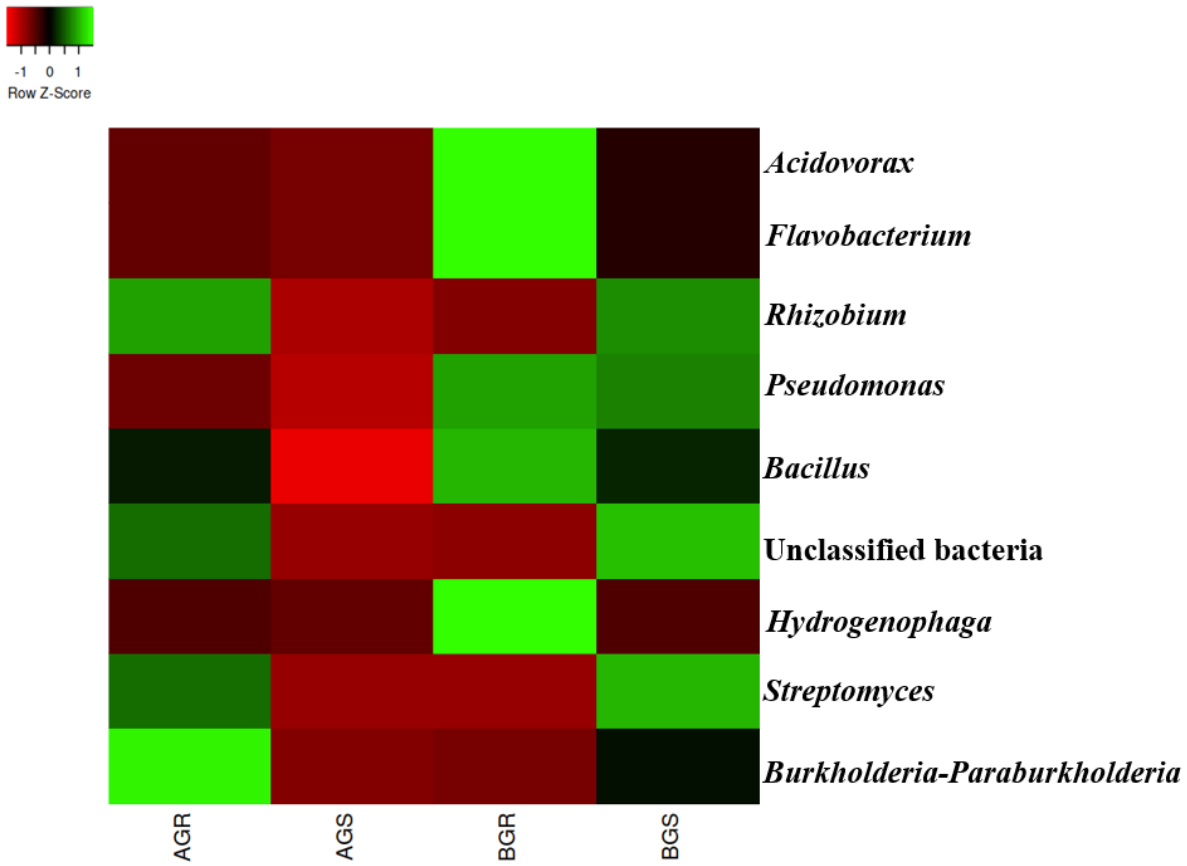


Figure 10.7: Taxonomic classification of bacterial genera from roots and stem of growing sunflower. Key: AGR = root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR – root samples from Lichtenburg, BGS - stem samples from Lichtenburg

Bacillus predominantly shows high percentage composition of 75.04% in BGR, followed by BGS with a percentage composition of 49.64%, then, 47.61% in AGR, while the least percentage composition of 0.84 was observed in AGS.

10.3.7 Environmental factors influence the endophytic bacterial community structure

The correlation between environmental variables on the distribution of bacterial endophytes at the phylum level was analyzed using the CCA. The soil parameters analysis (Table 10.1), was considered for the CCA plot (Figure 10.8). The results revealed that the bacterial community structure was induced by the soil parameters, with a CCA permutation test = 0.02. Sand, calcium, potassium, sodium, organic matter, and N-NH_3 showed prominent correlations with bacterial diversity than other environmental variables tested.

Below the mid-point, Actinobacteria, Cyanobacteria, Proteobacteria, and unassigned bacteria positively correlated with Na, while negative correlation of Bacteriodetes with sand, Mg, and resistivity conductivity were observed compared to the soil parameters above the mid-point (Figure 10.8). The constraints environmental variables and testing with 499 random permutations were employed to deduce environmental variables and influence on bacterial diversity.

10.3.8 Predictive functional abundance information

The predictive functions of endophytic bacterial across the sites were analyzed using PICRUSt. The predicted functional information of bacterial communities at a different level from each cluster is presented in Table 10.4. At level 1 selection, bacterial possess predictive functional information, such as cellular metabolism, and environmental information processing relating to plant growth promotion.

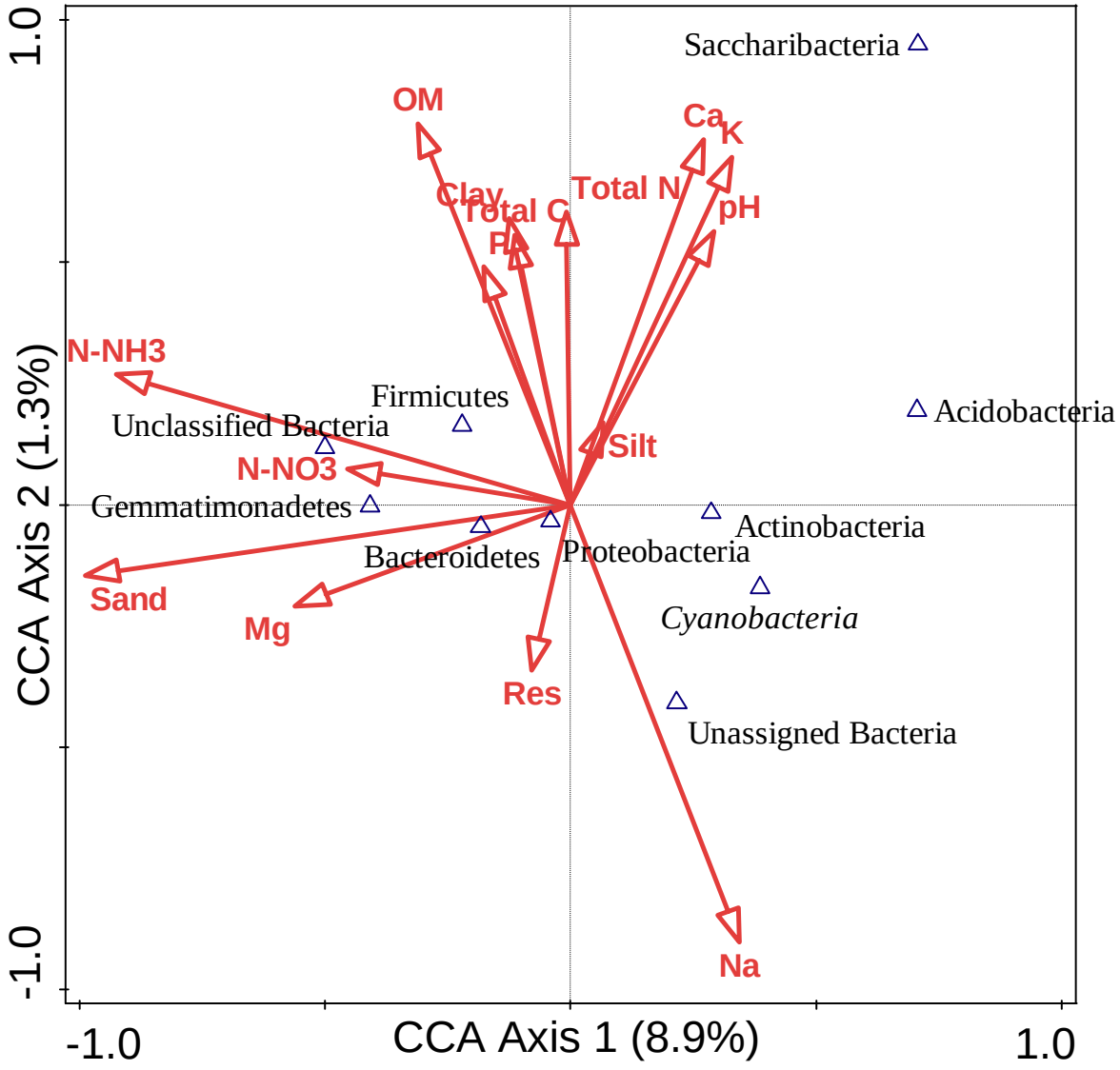


Figure 10.8: CCA plot of bacterial phyla and soil environmental variables

Table 10.4: Predicted functions of bacteria in sunflower at the growing stage

Predicted metabolism (1st level)	**Activity (2nd level)**	**Predicted function (3rd level)**
Environmental information processing	Membrane transport	ABC transport
Metabolism	Amino acid	Tryptophan metabolism
	Biosynthesis of other secondary metabolites	Streptomycin biosynthesis
	Energy metabolism	Nitrogen metabolism
	Metabolism of other amino acids	Phosphonate and phosphinate metabolism
	Metabolism of terpenoids and polyketides	Biosynthesis of siderophore group non-ribosomal peptides

The predicted functions revealed at third level classification include ABC transport, tryptophan metabolism (a precursor for indole-3-acetic acid production), streptomycin biosynthesis (antibiosis), nitrogen metabolism (nitrogen fixation potential), phosphonate and phosphinate metabolism, and biosynthesis of siderophore and other secondary metabolites (Table 10.4 and Figure 10.9). High-predicted functional profiling of bacteria in the root samples collected from Itsoseng was recorded across the sites, while a lower tryptophan metabolism rate of 15.23% was recorded from the bacteria in stem samples from Itsoseng (Figure 10.9).

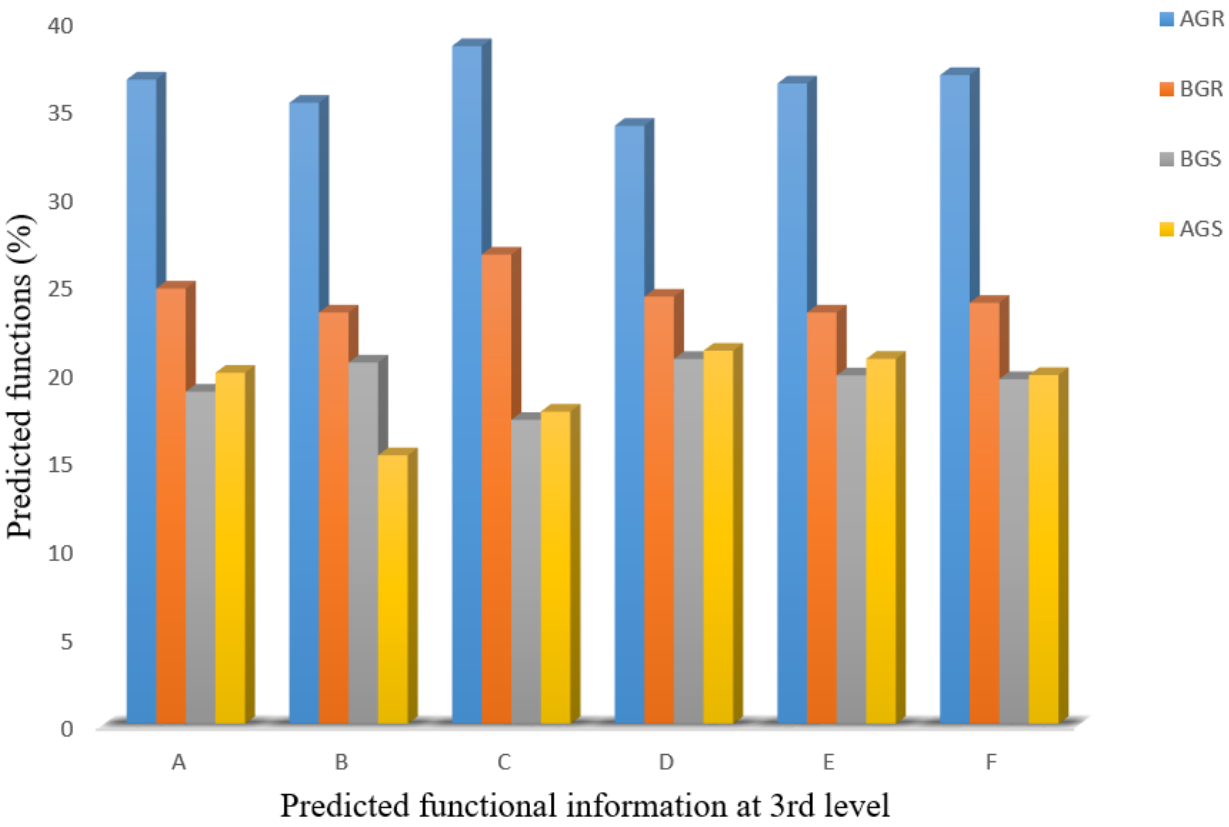


Figure 10.9: Percentage predicted functional information of bacteria in sunflower at the growing stage across different sites. Key: A – ABC transport, B – tryptophan metabolism, C - streptomyces metabolism, D – nitrogen metabolism, E – phosphonate and phosphinate metabolism, F – biosynthesis of siderophore group non-ribosomal peptides, AGR = root samples from Itsoseng, AGS - stem samples from Itsoseng, BGR – root samples from Lichtenburg, BGS - stem samples from Lichtenburg

10.4 Discussion

In a bid to understand plant endosphere biology, we present a next-generation sequencing approach to determine endophytic bacteria community structure in the root and stem of sunflower at the growing stage. In our view, this is the first report on the use of Illumina-based sequencing for determining bacterial community structure in sunflower cultivated in Southern Africa. Based on the farm history across the sites, we hypothesized that the diversity of bacteria in the sunflower endosphere may be influenced by soil parameters and agricultural practices (mono-cropping, and mixed cropping, as well as the use of chemical fertilizer and organic manure), which justify the choice for the sampling sites. The use of chemical fertilizer and organic manure in boosting soil nutrients and plant growth, on the other hand, may induce a shift in the soil parameters and bacterial community structure in its entirety (Sun et al., 2015b). Research findings on the use of next-generation amplicon-based approach have been employed in determining bacterial diversity in rice, soybean, sunflower, and melon with success (Wen et al., 2016, Jayanti and Joko, 2020, Li et al., 2020a, Tokgöz et al., 2020).

In this study, dominant bacterial phyla, such as Saccharibacteria, Gemmatimonadetes, Acidobacteria, Bacteriodetes, and Proteobacteria were identified from the root and stem of sunflower at the growing stage. The presence of these bacteria might be due to their affinity to form a community within the sunflower endosphere. Similar bacterial phyla have been reported in the root, stem, and leaf endosphere of maize and peony (Yang et al., 2017, Fadiji et al., 2020a). The unclassified bacteria phyla identified in the AGR (root sample from Itsoseng) and BGS (stem sample from Lichtenburg) may create insights for further research in determining their novel identity and exploration for agricultural and industrial use. The bacteria phyla identified in this study corroborate the findings of Dang et al. (2020) who reported identifiable

endophytic bacteria phyla, Acidobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria from the root of three species of medicinal licorices (*Glycyrrhiza uralensis, Glycyrrhiza glabra*, and *Glycyrrhiza inflata*) grown on ploughed farmlands in China. The populations of Acidobacteria, Bacteriodetes, and Proteobacteria in sunflower samples have been reported to contribute to plant growth promotion and health sustainably (Leff et al., 2017). The higher bacterial diversity in BGR and BGS can be linked to soil nutritional profile, organ differentiation, geographical location, and farming practices (Leff et al., 2017). Similarly, the lower bacteria diversity in AGR (root sample from Itsoseng) and AGS (stem sample from Itsoseng) can be attributed to root differentiation, low bacterial metabolic activity, weak infiltration from the external root environment, prevailing environmental factors, and agricultural practices (Babin et al., 2019). A difference in the relative abundance of endophytic bacteria community composition between roots and stems of sorghum plants has been reported by Maropola et al. (2015). Additionally, the effect of different agricultural practices and climatic conditions have been reported to influence the distribution of endophytic bacteria communities in the root endosphere (Puri et al., 2019). Interestingly, the difference observed in the bacterial community in the root and stem of sunflower at the growing stage in Lichtenburg compared to the stem of sunflower at the growing stage in Itsoseng validate the hypothesis of this study on the effect of crop rotational and mixed farming system on the bacterial diversity under different agricultural practices.

In this study, the diversity indices at phylum and class level showed a significant difference in the bacterial distribution across sampling sites and this can further explain how crop rotation systems and mixed cropping systems showed higher bacterial diversity in Lichtenburg than the mono-cropping system in Itsoseng. The crop rotation system in maintaining stable biodiversity

and activities of endophytic bacteria has been documented by Dube et al. (2019). The successional mixed cropping system can enhance nutrient bioavailability and endo-rhizosphere competence, and this may selectively reintroduce different plant growth-promoting bacteria into the soil (Tiemann et al., 2015).

The bacteria class identified in this study, such as Flavobacteriia in BGR (root sample from Lichtenburg) has not been documented in the root of any oilseed crops, thus revealing its bioprospecting in agriculture. Nevertheless, a study by Furtado et al. (2019) revealing dominant Gammaproteobacteria and Flavobacteriia in the root of *Salicornia europaea* has been documented. The identifiable Gammaproteobacteria from the stem of sunflower (AGS) corroborate with Romero et al. (2014) who reported similar bacterial phyla from the leaf of tomato plants. Interestingly, the diverse classes of endophytic bacteria in the sunflower root can underline their significance in agriculture in enhancing plant growth.

At the genus level, the dominant *Acidovorax, Bacillus, Flavobacterium, Hydrogenophaga,* and *Pseudomonas* in BGR (root sample from Lichtenburg) compared to other samples may suggest agricultural importance of this bacterial genera. The agricultural importance of *Pseudomonas, Rhizobium,* and *Streptomyces* identified in this study may suggest their future exploration as bioinoculants in enhancing plant growth and control of plant pathogens. The biological relevance of *Pseudomonas* and *Bacillus* isolated from a medicinal plant *Echinacea purpurea* and their contribution has been reported in sustainable crop production and plant health (Presta et al., 2017). Due to the scanty information on the identification of plant growth-promoting endophytic bacteria genera from sunflower using amplicon sequencing, results from this study can serve as a model in future studies of endophytic bacteria associated with sunflower and other oilseed crops.

Subjecting agricultural soils to long-term fertilization may cause a change in the nutritional profile and bacterial diversity (Sun et al., 2015b). In this study, the analyzed soil parameters showed a significant correlation to bacteria diversity. Sodium, organic matter, and ammonium nitrate best explain the bacterial diversity across the sites. The influence of these factors on bacteria diversity, however, may shape the bacterial structure and soil selection for agricultural purposes. The influence of sodium, phosphorus, magnesium, and potassium on the distribution of beneficial endophytic bacteria in the root of wheat has been documented (Minervini, 2019). pH is one of the key determinant factors influencing bacterial community structure in the soil (Qu et al., 2020). The pH values of sunflower rhizosphere soil ranged from 6.37 to 7.06 and this corroborates with the findings of Alawiye and Babalola (2021) who reported pH values, 6.0, 6.5, 6.6, and 5.8 on sunflower rhizosphere soil collected from four different locations in South Africa. The influence of soil pH and organic matter have been reported to influence bacteria community structure in plants (Wang et al., 2019b, Chen et al., 2021). From this study, the soil parameters assessed and their positive influence on bacterial distribution across the sites can influence bacteria potential in the soil. Hence, emphasis on the use of 16S rRNA amplicon metataxonomics in assessing diverse endophytic bacterial communities in sunflower can be promising in agriculture sustainably. In addition, endophytic bacteria identified in this study may play significant roles in recycling soil nutrients, improving plant growth, and soil health. Furthermore, the sunflower endophytic bacteria community structure as confirmed in this study and their higher distribution in root compared to stem reveal similar predictive functional attributes in the below-and-above ground levels.

The bacterial predictive functions at third level classification include ABC transport, tryptophan metabolism (a precursor for indole-3-acetic acid production), streptomycin

biosynthesis (antibiosis), nitrogen metabolism (nitrogen fixation potential), phosphonate and phosphinate metabolism, and biosynthesis of siderophore and other secondary metabolites, and these predicted functions can be linked to the interdependence of endophytic bacteria with the host plants across the sites (Itsoseng and Lichtenburg). The predicted functional traits of endophytic bacteria across the sites may underline their effects on plant growth promotion and enhancing soil fertility. Production of certain secondary metabolites has been attributed to the Actinobacteria and Acidobacteria found in the root-soil environment, which enhances their ability to metabolize organic substrates and control plant pathogens (Crits-Christoph et al., 2018, van Bergeijk et al., 2020, Zhang et al., 2021). Other predicted functions involved indole-3-acetic acid and siderophore production, antibiotic synthesis, and phosphate solubilization may enhance plant growth and health through nitrogen fixation, root development, the bioavailability of soil nutrients for plant uptake, and synthesis of biocontrol agent against phytopathogens (Patel et al., 2018). In line with this study, the predicted functional analysis of endophytic bacteria associated with sunflower root and stem corroborate the findings of Yuan et al. (2021) who reported similar potential functions exhibited by endophytic bacteria associated with flowering and nonflowering moso bamboo (*Phyllostachys edulis*). The predicted functional reports on endophytic bacteria colonizing the sunflower endosphere have not been documented. Hence, the observed predicted functions exhibited by endophytic bacteria in this study can serve as a guide for future studies.

10.5 Conclusion

In the present study, 16S rRNA gene amplicon sequencing was employed to evaluate the bacterial communities in the root and stem of sunflower at the growing stage across the sampling sites (Itsoseng and Lichtenburg). The results obtained at the phyla level revealed a

significant difference in bacterial diversity across the sites, with the most dominant in sunflower root collected from Lichtenburg. The predominance of unclassified bacteria in the stem sample from Lichtenburg suggests further studies in devising culturable means for the characterization and detection of novel traits that can be harnessed as bioinoculants in developing eco-friendly agriculture. Environmental variables showed positive and negative influences on bacteria diversity across the sites. The predicted functions of these bacteria suggest their agricultural importance, which can be explored in developing bioproducts as an alternative to chemical fertilizer. Promisingly, due to the economic value of sunflower, it is recommended to use culture-dependent techniques and practicable *in vitro* seed inoculation and planting under greenhouse to further experiment with the potential of endophytic bacteria on sunflower plants.

Furthermore, understanding plant-associated microbes under different farming systems will help determine their functional roles in plant nutrition, growth, and health; although, information on sunflower-associated endophytic bacteria is limited. Interestingly, this study provides clear evidence on the influence of crop rotation, agricultural practices, and soil parameters on the bacteria diversity in sunflowers from the two sites. In conclusion, this study may serve as a model in future endosphere biology under different agricultural systems to enhance plant performance, comparing the plant growth stages, locations, and plant organs.

CHAPTER ELEVEN

11.0 CONCLUSION AND RECOMMENDATIONS

11.1 General conclusions

Given that the endosphere of crop plants is composed of several taxonomic classes of bacteria, a major challenge is to identify beneficial bacterial species influencing plant growth. Mining this reservoir holds exciting opportunities for understanding bacterial candidates with biotechnological applications in current agricultural practices. Extending beyond the traditional microbiome survey approach, in this research, a combination of culture-independent techniques in determining the novel functions and structural diversity of endophytic bacteria, and culture-dependent techniques involving sampling, isolating, screening, and whole-genome sequencing of resident endophytic bacteria from sunflower, have demonstrated its valuable approach for identifying endogenous host-associated bacterial strains. Interestingly, this work provides a useful resource for further experimental and comparative genomics analyses in understanding the co-evolution of bacteria with sunflower.

This study presents a genomic assessment of copious plant growth-promoting endophytic bacteria *B. cereus* T4S, *S. indicatrix* BOVIS40, and *S. maltophilia* JVB5 isolated from the sunflower endosphere. Also, through a combination of *in-vitro* and *in-vivo* experiments, this study reveals novel endophytic bacterial strains with potential growth-promoting traits in sunflower. Furthermore, the genomic assessment of *B. cereus* T4S, *S. indicatrix* BOVIS40, and *S. maltophilia* JVB5 revealed functional genes contributing to the bacterial attachment to the host plant. Multiple genes involved in various metabolic pathways were also predicted in the bacterial genome. In addition, the structural analysis revealed that the bacteria community was more dominant in the root than the stem, thus suggesting their active participation in various

plant physiology and ecological functions. The difference observed in the bacterial composition may help determine endophytic bacterial affinity with plants in ensuring endosphere competence for sunflower improvement.

11.2 Recommendations

From this study, I recommended that:

- Based on the functional efficacy of the bacterial strains as revealed from the whole genome sequencing analysis, exploration and commercialization of these bacterial strains should be harnessed as a roadmap in producing safe and ecofriendly soil adjuvants.
- The molecular insights of endophytic bacteria and their effects on sunflower should form the basic theoretical background for possible application of these strains in growing oilseed and medicinal crops; to strategically help in mitigating agricultural problems in terms of food insecurity, pest infestation, low crop yield, and ecological disturbances in Southern Africa, and other developed and developing countries of the world.
- The expression of phytohormone genes in the bacterial genome and the diverse identifiable bacterial endophytes in the root and stem of sunflower dictating their interactions within the plant tissues can suitably serve as a precursor in the synthesis of bioinoculants.
- Studies on the comparative assessment of sunflower rhizosphere and endosphere microbiota, mechanisms of interactions, and further application under field experiment for sustainable sunflower production is recommended.
- Shotgun metagenomics approach in determining the functional attributes and metabolic pathways should be employed in further sunflower bacterial endophytic studies. In addition, more knowledge will be added to science using metabolomics, metatranscriptomics, and

transcriptomics with specificity in determining metabolites, gene expression profiling, transcriptome, and gene functions from bacteria associated with sunflower plants.

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APPENDICES

Appendix 1: Published Review article from this study

International Microbiology (2021) 24:1–17
https://doi.org/10.1007/s10123-020-00140-2

REVIEW

The endosphere microbial communities, a great promise in agriculture

Bartholomew Saanu Adeleke[1] · Olubukola Oluranti Babalola[1]

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Abstract
Agricultural food production and sustainability need intensification to address the current global food supply to meet human demand. The continuous human population increase and other anthropogenic activities threaten food security. Agrochemical inputs have long been used in conventional agricultural systems to boost crop productivity, but they are disadvantageous to a safe environment. Towards developing environmentally friendly agriculture, efforts are being directed in exploring biological resources from soil and plant microbes. The survival of the rhizosphere and endosphere microbiota is influenced by biotic and abiotic factors. Plant microbiota live interdependently with the host plants. Endophytes are regarded as colonizer microbes inhabiting and establishing microbial communities within the plant tissue. Their activities are varied and include fixing atmospheric nitrogen, solubilizing phosphate, synthesis of siderophores, secretion of metabolite-like compounds containing active biocontrol agents in the control of phytopathogens, and induced systemic resistance that stimulates plant response to withstand stress. Exploring beneficial endophyte resources in the formulation of bio-inoculants, such as biofertilizers, as an alternative to agrochemicals (fertilizers and pesticides) in developing environmentally friendly agriculture and for incorporation into crop breeding and disease control program is promising. Therefore, in this review, endosphere microbial ecology, associating environmental factors, and their roles that contribute to their effectiveness in promoting plant growth for maximum agricultural crop productivity were highlighted.

Keywords Beneficial microbes · Endophytes · Environmental factors · Microbial communities · Sustainable agriculture

Appendix 2: Published Review article from this study

Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-021-10406-2

Roles of Plant Endosphere Microbes in Agriculture-A Review

Bartholomew Saanu Adeleke[1] · **Olubukola Oluranti Babalola**[1]

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Abstract
Despite the importance of diverse plant growth-promoting endophytes in agricultural production, their biotechnological and agricultural applications are not well-documented. The diversity of microbial communities interacting with the endosphere contributes to plant functions and immunity, leading to higher productivity. Plant-microbe interactions range from beneficial in terms of influencing plant growth to harmful, as they also cause plant diseases. Microbial survival in the internal tissues of plants depends on their colonization tendencies and their ability to compete with the indigenous plant microflora. The infiltration of microbes through the external soil-root environment into the plant endosphere significantly enhances growth-promoting attributes of plants such as antibiosis, siderophore production, induced systemic resistance, bioremediation and growth hormones synthesis. However, the growth and diversity of endophytic microbes are influenced by the availability of soil nutrients, presence of pathogens, plant growth stages, plant genome, and other abiotic factors. Knowledge and understanding of the possible use and biotechnological relevance of endosphere communities in sustainable agriculture cannot be overemphasized. Hence, this review discusses the importance of endophytic microbes in agriculture for enhancing crop productivity.

Keywords Agriculturally important microorganisms · Crop productivity · Eco-friendly agriculture · Endosphere microbial communities · Plant growth-promoting endophytes (PGPE)

Appendix 3: Published Research article from this study

Current Genetics (2021) 67:891–907
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ORIGINAL ARTICLE

Genomic assessment of *Stenotrophomonas indicatrix* for improved sunflower plant

Bartholomew Saanu Adeleke[1] · **Ayansina Segun Ayangbenro**[1] · **Olubukola Oluranti Babalola**[1]

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Abstract
Diverse agriculturally important microbes have been studied with known potential in plant growth promotion. Providing several opportunities, *Stenotrophomonas* species are characterized as promising plant enhancers, inducers, and protectors against environmental stressors. The *S. indicatrix* BOVIS40 isolated from the sunflower root endosphere possessed unique features, as genome insights into the *Stenotrophomonas* species isolated from oilseed crops in Southern Africa have not been reported. Plant growth-promotion screening and genome analysis of *S. indicatrix* BOVIS40 were presented in this study. The genomic information reveals various genes underlining plant growth promotion and resistance to environmental stressors. The genome of *S. indicatrix* BOVIS40 harbors genes involved in the degradation and biotransformation of organic molecules. Also, other genes involved in biofilm production, chemotaxis, and flagellation that facilitate bacterial colonization in the root endosphere and phytohormone genes that modulate root development and stress response in plants were detected in strain BOVIS40. IAA activity of the bacterial strain may be a factor responsible for root formation. A measurable approach to the *S. indicatrix* BOVIS40 lifestyle can strategically provide several opportunities in their use as bioinoculants in developing environmentally friendly agriculture sustainably. The findings presented here provide insights into the genomic functions of *S. indicatrix* BOVIS40, which has set a foundation for future comparative studies for a better understanding of the synergism among microbes inhabiting plant endosphere. Hence, highlighting the potential of *S. indicatrix* BOVIS40 upon inoculation under greenhouse experiment, thus suggesting its application in enhancing plant and soil health sustainably.

Keywords Agricultural sustainability · Bacterial endophytes · Crop improvement · Plant growth-promoting genes · Whole-genome sequencing

Appendix 4: Published Research article from this study

plants

MDPI

Article

Genomic Analysis of Endophytic *Bacillus cereus* T4S and Its Plant Growth-Promoting Traits

Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola *

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Abstract: Insights into plant endophytic microbes and their exploration in agriculture have provided opportunities for sustainable plant health and food safety. Notable endophytic *Bacillus* species with plant growth-promoting traits have been documented; nevertheless, information on genome analysis of *B. cereus* associated with the sunflower in South Africa has not been studied. Therefore, we present whole-genome sequence of agriculturally important *B. cereus* strain T4S isolated from sunflower plants. The NextSeq Illumina sequencing yielded 7,255,762 bp sequence reads, 151 bp average read length, 5,945,881 bp genome size, 56 tRNA, 63 rRNA, and G + C content of 34.8%. The phylogeny analysis of strain T4S was similar to *B. cereus* NJ-W. Secondary metabolites, such as petrobactin, bacillibactin, bacitracin, molybdenum factor, zwittermicin, and fengycin underlining bacterial biocontrol efficacy against phytopathogens were found in the T4S genome. The predicted novel genes in the bacterial genome mediating the complex metabolic pathways can provide a genetic basis in understanding endosphere biology and their multiple functions thereof in crop improvement. Interestingly, seed and root inoculation with strain T4S contributed to sunflower yield under greenhouse experiments. Hence, the detection of notable genes specific for plant growth promotion as validated under in vitro screening, promisingly, suggests the relevance of strain T4S in agricultural biotechnology.

Keywords: bacterial endophytes; greenhouse experiment; Illumina sequencing; oilseed crop; plant growth promotion; secondary metabolite genes

check for updates

Citation: Adeleke, B.S.; Ayangbenro, A.S.; Babalola, O.O. Genomic Analysis of Endophytic *Bacillus cereus* T4S and Its Plant Growth-Promoting Traits. *Plants* **2021**, *10*, 1776. https://doi.org/10.3390/plants10091776

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1. Introduction

In recent times, agricultural sustainability to maximally meet the nutritional food demand of the growing population needs a boost, more importantly, to avert future food scarcity and insecurity, and hunger. Notably, one of the promising measures is through

Appendix 5: Published Research article from this study

PLANT SIGNALING & BEHAVIOR
https://doi.org/10.1080/15592324.2021.1974217

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RESEARCH PAPER

OPEN ACCESS Check for updates

Bacterial community structure of the sunflower (*Helianthus annuus*) endosphere

Bartholomew Saanu Adeleke, Ayansina Segun Ayangbenro, and Olubukola Oluranti Babalola

Food Security and Safety Niche Area, Faculty of Natural and Agricultural Sciences, North-West University, Mmabatho, South Africa

ABSTRACT

Agrochemical applications on farmland aim to enhance crop yield; however, the consequence of biodiversity loss has caused a reduction in ecological functions. The positive endosphere interactions and crop rotation systems may function in restoring a stable ecosystem. Employing culture-independent techniques will help access the total bacteria community in the sunflower endosphere. Limited information is available on the bacteria diversity in sunflower plants cultivated under different agricultural practices. Hence, this study was designed to investigate the endophytic bacterial community structure of sunflower at the growing stage. Plant root and stem samples were sourced from two locations (Itsoseng and Lichtenburg), for DNA extraction and sequenced on the Illumina Miseq platform. The sequence dataset was analyzed using online bioinformatics tools. Saccharibacteria and Acidobacteria were dominant in plant roots, while the stem is dominated by Proteobacteria, Bacteriodetes, and Gemmatimonadetes across the sites. Bacterial genera, *Acidovorax, Flavobacterium, Hydrogenophaga*, and *Burkholderia-Paraburkhoderia* were found dominant in the root, while the stem is dominated by *Streptomyces*. The diverse bacterial community structure at phyla and class levels were significantly different in plant organs across the sites. The influence of soil physical and chemical parameters analyzed was observed to induce bacterial distribution across the sites. This study provides information on the dominant bacteria community structure in sunflowers at the growing stage and their predictive functions, which suggest their future exploration as bioinoculants for improved agricultural yields.

ARTICLE HISTORY
Received 5 August 2021
Revised 23 August 2021
Accepted 25 August 2021

KEYWORDS
Agricultural sustainability; bacterial diversity; culture-independent techniques; amplicon sequencing

Appendix 6: Published Genome Short Report from this study

Check for updates

Whole Genome Sequencing of Sunflower Root-Associated *Bacillus cereus*

Olubukola Oluranti Babalola, Bartholomew Saanu Adeleke and Ayansina Segun Ayangbenro

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Evolutionary Bioinformatics
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SAGE

ABSTRACT: In recent times, diverse agriculturally important endophytic bacteria colonizing plant endosphere have been identified. Harnessing the potential of *Bacillus* species from sunflower could reveal their biotechnological and agricultural importance. Here, we present genomic insights into *B. cereus* T4S isolated from sunflower sourced from Lichtenburg, South Africa. Genome analysis revealed a sequence read count of 7 255 762, a genome size of 5 945 881 bp, and G + C content of 34.8%. The genome contains various protein-coding genes involved in various metabolic pathways. The detection of genes involved in the metabolism of organic substrates and chemotaxis could enhance plant-microbe interactions in the synthesis of biological products with biotechnological and agricultural importance.

KEYWORDS: Bacterial endophytes, plant-microbe interactions, root architecture, secondary metabolites, siderophore

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Appendix 7: Published Genome Announcements from this study

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Resource Announcements

GENOME SEQUENCES

Draft Genome Sequencing of *Stenotrophomonas indicatrix* BOVIS40 and *Stenotrophomonas maltophilia* JVB5, Two Strains with Identifiable Genes Involved in Plant Growth Promotion

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ABSTRACT Here, plant growth-promoting *Stenotrophomonas* strains isolated from the sunflower root endosphere were studied. Bacterial DNA was sequenced on Illumina's NextSeq platform. The gene prediction reveals diverse functional genes involved in plant growth promotion from each bacterial genome. The exploration of bacterial resources as bioinoculants is promising for agricultural biotechnology.

Stenotrophomonas species are free-living, Gram-negative nonsporeformers that are commonly found in the soil and plant environments (1). *Stenotrophomonas* species can be involved in plant growth promotion (PGP) (2), although some are yet to be explored. Therefore, genomic elucidation can help predict the diverse genes responsible for bacterial functions in agricultural biotechnology.

Bacteria isolated from sunflower roots were sourced from farmlands in Lichtenburg, South Africa (26°4′31.266″S, 25°58′44.442″E), in February 2020. The healthy sunflower plants were carefully uprooted, placed inside sterile ziplock bags, transported to the laboratory, and stored at 4°C. The root samples were cut into small pieces with a sterile scalpel and washed in sterile distilled water. To ensure complete removal of the epiphytic bacteria, surface sterilization was achieved by soaking the samples in 70% ethanol for 3 min, followed by 3% sodium hypochlorite for 3 min, 70% ethanol for 30 s, and rinsing with sterile distilled water. The level of sterility of the samples was assessed by pour plating onto Luria-Bertani (LB) medium using the last water used to rinse the plant samples. One gram of plant material was weighed, suspended in 1 M phosphate-buffered solution, and manually macerated in a mortar and pestle until a smooth suspension was obtained. Sample suspensions were serially diluted up to 10^{-9} dilutions, and 0.1 ml of an aliquot from dilutions 10^{-5} and 10^{-6} was pipetted into petri dishes and pour plated with sterilized LB agar. The inoculated petri plates were incubated at 28°C for 24 h. Distinct bacterial colonies that formed on the plates were counted and selected based on their morphological appearance. A pure culture of the bacterial isolate was obtained by repeated streaking onto sterile LB agar and incubated at 28°C for 24 h. The pure bacterial strains were kept on an agar slant and stored at 4°C for further use.

The pure strains were used for DNA extraction, using a commercial Quick-DNA miniprep kit specific for fungi or bacteria (Zymo Research, Irvine, CA, USA; catalog number D6005). Whole-genome sequencing was performed on Illumina's NextSeq platform at Inqaba Biotechnical Industries (Pty.) Ltd. (Pretoria, South Africa). The sample preparation (DNA library) was performed using a NextSeq midoutput kit, and a data set (2 × 150-bp paired-end reads) was generated for each sample. Genomic sequences were analyzed on the KBase platform (https://kbase.us/) (3). The quality of the sequence reads was evaluated using FastQC version 0.11.5 (4), while sequence adaptors and low-quality bases were removed using Trimmomatic version 0.36 (5). Furthermore, the sequence reads were assembled using SPAdes version 3.13.0 (6). Gene annotation and prediction were performed using the RASTtk (Rapid Annotations using Subsystems Technology) toolkit version 1.073 and the publicly

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Appendix 8: Published Genome Data from this study

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Data in Brief

journal homepage: www.elsevier.com/locate/dib

Data Article

16S rRNA gene amplicon sequence data from sunflower endosphere bacterial community

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ABSTRACT

Insights into plant endosphere bacterial diversity and exploration of their bioincentives in the formulation of biofertilizers promise to avert ecological disturbances. Here, we presented the sequence dataset of the endophytic bacterial community from the roots and stems of sunflower obtained from farmlands in Itsoseng and Lichtenburg, North West Province of South Africa, using 16S rRNA gene amplicon sequencing. The climatic conditions of this region are characterized by an annual rainfall of about 600 mm and a temperature range of 3 to 21°C during winter and 22 to 34°C during summer. The genomic DNA was obtained from 1 g of each macerated sample using commercial DNA kits (DNeasy® Plant Mini kit, Qiagen, USA). The DNA was amplified through polymerase chain reaction at the V4 region using the specific forward and reverse primers. Amplicon sequencing was performed on the Miseq Illumina platform. Sequence read processing was performed using QIIME 1 16S-based pipeline implemented on Nephele microbial bioinformatics platform using default parameters. The sequence has been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) with assigned Bioproject accession numbers. The data reveals the endophytic bacterial community structure and functions in sunflower cultivated in organic and nonorganic soils at growing and flowering stages.

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Appendix 9: Published Review article from this study

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REVIEW

Food Science & Nutrition Open Access WILEY

Oilseed crop sunflower (*Helianthus annuus*) as a source of food: Nutritional and health benefits

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Abstract

The use of biofertilizers in developing environmentally friendly agriculture as an alternative to chemical-based fertilizers in enhancing food production is promising in sustainable agriculture for the improvement in the yield of some commercial crops such as sunflowers and other oilseed crops in terms of quality and quantity. Sunflower is an important oilseed crop native to South America and currently cultivated throughout the world. Generally, the sunflower is considered important based on its nutritional and medicinal value. Due to its beneficial health effects, sunflower has been recognized as functional foods or nutraceutical, although not yet fully harnessed. Sunflower contains mineral elements and phytochemicals such as dietary fiber, manganese, vitamins, tocopherols, phytosterols, triterpene glycosides, α-tocopherol, glutathione reductase, flavonoids, phenolic acids, carotenoids, peptides, chlorogenic acid, caffeic acid, alkaloids, tannins, and saponins; and these compounds contribute to their functional and nutraceutical development. The extract from sunflower is known to be a potential source of antimicrobial, anti-inflammatory, antitumor, and antioxidants agents that protect human cells against harmful reactive oxygen molecules and pathogenic microorganisms. Also, the pharmacological survey on sunflower had revealed its curative power to different kinds of diseases. The health benefits of sunflower include blood pressure and diabetic control, skin protection, and lowering cholesterol and other functions. This review is written with appropriate referencing to previously published work and provides updated information regarding the new method of organic farming for sunflower production, nutritional and health benefits, and its by-products as human diet and livestock feed. Also, the constraints of sunflower production are elucidated.

KEYWORDS
food security, human nutrition, oilseeds, sunfoil, sustainable agriculture

Appendix 10: Published Review article from this study

Rhizosphere 20 (2021) 100433

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Rhizosphere

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Plant growth-promoting root-colonizing bacterial endophytes

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ARTICLE INFO

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ABSTRACT

The development of an environmentally friendly agricultural system as opposed to conventional methods using chemical fertilizers and pesticides for improved crop productivity is a promising aspect of modern agricultural biotechnology. Current research has focused on using free-living microbes that can colonize the plant endosphere as a means of enhancing crop productivity. In the plant rhizosphere, the complex root matrix facilitates microbe-microbe, microbe-plant, and soil-microbe interactions in establishing microbial communities, which precede endophytic colonization of the plant by some of these microbes. Endophytic microbes play an important role in plant growth promotion, as they employ direct or indirect mechanisms to facilitate plant growth by producing phytohormones and various secondary metabolites. The roles of endophytic microbes in sustaining plant growth under biotic and abiotic stresses through these mechanisms can provide insights into their envisaged putative functions in establishing host plant interactions for maximum use in the agricultural sector as an ecofriendly alternative tool to improve crop yield. In addition, a better understanding of endophytic bacteria functions in agriculture, medicine, biotechnology, and industry may enable scientists to unlock several opportunities by exploring valuable endophytic bioproducts in the recent application as bioinoculants, biostimulants, and environmental safety in pollution control and phytoremediation. Furthermore, the genomic insights into endosphere biology can provide detail structural diversity and functional profiling of endophytic microbiome for possible recommendations in future agriculture as a source of the organic amendment. Hence, this review emphasis on the root-colonizing endophytic bacteria and their importance in modern agricultural biotechnology.

Appendix 11: Accepted (In Press) Research article from this study



Articles in press

171/2021-PPS

Effect of endophytic bacterium, Stenotrophomonas maltophilia JVB5 on sunflower

Bartholomew Saanu Adeleke , Ayansina Ayangbenro , Olubukola Oluranti Babalola

abstract

Effect of endophytic bacterium, Stenotrophomonas maltophilia JVB5 on sunflower

Bartholomew Saanu Adeleke , Ayansina Ayangbenro , Olubukola Oluranti Babalola

Identifiable endophytic bacteria with plant growth-promoting traits promise to ensure sustainable agriculture. However, information on the versatility and exploration of sunflower-associated bacteria as bioinoculants is less studied. Here, we present the whole-genome sequence and annotation of *S. maltophilia* JVB5 isolated from the sunflower root endosphere from North West Province, South Africa. Whole-genome analysis revealed a genome size of 4,771,305 bp, sequence read count of 8,764,890, 66% GC content, 57 tRNAs, 268 contigs, and 4,160 protein-coding genes with functions in various metabolic pathways. Pathways involved in IAA production were found in the *S. maltophilia* JVB5 genome. The whole-genome annotation predicted notable genes involved in bacterial colonization, antibiosis, and plant growth promotion. The predicted genes involved in sulfur metabolism, and oxidative stress may enhance plant growth promotion and boost plant resistance to stress. Upon inoculation, *S. maltophilia* JVB5 efficiently colonized sunflower root under greenhouse with significant improvement on the wet plant weight of 437.20 g compared to uninoculated with 331.04 g wet weight. The genomic analysis revealing specific functional genes in the bacteria genome suggests its bioprospecting in agriculture. Hence, understanding the mechanisms employed by *S. maltophilia* JVB5 based on the predicted multifunctional genes will help harness their bioresource in sustainable plant health.

Keywords:

bacterial genome; crop improvement; putative phytohormone genes; root adherence; sunflower; sustainable agriculture