Metagenomic Analysis of Endophytic Microbiome Inhabiting Maize Plant

A.E. Fadiji

orcid.org/0000-0002-2893-6658

Thesis accepted in fulfilment of the requirements for the degree
Doctor of Philosophy in Biology
at the North-West University

Promoter: Prof O.O. Babalola

Graduation ceremony: December 2021
Student number: 33462623

DECLARATION AND APPROVAL

Declaration by candidate

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Natural and Agricultural Sciences, and the work contained herein is my original work with exception of the citations and that this work has not been submitted at any other University in part or entirety for the award of any degree.

Ayomide Emmanuel FADIJI

SIGNATURE…. **……**

DATE……………………………………...

Declaration by supervisor

I confirm that the work reported in this thesis was carried out by the candidate under my supervision.

Professor Olubukola Oluranti BABALOLA

SIGNATURE…………………………….

DATE………………………………….

DEDICATION

This work is dedicated to Almighty God, for His faithfulness, guidance and infinite mercy over my life.

ACKNOWLEDGEMENTS

My special gratitude goes to my wonderful mentor and supervisor, Prof Olubukola Oluranti Babalola. Thank you for your encouragement, support, guidance, valuable advice, constructive criticism and constant motivation towards the timely completion of this work. It was a great pleasure working with you ma. I appreciate Dr A.S. Ayangbenro for his encouragement and support at all times. I deeply appreciate Prof. Helen Drummond for her ceaseless support.

I acknowledge with gratitude the support of National Research Foundation, South Africa/The World Academy of Science (NRF-TWAS) African Renaissance PhD scholarship (Ref: UID116107) which greatly supported me, and North-West University for offering me bursary award to pursue the PhD degree.

My special thanks also goes Prof O.S Bello of the Department of Pure and Applied chemistry, Lautech, Ogbomoso, Oyo State for showing me the way and encouraging me to go for my PhD, I appreciate you and pray God will continue to uphold and increase you sir. I sincerely appreciate my brother Mr Seyi Fadiji for supporting me both morally and financially in making my dream come true, I pray your fountain will never run dry in Jesus Name. My thanks also go to my master's supervisor Dr Mrs I.O. Omomowo and her husband Mr I.O Omomowo for standing by me at all times, may God continue to increase you.

My special thanks to, Prof O.A. Afolabi and Dr Segun Omolewa, for their fatherly counsel and encouragement may God continue to guide and uphold you. I would like to express my indebtedness to all those who contributed directly or indirectly to the success of this project; Prof S.S. Babalola, Prof. and Mrs Abiodun Salawu, Prof. and Prof. (Mrs). Akpovire Oduaran, Dr Ruth Adebayo, Dr A. Ayandele, Dr O.N. Majolagbe, Dr. A.E. Amoo, Mr O.O. Aboyeji, Dr Bernard Ojuederie, Dr Uju Emmnauel, and Mrs Fumilayo Adeoye, I will forever be grateful.

My appreciation also goes to the members and staff of the Department of Biological Sciences, the present HODs, Dr Hazel Mufhandu as well as all faculty and staff members of the Faculty of Natural and Agricultural Sciences, North West University, for their immense support. My sincere thanks goes to my wonderful family and friends in North West University, for their love encouragement and support. I would like to specially thank all my colleagues in the Microbial Biotechnology Research Group, Dr K.A.Odelade, Dr Tayo Alawiye, Dr Atilade Adedeji, Dr Ben Enagbonma, Mr B.S. Adeleke, Mrs O. Fasusi, Dr Matthew Enebe, Mrs Fortune Chukwuene, Mrs Pelumi Omotayo, and Mrs Tobi Ayiti.

I thank my parents, Mr Femi and Mrs Grace Fadiji, for investing so much in me and for their prayers, encouragement and support both spiritually and morally. May you live long to reap the fruit of your labour. I am also indebted to all my wonderful siblings, Mr Tosin Fadiji, Miss Damilola Fadiji, and Mr Fortunate Fadiji for being so supportive. I sincerely appreciate my brother and confidant Mr Kayode Ogunwole and his wife for being there for me, I thank you so much.

I will also like to appreciate my pastors Pst. Theophilus Bajulaiye, Pst. Abiodun Olatunji, Pst. Stephen Adeyeba, Prophet Sunday Fagbamila, Prophetess Elizabeth Fajinmi and Pst Ibiyomi for their prayers and spiritual support. Similarly, I appreciate the pastorate and members of Living Faith Church, Irewolede, Ilorin, Kwara State, Nigeria and the Redeemed Christian Church of God (GHA parish), Mafikeng Area II, South Africa.

Above all, I give all the glory, honour and adoration to almighty God for His mercies and abundant grace in my life.

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GENERAL ABSTRACT

Endophytes are promising microbes which support the plant in many beneficial ways such as growth promotion and health improvement with no harmful effects to their host. However, to have better insights in to important microbes, diverse next-generation sequencing (NGS) methods have been adopted to study the diversity of microbes in the maize plant, with limited understanding of the functions. Presently, shotgun metagenomics is one of the novel sequencing techniques which allows the profiling of the entire microbial communities and their functions in a given environment. Therefore, this study is aimed at profiling the influence of different farming practices on the diversity, community structure, and functions of the endophytic microbiome associated with the root of maize plant (*Zea mays* L.) using shotgun metagenomic sequencing. The study was carried out on the experimental plots of North-West University School Farm, Molelwane, Mafikeng, North West Province, South Africa. Three experimental sites were adopted within the farm representing different farming practices namely inorganic fertilizer (NK) site, organic fertilizer (FK) site, and no fertilizer (CK) site. These sites have been in operation for more than 15years following standard procedures. Maize seed WEMA (WE 3127) was used in the experiment, these seeds were planted on the 3 sites respectively. Each farming site was divided into three regions representing 3 replicates for each farming site for the sequencing. The plants were harvested at the fruiting stage of growth. The total DNA extraction for each biological replicate was carried out and sequenced using shotgun sequencing approach. The sequenced data obtained in fastq format were uploaded on to an online database called MG-RAST where quality control (QC) assessments were carried out. Our taxonomic annotation results using the Subsystem database revealed a total phyla of 28 endophytic bacteria, 3 endophytic archaea and 2 endophytic fungi in all the samples. The major endophytic bacteria phyla observed were Firmicutes, Bacteroidetes, Actinobacteria, Bacilli, Proteobacteria, Acidobacteria, Chloroflexi, Verrucomicrobia, Tenericutes, Planctomycetes, Cyanobacteria, and Chlorobi. Bacteroidetes dominated maize from organic fertilizer sites, Firmicutes dominated the no fertilizers site while Proteobacteria dominated inorganic fertilizer sites. The three identified endophytic archaea phyla are Crenarchaeota, Euryarchaeota, and Thaumarchaeota while the identified fungi phyla were Ascomycota and Basidiomycota in the samples across sites. The diversity analysis revealed that the abundance of endophytic bacteria, archaea and fungi in all the sites are in the order organic fertilizer (FK) > no fertilizer (CK) > inorganic fertilizer (NK) with higher abundance in samples from organic fertilizer site. Our functional annotation results further revealed a total of 28 functional groups within the endophytic microbiomes across the farming sites. Some functional groups and metabolisms associated with plant growth promotion such as secondary metabolism, nitrogen metabolism, iron acquisition and metabolism alongside phosphorus metabolism were observed in the endophytes across the sites. Our results further showed the presence of putative functional genes associated with plant growth-promotion (PGP) and endophytic behaviors. Taken together, using the shotgun metagenomic approach, this study showed that organic farming has a positive influence on the diversity, abundance, and functions of the endophytic microbiome in maize plant.

Keywords: Illumina HiSeq, Microbial communities, Shotgun sequencing, Symbiosis, *Zea mays* L., Endophytes, Fertilizer application, Plant associated microbes, Metagenomes, SEED subsystems

LIST OF PUBLICATIONS

-Chapter 2: Metagenomics methods for the study of plant-associated microbial communities: A review.

This chapter has been published in *Journal of Microbiological Methods* 2020;170:105860. https://doi.org/10.1016/j.mimet.2020.105860

Authors: Ayomide Emmanuel Fadiji and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches and wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

-Chapter 3: Exploring the potentialities of beneficial endophytes for improved plant growth.

This chapter has been published in *Saudi Journal of Biological Sciences* 2020; 27:3622-3633. https://doi.org/10.1016/j.sjbs.2020.08.002

Authors: Ayomide Emmanuel Fadiji and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches, wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

-Chapter 4: Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects.

This chapter has been published in *Frontiers in Bioengineering and Biotechnology* 2020; 8:467. https://doi.org/10.3389/fbioe.2020.00467

Authors: Ayomide Emmanuel Fadiji and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches, wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

-Chapter 5: Metagenomic profiling of the community structure, diversity, and nutrient pathways of bacterial endophytes in maize plant.

This chapter has been published in *Antonie van Leeuwenhoek* 2020; 113:1559-1571. https://doi.org/10.1007/s10482-020-01463-w

Authors: Ayomide Emmanuel Fadiji, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches, carried out the planting and laboratory works, performed the analyses, interpretation of results, wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

-Chapter 6: Organic farming enhances the diversity and community structure of endophytic archaea and fungi in maize plant: a shotgun approach.

This chapter has been published in *Journal of Soil Science and Plant Nutrition* 2020; 20:2587–2599. https://doi.org/10.1007/s42729-020-00324-9

Authors: Ayomide Emmanuel Fadiji, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches, carried out the planting and laboratory works, performed the analyses, interpretation of results, wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

-Chapter 7: Shotgun metagenomics reveals the functional diversity of root-associated endophytic microbiomes in maize plant

This chapter has been published in *Current Plant Biology* 2021; 25:100195. https://doi.org/10.1016/j.cpb.2021.100195

Authors: Ayomide Emmanuel Fadiji, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches, carried out the planting and laboratory works, performed the analyses, interpretation of results, wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

-Chapter 8: Unveiling the putative functional genes present in root-associated endophytic microbiome from maize plant using the shotgun approach

This chapter has been published in *Journal of Applied Genetics* 2021; 62:339–351. https://doi.org/10.1007/s13353-021-00611-w

Authors: Ayomide Emmanuel Fadiji, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's contributions: managed the literature searches, carried out the planting and laboratory works, performed the analyses, interpretation of results, wrote the first draft of the manuscript and revised the manuscript based on reviewers' comments.

ACRONYMS AND ABBREVIATIONS

NK	Samples from inorganic fertilizer site
FK	Samples from organic fertilizer site
CK	Samples from no fertilizer site
USA	United States of America
MG-RAST	Metagenomics rapid annotation
PAST	Paleontological Statistics
ANOSIM	Analysis of similarities
PCA	Principal component analysis
PCoA	Principal coordinate analysis
NCBI SRA	National Center for Biotechnology Information's Sequence Read Archive
QC	Quality control
YEM	Yeast extract-mannitol medium
NaOCl	Sodium hypochlorite
DNA	Deoxyribonucleic acid
e^H/S	Evenness
α	Alpha
β	Beta

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Maize (*Zea mays* L.) is one of the major cereal crops in Africa and is produced almost all over the continent under various environmental conditions (Alori et al., 2019). Adequate application of production inputs that will ensure environmental sustainability is necessary for maize production to be successful. Some of the inputs are, adapted cultivars, soil tillage, plant population, weed, soil tillage, insects, fertilization, financial resources, disease control, marketing, and harvesting (Du-Plessis, 2003). In advanced countries of the world, maize is most times eaten as second-cycle produce, majorly in meat forms, dairy products, eggs etc (Du-Plessis, 2003, Alori et al., 2019). However, in most developing countries, it is often eaten directly and is a major staple diet for more than 200 million people. Most people in these countries adopt maize as their major breakfast cereal. When processed, maize can also be used as fuel (ethanol) and starch sources.

In South Africa, about eight (8) million tons of maize grain are produced yearly on almost 3.1 million hectares of land. Half of this production is white maize, which is majorly consumed by humans (Du-Plessis, 2003). Maize being a household food crop can be grown in almost all soil types (Iken and Amusa, 2004), with different degrees of yield. More than fifty species are cultivated depending on the region; the species vary in taste, texture, sizes and shapes.

The plant microbiome research has gained prominence and attention in the scientific world since last few decades. Many studies have shown that microbial communities associated with higher plants performed notable functions such as supporting health, enhancing growth and sustenance of their hosts (Hardoim et al., 2015, Bulgarelli et al., 2013). These microbes comprising of archaea, fungi, bacteria, protists, and viruses inhabit land plants. Some inhabit the root surroundings (rhizosphere), surface of the root (rhizoplane), the the leaf plant surface (phyllosphere), stem (caulosphere), leaves (phylloplane), flowers (anthosphere) and fruits (carposphere) (De Tender, 2017). Microorganisms are always attracted to plants based on the rich source of nutrients attributed to the plant (Compant et al., 2019). Some of these microorganisms originate from close environments such as water, soil and air. The plant exudates are released into the soil environment surrounding the roots, and these attract microbes that have the tendency of competing with each other. Plant microbiome studies have rapidly developed due to the fact that these microbiomes have been shown to have tremendous benefits on plant's health, productivity, and growth (Mayak et al., 2004, Niu et al., 2017).

Plant associated microbiota are also components of the plant genome and metagenome, considering the fact that different species of microorganisms are involved in performing distinct beneficial functions to a plant (Kristin and Miranda, 2013). Many notable functions have been attributed to beneficial plant microbiota, some of which include, plant protection from pathogens and pests, as well as plant growth promotion and stress tolerance (Singh and Dubey, 2018, Kumar et al., 2017). These notable functions can be explored to combat some of the challenges currently facing agriculture, especially food sustainability.

The world population is increasing daily and there is an urgent need to ensure increased food production (Omomowo and Babalola, 2019). A recent study conducted by Sessitsch et al. (2019), reported that about 52% of all fertile soils used for food production globally are now degraded, and it has been projected that if alternative means of boosting soil nutrients are not discovered it will lead to a decline in food production globally by about 12% in the next 25 years. Furthermore, our society seriously requires a sustainable eco-friendly agricultural practice. The application of beneficial plant-associated microbiota stands a chance to improve plant production, especially when cultivated under extreme conditions, and also of reducing our dependence on synthetic fertilizers (Xia et al., 2015, Mefteh et al., 2019). Notable among these beneficial plant-associated microbiota are the endophytes.

Endophytes are one of the major beneficial plant-associated microbiota. According to a definition given by Petrini (1991), endophytes encompass all microorganisms that inhabit the organs of a plant at one point or the other in their life cycle, which can colonize the internal tissues without causing any harm to their host. Most plants develop an association with beneficial endophytes and this can be traced back to co-evolution, which occurred millions of years ago (Nair and Padmavathy, 2014, Hardoim et al., 2015). Endophytes are advantageous to their host due to the symbiotic association they develop with their host, but recent studies have shown that some can be pathogenic or saprophytic at certain stages of their host growth cycle and/or under certain environmental stresses (Jia et al., 2016, Brader et al., 2017a, Fadiji and Babalola, 2020b). They have been reported to enhance plant growth through, phytostimulation, nutrient recycling, antimicrobial properties, and biocontrol activities among others (Fadiji and Babalola, 2020a). These activities are carried out through different mechanisms of action, majorly direct and indirect mechanisms.

Aside from their benefits to plants, they are also important to human. Endophytes have been discovered as an important source of bioactive compounds which are of immense importance to humans. Some of the bioactive compounds produced by endophytes have the prospect of serving as anticancer, antidiabetic, antimalarial, antitumor, antituberculosis, antiviral, and antiarthritis agents among others (Gouda et al., 2016, Fadiji and Babalola, 2020a). Considering, its immense importance to humans, the internal microbes' colonization of maize needs to be precisely analyzed for the potential utility of these endophytes as a delivery system of bioactive metabolites for controlling plant diseases as well as enhancing its growth and yield (Rai et al., 2007). Knowing all these prospects of endophytes, in this study, the endophytic microbiome associated with the root of maize plant were assessed.

It is important to state that different farming practices exist in crop production with varying degrees of effects on plant microbiome, however recent studies have revealed that organic farming is now being embraced globally (Xia et al., 2019). An organic farming systems is the combination of different practices to increase soil quality and nutrient optimization while eliminating the use of chemical pesticides and inorganic fertilizers alongside reducing their effects on non-target microorganisms (Xia et al., 2015). This farming practice has been designed to embrace methods such as cover cropping, manure and compost amendments to enhance soil nutrient in a bid to improve the biodiversity and health of soil microbes (Heckman, 2006, Zarb et al., 2005). Many studies have been carried out to unravel the differences between organic and conventional farming practices, and their effects on microbial community populations and diversity in soil and plants (Xia et al., 2019, Araújo et al., 2009, Li et al., 2012c, Xia et al., 2015).

To have bettter undestanding of these beneficial microbes, it is important to state that in any ecosystem either artificial or natural, three major questions are often asked as regards the identification and characterization of the community of microbes: (1) what type of microorganisms inhabit the environment? (2) what are the functions of these microorganisms? and (3) how does the ecosystem affect the functions performed by these microbes? (Rastogi and Sani, 2011). Culturing microorganisms using a commercial culturing kit makes it easier to identify and characterize these microbial species (De Tender, 2017). But, more than 99% of microbes in any given environment are still not culturable using existing standard techniques of cultivation, which makes numerous microbial communities underexplored (Staley and Konopka, 1985, Fadiji and Babalola, 2020c). Interestingly, the discovery of culture-independent techniques now makes it easier to analyze the non-culturable endophytic microorganisms inhabiting the tissue of plants (Fadiji and Babalola, 2020c).

The development of advanced molecular techniques introduced in the 1980s, which employed the direct extraction and characterization of DNA from environmental samples (e.g. plants, water, and soil), transformed the studies of microbiomes in the environment (Fox et al., 1980, De Tender, 2017). Most studies involving plant microbiomes often employ amplicon-based techniques to analyze the community structure which only focuses on a few sets of representative pathogens or beneficial microbiome in plants. The 16S ribosomal RNA (rRNA), Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) techniques are often used as a molecular marker for identification of bacteria (Fadiji and Babalola, 2020c). The 16S rRNA gene is an extremely conserved region among the numerous species of archaea and bacteria which consists of 2 types of regions: (1) the extremely conserved regions which are mainly for primer design and (2) the hypervariable region produces species-specific sequences which are of great importance in the identification of bacteria (De Tender, 2017). Similar to the 16S rRNA gene is the internal transcribed spacer (ITS) region, often used across a wide class of fungi but contains detailed variation which makes it easier for phylogenetic analysis (Lindahl et al., 2013). Like in many molecular techniques, only a few parts of both phylogenetic markers can be utilized.

However, all these limitations were overcome with the development of methods that can be used for community evaluation such as high throughput sequencing (HTS) techniques (Fadiji and Babalola, 2020c). To evaluate the whole microbiome in a particular environment, two (2) major types of HTS techniques can be used namely: shotgun metagenomics and metatranscriptomics. However, in this study, shotgun metagenomics, which is one of the novel techniques in the study of plant microbial communities, was used to answer questions that have to do with profiling of endophytic microbiome (bacteria, fungi and archaea) inhabiting maize plant, giving insight into the microbial community composition, their functional potentials alongside assessing the impact of farming practices on their existence.

1.2 Statement of problem

Microorganisms represent a major fragment of the overall biomass and sources of biodiversity on earth (Vieites et al., 2010). The progression of structure and functions of microbiome remain a major topic in environmental microbiology (Cong et al., 2015). Association between plant and microorganisms can be influenced by several factors, some of the factors include the symbiont's genetic diversity; how they are acquired from environment and individual's ability to colonize hosts (De Tender, 2017). Large biological diversity exists among endophytes, with some plant species harbouring over 100 different endophytic species (Correa-Galeote *et al.*, 2018). Many attempt have been made to characterize endophytic microbial communities inside maize plant using advanced molecular methods such as 16S rRNA, ITS, DGGE, and T-RFLP, but due to their limitation their results are often not reliable (Woźniak et al., 2018, Maropola, 2014, Xia et al., 2019). Characterization of endophytic microbial communities through these conventional methods or protocols is often biased, as they do not allow for total community analyses. Also, only less than 2% of the overall population of the microbiome can be identified with these methods, in which only organisms with high abundance tend to be detected (De Tender, 2017, Akinsanya et al., 2015b**.** However, the introduction of HTS techniques have enabled us to access and identified more microbial communities resident in maize plant while their roles and functions in the tissues of plants have not been effectively profiled and are not fully understood (Fadiji and Babalola, 2020c). Furthermore, the effect of different farming practices on the structure, diversity and functions endophytic microbiome in maize plant have not been thoroughly examined (Reinhold-Hurek and Hurek, 2011).

In South Africa, most maize farmers adopt conventional farming methods through the use of chemical fertilizer for enhancing plant yield. The country's reliance on chemical fertilizers has encouraged the industries manufacturing these chemicals, which have not only been reported to be dangerous for human consumption but can strongly disturb the balance of the ecosystem thereby affecting our farmlands (Savci, 2012). Excessive application of chemical fertilizers to plants not only generates adverse effects of being environmentally risky, but it may also affect the microbial communities, seed quality, and reduce yield through an increase of plant lodging (Xia et al., 2019). Similarly, inorganic fertilizers are not cost-effective and are a non-renewable source of nutrients available to plants (Scheiner *et al*., 2002). There is an urgent need to look at organic farming and microbial sources which have great plant growth and yield promoting qualities.

1.3 Justification

As a result of the introduction of HTS techniques, the expertise needed to characterize the spatial and temporal differences of plant microbial communities has become achievable (Fadiji and Babalola, 2020c). In this study, the shotgun metagenomic sequencing was emplored because it enhances the evaluation of the whole microbiome in environmental samples (plants) without having to go through stressful cultivation efforts (Vieites et al., 2010, Babalola et al., 2020). In shotgun metagenomic sequencing, all DNA samples pulled out from a particular environment will be analyzed, instead of focusing on a distinct genomic locus. This novel globally recognized technique is dependent on two major steps. Firstly, the splitting of the DNA molecules into some tiny gene fragments, followed by independent sequencing. Secondly, the gene fragments are then reassembled (Bouchez et al., 2016, Fadiji and Babalola, 2020c, Fadiji et al., 2020b). Some of the reads obtained are then used to provide taxonomically relevant information, while others will be used to give necessary information on the biological function concealed in the genome (Sharpton, 2014, Fadiji et al., 2020a, Fadiji et al., 2021). Shotgun metagenomics is advantageous over other similar techniques such as amplicon sequencing and other previous sequencing techniques for 2 main reasons: (1) extensive sequencing of the entire metagenome which enables the diversity, community structure, and functional evaluation of microbial communities in a given environment, and (2) if they exist in the database before, new functions can be allocated to the particular environment in which they were formally not recognised (Bouchez et al., 2016).

Furthermore, organic farming was designed to lessen the effects of chemical pollution, enhance the diversity and abundance of microorganisms in the soil, alongside improving the value, yield and food security (Van Quyen and Sharma, 2003, Xia et al., 2019). Some studies have examined the structural and functional association among culturable endophytic microbiome of plants grown using inorganic and organic farming practices (Xia et al., 2015, Xia et al., 2019). However, a well-structured study on the effects of different farming methods on endophytic microbiomes in the maize root is rare. The roots of a plant have been reported to be the part of a plant that harbors the highest population of endophytes (Das and Varma, 2009, Sessitsch et al., 2012), therefore, roots of maize plant were used in this study. To the best of our understanding, no study exists presently on the impacts of different farming methods on the structure, functions and diversity of endophytic microbiomes in roots of maize plant using the shotgun metagenomic technique. Hence, this study presents the first report profiling the structure and functions of endophytic microbiomes in the roots of maize plant using the shotgun metagenomic approach. This culture independent approach will enable us to profile the effect of farming practice on the diversity, abundance, and functions of endophytic microbiomes inhabiting maize plant, which are known to play a major role in the growth, development, and health of their host (Basha and Ramanujam, 2015, Berg et al., 2014a).

1.4 Aim and Objectives

1.4.1 Aim

The aim of this thesis is to profile the influence of different farming practices/methods on the community structure, diversity and functions of endophytic microbiome associated with the roots of maize plant (*Zea mays* L.) using shotgun metagenomic sequencing.

1.4.2 Specific objectives

Specific objectives are to:

(i) determine the structural and compositional diversity of endophytic microbial communities associated with the root of maize plant;

(ii) investigate the functional diversity of endophytic microbiome associated with the root of maize plant;

(iii) assess the effect of different farming practices on the community structure, diversity and function of endophytic microbiomes associated with the root of maize plant;

(iv) to determine the plant growth-promoting and endophytic genes in the identified endophytic microbiome.

CHAPTER TWO

2.0 METAGENOMICS METHODS FOR THE STUDY OF PLANT-ASSOCIATED MICROBIAL COMMUNITIES: A REVIEW

Abstract

Plant microbiota have different effects on the plant which can be beneficial or pathogenic. In this study, we concentrated on beneficial microbes associated with plants using endophytic microbes as a case study. Detailed knowledge of the microbial diversity, abundance, composition, functional genes patterns, and metabolic pathways at genome level could assist in understanding the contributions of the microbial community towards plant growth and health. Recently, the study of microbial community has improved greatly with the discovery of next-generation sequencing and bioinformatics technologies. Analysis of next generation sequencing data and a proper computational method play a key role in examining the microbial metagenome. This review presents the general metagenomics and computational methods used in processing plant associated metagenomes with concentration on endophytes. This includes 1) introduction of plant-associated microbiota and the factors that drive their diversity. 2) plant metagenome focusing on DNA extraction, verification and quality control. 3) metagenomics methods used in community analysis of endophytes focusing on maize plant and, 4) computational methods used in the study of endophytic microbiomes. Limitations and future prospects of metagenomics and computational methods for the analysis of plant-associated metagenome (endophyte metagenome) were also discussed with the aim of fostering its development. We conclude that there is a need to adopt advanced genomic features such as k-mers of random size, which do not depend on annotation and can represent other sequence alternatives.

Keywords: Bioinformatics, Endophytes, Illumina HiSeq, Microbiomes, Shotgun metagenomics, *Zea mays*

This chapter has been published in *Journal of Microbiological Methods* 2020;170:105860. https://doi.org/10.1016/j.mimet.2020.105860

2.1 Introduction

Advances in molecular biology have led to the development of omics techniques, which recently gained prominence in the diversity and abundance study of microbes (Brader et al., 2017b, Alawiye and Babalola, 2019). The word ‘metagenomics’ was first introduced in the year 1998, and was defined as the evaluation of all the genetic materials isolated directly from the environmental samples (Handelsman, 2005). More importantly, it is one of the major methods employed in the investigation of the complex community of microbes that inhabit environmental samples through analysis of the content of the nucleotide sequence (Hugenholtz and Tyson, 2008). Metagenomic researches may employ either a targeted or shotgun sequencing approach, this depends largely on the type of environmental studies to be conducted. Genomic studies of environmental samples are gaining ground in recent years as a major tool for better understanding of the evolutionary history, structural, functional and ecological biodiversity (Shokralla et al., 2012). It completely discourages the need for laboratory isolation and culturing of specimens. Some environmental samples used for the study are water, plants, sediments, sometimes collected from aquatic, terrestrial specimens, gut contents, benthic specimens and faeces. The conventional Sanger DNA-sequencing method (Sanger et al., 1977) can only be used for individual DNA sequencing and thus information from it will be insignificant when complex environmental samples are to be processed. Samples of this nature do contain numerous DNA ranging from thousands to millions of individuals. Though conventional sequencing has created an avenue for the buildup of large barcode DNA reference libraries, yet the numbers of DNA in an environmental sample is more than what the capacity of conventional method can curtail (Hajibabaei et al., 2007)

Next-generation sequencing (NGS) has created an enabling platform to retrieve DNA sequence data straight from samples in the environment (Sogin et al., 2006). These data are reported to have numerous applications, some of which include a comparison of microbiota present in diseased and healthy individuals (Andersson et al., 2008, Zhang et al., 2009a); biodiversity studies of the ecosystem (Hajibabaei et al., 2011); evolutionary studies of DNA (Boessenkool et al., 2012) and the analysis of faeces or gut DNA fragments (Deagle et al., 2009). The comparison of the sequence data with a growing standard reference library of identified organisms will help in presenting the different taxa available in environmental samples with confidence. Recent findings in the computational techniques have aided the study of biodiversity across space and time through the use of annotation and DNA clustering employing phylogenetic and alignment techniques (Hajibabaei et al., 2011). Due to corresponding increases recorded in both breadth and number of data using NGS platforms, ecological researches are now been channeled towards the use of large volumes of sequence data. This approach has helped in reducing error and bias results attributed to PCR and the run time is significantly shorter. Many platforms have been discovered lately, each with advantages and disadvantages. These identified platforms use distinct templates in their preparation and different chemistries in their sequencing signal detection (Mardis, 2013, Van Dijk et al., 2014).

Plants are inhabited by a great number of microorganisms which includes bacteria, archaea, fungi, viruses and oomycetes (Brader et al., 2017c). The microbes associated with plants can be classified as pathogenic or nonpathogenic. The nonpathogenic association may be mutualistic, beneficial, commensal or neutral (Jones et al., 2019). Prominent among organisms involved in beneficial association with the plant is endophyte. Endophytes are simply defined as microbes that colonize plant tissues without showing any harmful effects (Bamisile et al., 2018, Odelade and Babalola, 2019). Many plants have different endophytic microbes specific for their colonization, either mutualistic or beneficial for plant growth improvement and health (Verma and White, 2018), though recent findings revealed some pathogenic species (Brader et al., 2017b). Endophytes gain access to the plant through the rhizospheric soil and colonize the tissues of plants through horizontal transmission and vertical seeding methods (Omomowo and Babalola, 2019). Whatever the colonization pattern adopted, endophytes are still known to be beneficial to the host plant (Huang et al., 2016). The abundance of endophytes is greater in the roots and decreases as it cuts across the stem to the leaves (Das and Varma, 2009). In a bid to sustain stable symbiosis, endophytes produce many compounds which help in growth promotion of the plants and better adaptation to the environment (Das and Varma, 2009). The review gives a detailed explanation of the metagenomics and computational methods used in studying plant-associated microorganisms with focus on endophytes taking advantage of genome sequences information. An introduction to plant-associated microbes and factor affecting diversity of microbiota is given. This is followed by a general introduction to plant metagenome, plant DNA extraction, storage and quality control. A detailed review of the metagenomics method for community analysis of endophytes is discussed using maize plant as a case study in the section. Also, a comprehensive review of the computational methods and tools used for the community analysis of endophytic microbes including taxonomic composition, alpha and beta statistical analyses, taxonomic similarity and differences, and functional analysis of microbial genes are discussed. In conclusion, the limitations and future prospects of metagenomics and computational methods for the analysis of endophytic microbial community are discussed.

2.2 Plant-associated microbiota

Most ecological niches contain numerous microorganisms. A plant contains different nutrients which aid in attracting microorganisms to it. Microorganisms found on the surface of the plant are called epiphytes while those who inhabit plant tissues are called endophytes (Whipps et al., 2008). Plants present three types of environments depending on the one found to be conducive for the microbiota (Figure 2.1). The first environment is called the rhizosphere, this is where microorganisms interact with the soil and roots, the environment also contains many exudates from the plant (Walker et al., 2003). Endosphere is the second environment and its means inside the tissues of the plant (Hardoim et al., 2008). The third environment is called the phyllosphere which comprises the surface of the leaves and stems. The phyllosphere is known not to be a conducive environment for microorganisms, this is because nutrients availability in this environment is limited, irradiation of the sun is strong and water availability is inconsistent (Berlec, 2012). Plant-associated microorganisms are regarded as mutualist, commensals or pathogens (Brader et al., 2017b), the pathogenic ones are of great concern to scientists because of the economic importance of plants. This study also focused on the mutualist found in the endosphere e.g. endophytes, which by definition, cause no harm to the plant. It is also important to state that under certain conditions, plants also benefit from the numerous genes and proteins present in these microbes.

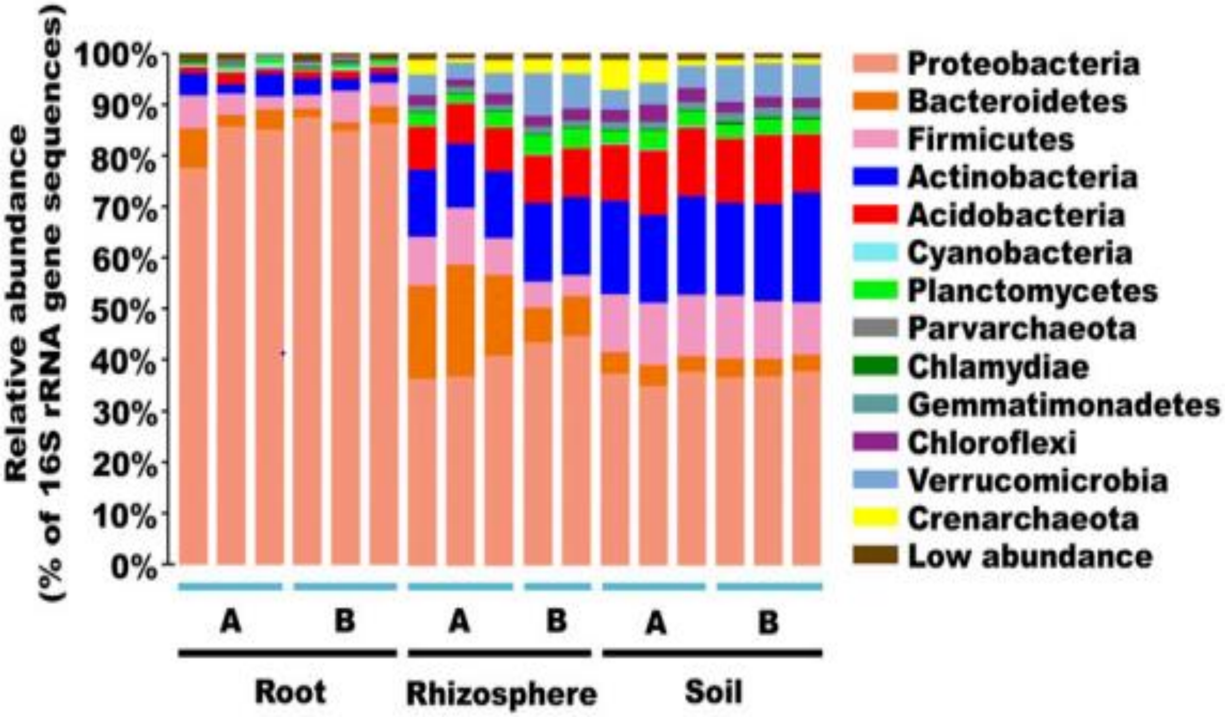


Figure 2.1: Histogram showing the abundance of endophytic bacterial phyla in different environments of maize plant (Niu et al., 2017)

2.2.1 Factors driving community diversity of microbiota

Designing the structure of a community (using the diversity estimates such as Unifrac distances or Bray-Curtis dissimilarity) depends greatly on variables from the environment, which assist in discovering which portion of the whole data will be linked to each environmental factor (Wagner et al., 2016, Dombrowski et al., 2017, Zgadzaj et al., 2016). The environmental factors involved in the variation of microbiota are calculated to give approximately 20-30% differences in the data when fixing the technical factors (Peiffer et al., 2013, Schlaeppi et al., 2014, Dombrowski et al., 2017, Wagner et al., 2016). However, these variables generally cannot stand alone and it is difficult to unravel their interactions and effects. For example, sampling using plants cultivated on natural soil confuse changes in climatic conditions with soil properties, which are most times combined as the same variable on a site (Wagner et al., 2016).

Also, profiling natural communities through sampling in the greenhouse or the field exposed to the controlled environment makes it difficult to differentiate between the soil chemical characteristics (such as nutrient availability, pH, etc.), physical properties and other local microbiota. This shows the limitation in an attempt to establish the relationships among environmental factors when a culture-dependent approach is employed in the evaluation of the microbial community (Garrido-Oter, 2018).

Microbes colonize plant roots successfully and assemble their communities, thereby forming a network of different interactions. The majority of these interactions are not restricted to each microorganism and the plant alone, but also involve associations among other microorganisms (Agler et al., 2016). The analysis of co-existence and removal pattern in the community of microbes includes but is not restricted to those related to eukaryotic hosts alone, it further buttress the roles of microbe-microbe interactions, structuring and stability (Edwards et al., 2015, Zhang et al., 2014b, van der Heijden and Hartmann, 2016, Lima-Mendez et al., 2015). Furthermore, analysis carried out on metagenomics sequences obtained from plant rhizosphere and roots (Ofek-Lalzar et al., 2014, Bulgarelli et al., 2015) has shown that some important genes are responsible for interaction and competition with other microorganisms and these genes also enhance their adaption or survival in the environment of the host e.g. endophytic genes (Santoyo et al., 2016).

Assessment of the diversity of the genome existing in individual host species has shown that the genotype of the host has limited effect on the variation in the community as compared to the organ of the host (stem, root or leaf), root fraction (rhizosphere, soil, endosphere or rhizoplane) and natural site (or soil type) (Garrido-Oter, 2018). Deconvolution of variance estimates from beta-diversity between genotypes of maize (Peiffer et al., 2013) showed that approximately 5%

of the variation recorded in the community structure can be linked to the genotype of the host. However, further research into the effect of genotype in the diversity of microbiota is required. A possible approach in addressing this question is the adoption of shotgun metagenome sequencing for the plant microbiome, this approach has the ability to give a high functional data as well as high-resolution taxonomic profiles (Mendes et al., 2014, Bulgarelli et al., 2015, Ofek-Lalzar et al., 2014).

2.3 Plant associated metagenome

Metagenomics can improve our understanding of the importance of microbes to plants and the associations that exist among them (Melcher et al., 2014). Presently, many public genomic databases for plant-associated microbial communities and metagenome are developed each year. DNA extraction from the plant tissues is important in plant metagenome studies. Diversity of endophytes in plants was recently studied by Porras-Alfaro and Bayman (2011). The authors reported that one major difficulty in the metagenomics analysis of endophytes is the technical challenge of how to separate microbes from the plant DNA. DNA of the plant is most times more dominant than that of endophytic microbe, thus making the isolation and sequencing of endophyte metagenome at high coverage tough. Figure 2.2 presents some of the molecular and omics methods that have been employed in understanding the diversity of endophytes. As reported by Porras-Alfaro and Bayman (2011), plant-associated microbes especially, endophytes are under-investigated sources of bioactive molecules. Metagenomics studies will help in unravelling more beneficial functions of endophytes and also give a pointer to possible culturability of beneficial species (Cuadros-Orellana et al., 2013). This section discussed the plant DNA extraction techniques, preprocessing methods and quality control process.

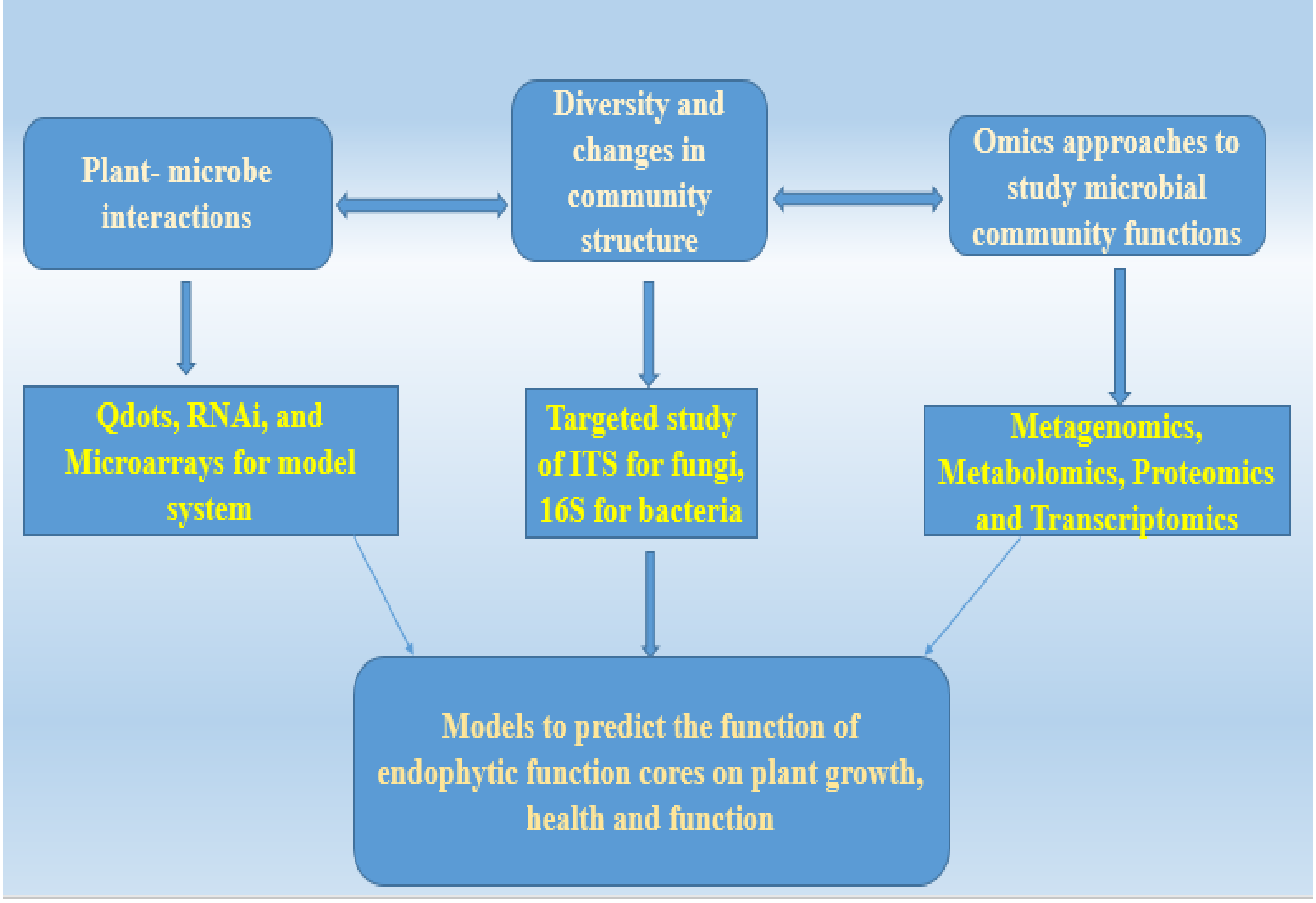


Figure 2.2: Current techniques employed in comprehensive studies of plant-associated microbiomes.

2.3.1 DNA extraction, verification and storage

Extraction of DNA is the basis for carrying out metagenomic and computational analyses of plant-associated microbial communities. Before microbes inhabiting a plant can be studied, extraction of the plant DNA is very important which will give the metagenome of the plant. The DNA quality is greatly influenced by different methods employed during sampling (Ju and Zhang, 2015)**.** In order to ensure the effectiveness of the experiment, the use of the same sampling method for the whole experiment is advocated. Also, repeated experiment trials should be carried out for even distribution of the microbes in the sample. Before DNA extraction is done, the plant samples should be surface sterilized. The sterilized plant tissues can then be used for the extraction of plant-associated microbial communities. Some frequently used DNA extraction methods/kits for plant-associated microbial communities (endophytes) are summarized in Table 2.1. As presented in Table 2.1, the most commonly employed DNA extraction is the use of commercial kits which have about 95% acceptance among all other related methods. Cetyltrimethyl ammonium bromide based DNA extraction method (CTAB) is also one of the methods used for the extraction of DNA. In terms of production and acceptability of commercial kits, MoBio Laboratories, Qiagen, USA, and Macherey Nagel, Germany are leading companies from our assessment. In all, the USA has a very large percentage of the commercial DNA kits market. Once the DNA extraction is completed, it is important that the quality (concentration and purity) of the extracted DNA be assessed by measuring its absorbance at 260 nm and 280 nm with equipment such as UV spectrophotometer and NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA) (Bareither et al., 2013). The final purified DNA samples can be stored at -20^{o}C or -80^{o}C for further processing.

Table 2.1: Commonly used DNA extraction methods/kits for plant-associated microbial communities

S/N	DNA extraction methods	Description	Manufacturer	References
1	MoBio Power soil DNA extraction kit	This kit is designed for isolating genomic DNA from environmental samples. It is intended for use with environmental samples containing a high humic acid content including difficult soil types such as compost, sediment, and manure.	Mobio, USA	Maropola (2014)
2	MoBio Power plant Pro kit	This kit is designed for fast and easy purification of total cellular DNA from plant cells, tissues and seeds. Also, for extraction for recovery of high quality DNA from the toughest sample types, such as strawberry leaf, cotton leaf, cotton seeds, and pine needles.	Mobio, USA	Maropola (2014)
3	Qiagen DNeasy mini plant DNA kit	The kit aids the isolation of pure total DNA (mitochondrial, genomic, and chloroplast). It also provides a fast and easy way to purify DNA from plant and fungal tissue.	Qiagen, USA	Gomes et al. (2018)
4	Genejet plant genomic DNA purification kit	The kit is designed for rapid and efficient purification of high quality genomic DNA from a wide variety of plant species and tissue types. It employs silica-based membrane technology in the form of a convenient spin column.	Thermofisher Scientific, USA	Maropola (2014)
5	CTAB (Cetyl Trimethyl Ammonium Bromide)	This is a cationic detergent which facilitates the separation of polysaccharides during purification. while additives, such as polyvinylpyrrolidone, can aid in removing polyphenols. This method is widely used for extracting and purifying DNA from plant tissues.	-	Maropola et al. (2015), (Chiong et al., 2017)
6	MoBio ultra clean® soil DNA kit	The kit is used for examining microbial communities in soil. This kit has the capacity to isolate cellular, PCR quality DNA from soil	MoBio USA	Maropola et al. (2015)

		and ensures removal of humic acid inhibitors.		
7	EZNA SP plant DNA kit	It is a rapid isolation kit for the extraction high-quality total cellular DNA from a variety of plant species and tissues (leaf).	Omega Bio-Tek, USA	Pipan et al. (2018)
8	EZNA plant DS mini kit	This kit is designed for effective recovery of genomic DNA from frozen, fresh, or dried plant tissue samples rich in polyphenols, polysaccharides, or having a lower DNA content.	Omega Bio-Tek, USA	Pipan et al. (2018)
9	Nucleospin Plant II-Lysis Buffer PL1&PL2	This method is designed for isolation of DNA from plant material, fungi, and other biological samples. The kit includes two optimized, alternative lysis buffers based on the established CTAB and SDS lysis methods.	Macherey Nagel, Germany	Pipan et al. (2018)
10	Qiagen DNeasy Plant pro Kit	The kit is used for isolation of genomic DNA from plant cells, tissues and seeds. It has a number of innovative features which enables recovery of high-quality DNA from the toughest sample types, including strawberry leaf, grapevine leaf, pine needles and diverse seed types.	Qiagen, USA	Pipan et al. (2018)
11	Masterpure^{TM} Plant Leaf DNA purification kit	This kit is specially designed for extraction and purification of DNA from of fresh plant-leaf tissue.	Epicentre,USA	Marsal et al. (2013)
12	Invisorb Spin Plant Mini Kit	This kit is designed for isolation of high quality total cellular DNA from different varieties of tissue types and plant species.	Invitek Molecular, Germany	Pipan et al. (2018)

2.3.2 Pre-processing and quality control

A real metagenomics data flowchart starts with pre-treatment and quality control of the raw reads of the high-throughput sequence data. This is important in order to ensure high-quality metagenomic dataset that shows the actual abundance and diversity of microbiota present in the sample. The quality control process involves quality score, length check, GC content, and complexity distribution of the sequences data. Although there are different challenges attributed to researches using NGS technologies, some of them are highlighted in this study. Here we intend to point out some preprocessing challenges linked with plant-associated metagenomics: Filtering can be used to reduce the activities of sequences that contaminate the raw metagenomes. Filtering is most times carried out in order to get pure metagenome that contains only the required sequences. Filtering steps reduce the challenge of misassemblies, which limits the size of the dataset to enhance fast process of downstream analysis. There are two notable means of contamination: (i) genomic material from organisms and plant DNA present in the sample which are not the main focus of the metagenomic studies**,** (ii) primers and concatenations which are produced when metagenomes are established by pre-amplification through primer-based methods.

2.4 Metagenomic techniques in community analysis of endophytes

Advances in molecular biology have created an avenue to analyze the phylogenetic assemblages of endophyte communities straight from environmental samples and also enhances investigation into the functional roles performed by the entire community inhabiting the plant (Sessitsch et al., 2012). Profiling of the microbial community can be done with the aid of genomic samples of the whole population of microbes drawn out of their natural habitat. Metagenomic DNA produced from robust environments such as endophytic environments has been employed in the effective determination of the composition and diversity of microbiomes inhabiting such environments using metagenomics techniques (Maropola, 2014). In this section we focused on the endophytes from maize plant. Maize (*Zea mays* L.) is a common cereal that is cultivated all over the world (Alori et al., 2019). It is widely cultivated in western, central, eastern and southern Africa and germinates in almost all soil types recording varying yields (Babalola and Glick, 2012). Since maize is one of the foremost cereal crops, its protection against devastating pests is crucial. Hence, the internal microbes colonizing maize plant need to be critically analyzed for the potential utility of these endophytes as delivery system of bioactive metabolites for controlling damage (Rai et al., 2007). The distribution of genomic, structural and functional biomolecules in an environment can be used in evaluating the composition of microbes present in the given environment and the determination of the different biological activities arising there. Some of the used methods are highlighted below:

2.4.1 Denaturation Gel Gradient Electrophoresis (DGGE)

In DGGE, PCR is used for the amplification of phylogenetic marker sequence gotten from metagenomic DNA in the environment with the aid of GC-clamped (chemiclamp) primers and electrophoresis is applied on the amplicons through a denaturing acrylamide gel (Schäfer and Muyzer, 2001). There is a horizontal increase in the aggregation of denaturants inside the gel, which is parallel to the electric field. As the DNA proceeds to the cathode, they are trapped in the denaturants, leading to melting and separation of the DNA (Maropola, 2014). From theory, each endophytic bacteria species will be indicated through a unique band on the gel, the intensity of which is proportional to the richness of that particular species present in the community. So, the data gotten can be used to profile the diversity and abundance in the microbial population (Maropola, 2014).

The structure of the endophytic communities of two genotypes of transgenic maize namely MON810 and TC1507 was evaluated using denaturing gradient gel electrophoresis (DGGE) and the construction of clone libraries, respectively. The results from the analysis of the DGGE and the clone libraries of bacterial endophytes showed that genotype TC1507 had the highest diversity when compared with other genotypes. Though many bacterial genera were reported to be present in the genotypes, the dominant ones are *Achromobacter, Burkholderia* and *Stenotrophomonas,* the majority are found in TC1507 (da Silva et al., 2014). The diversity of endophytic bacteria in maize using DGGE was also studied by Woźniak et al. (2018), the result showed the presence of *Rhizobium, Achromobacter, Stenotrophomonas, Brevundimonas, Novosphingobium*, *Variovorax,* among others. The limitation with DGGE is that some DNA fragments from different species of an organism may have a similar melting pattern, thus leading to comigration within the gel. On the hand, some endophytes have different mixes of genes which are heterologous and codes for 16S rRNA. But when they are merged in the PCR product mix, they sometimes get separated in the DGGE gel, showing up as many samples. This technique has been reported to have low resolution and it is only bacterial groups that can be assessed (Maropola, 2014).

2.4.2 Terminal Restriction Fragment Length Polymorphism (t-RFLP)

The first method used for fingerprinting was designed by Avaniss-Aghajani et al. (1996), which was used widely used in medical microbiology for the identification of mycobacteria. Primers that are fluorescently-labelled are employed in the amplification of a phylogenetic marker, which can sometimes be the 16S rRNA gene of bacteria (Maropola, 2014) or nifH which is an example of functional genes (Sessitsch et al., 2012), from metagenomic DNA. Amplicons that have undergone purification process are then absorbed using restriction enzymes and the labelled terminal restriction fragments (t-RFs) with different sizes are divided through capillary electrophoresis. Each separated amplicon stands for an operational taxonomic unit (OTU). T-

RFLP has been applied extensively especially in the study of bacterial endophyte communities. This technique has wide application in understanding the diversity of endophytic bacteria communities inhabiting environmental samples. Ding et al. (2013) utilize t-RFLP to show (i) structural differences that exist among endophyte communities inhabiting different plant species, (ii) shifts of bacterial endophyte community, (iii) influence of sampling site on the abundance and diversity of endophyte communities.

Ten different varieties of maize plant were assessed for bacteria endophytes through culturing, cloning and DNA fingerprinting using terminal restriction fragment length polymorphism (TRFLP) of 16S rDNA. Results from Principle component analysis of TRFLP showed that the composition of the endophyte community present in the seed varied when compared to host phylogeny. Also, TRFLP signals predicted the presence of *Paenibacillus and Clostridium* and species across all maize genotypes (Johnston-Monje and Raizada, 2011). Johnston-Monje et al. (2016) reported that 16S rDNA fingerprinting of maize rhizospheres possess diverse bacteria, which are influenced by seed treatment or the genotype, and dominated by species of *Actinobacteria, Bacteroidetes, Firmicutes*, and *Proteobacteria*. These techniques of community profiling are vital in the determination of the bacterial community structure in a complex environment. However, the technique is unable to predict the makeup of microbial communities up to species level and some novel constituents are often overlooked in this analysis (Maropola, 2014). Introduction of a high-resolution model of these tools will enable researchers to conduct detailed structural and functional characterization of endophytic microbe communities (Maropola, 2014).

2.4.3 Pyrosequencing

Pyrosequencing is a high-throughput sequence-by-synthesis technology in which nucleotides are pointed out by the secretion of pyrophosphates when added to the template during the process of

DNA synthesis. Pyrosequencing approach exhibit a higher resolution than the t-RFLP fingerprinting approach in which each species of microbes can be correctly identified until species level (Charles and Marco, 2010). In whole-genome shotgun sequencing, the DNA from environmental samples e.g. plant metagenomics DNA, there can be shredding of plant metagenomics DNA and all other fragments sequenced to give an array of data of gene sequences present in an environment (Petrosino et al., 2009). The possible application of pyrosequencing in pathology was exhibited in a metatranscriptomic study which reported that pathogenic fungi and bacteria alongside virus do inhabit the tissues of plants (Molina et al., 2012). The bacterial endophyte communities inhabiting roots of maize plants grown on soils from the Quechua maize belt (Peruvian Andes) were studied employing tags pyrosequencing spanning the V4 and V5 hypervariable regions of the 16S rRNA. The result showed that the genera *Burkholderia, Bacillus, Candidatus, Glomeribacter, Chitinophaga, Staphylococcus*, and *Variovorax* were most abundant among the libraries (Correa-Galeote et al., 2018). Also, 39 bacterial endophytes were isolated from the roots and seeds of maize plants cultivated under drought condition using pyrosequencing. The endophytes were reported to exhibit plant growth promoting and biocontrol activities (Sandhya et al., 2017). Bacterial isolates from transgenic (Bt) and non-transgenic (non-Bt) maize at 50 and 90 days of development were studied. The result showed that the diversity of genera *Acinetobacter*, *Bacillus*, *Enterobacter, Pantoea, Pseudomonas, Serratia, Stenotrophomonas and Yersinia*. The study concluded that there was no significant difference in the diversity and functional analysis of endophytes isolated at the two developmental stages (Mashiane et al., 2018).

This important approach encompasses the uncultured species of bacteria in the environment and the discovery of novel natural product in the tissues of the plant (Berlec, 2012). Pyrosequencing approach can be employed in sequencing the whole genome of important bacteria. For instance, the genome of *Variovorax paradoxus*, a bacterial endophyte capable of soil pollutant, pesticides and chemical fertilizers degradation was detected through using pyrosequencing (Han et al., 2011). In the study, 6279 proteins were predicted, indicative of the fact that the important metabolic prospects of this microorganism are yet to be unravelled. This becomes interesting in the sense that, a sequenced genome of bacteria becomes a reliable reference for any other studies carried out on that particular organism, and a reference organism when other species are studied (Bertalan et al., 2009, Han et al., 2011).

2.4.4 Internal Transcribed Spacers (ITS) Sequencing

Mycobiomes play an important role in most biological processes. Therefore, understanding the role fungi plays in an environment and among microbial communities becomes imperative. However, as a result of the present culturing difficulties, fungi can be very hard to come by and are most times overlooked in studies involving metagenomics. In a bid to find a solution to this challenge, many researchers have adopted culture-independent methods like DNA sequencing for metagenomics studies. The internal transcribed spacer 1 (ITS1) region from the rRNA cistron is a popular DNA marker used for the identification of different species of fungi in metagenomics samples (Schoch et al., 2012). Amplification of the ITS1 region is an effective method for characterization and identification of fungal biota from culture-independent complex and diverse samples from the environment such as soil, plant, water among others (Schoch et al., 2012). The region is most times the combination of ITS and ribosomal sequences (partial) 18S rRNA sequence, an internally transcribed region (ITS1), an internally transcribed region (ITS2), the whole sequence of 23S rRNA, and a partial sequence of 28S rRNA. A total of 17 endophytic fungi were isolated from the leaf, stem and root of maize plant collected at 30, 60 and 90 days respectively after planting using ITS. Some fungal endophytes were identified using ITS as

Cladosporium oxysporum, Colletotrichum boninense, Colletotrichum gloeosporioides, Curvularia lunata, Fusarium fujikuroi, Curvularia lunata, Epicoccum sorghinum, Gibberella moniliformis, Penicillium sp*., Nemania* sp*., Rigidoporus vinctus, Scopulariopsis gracilis* and *Sarocladium zeae* (Renuka and Ramanujam, 2016).

Xia et al. (2019) isolated and identified 740 fungal isolates from the organic field and conventional fields, of which 550 and 190 fungal species were isolated from conventional and organic fields respectively using ITS. These endophytic fungi were classified into eight orders and 22 species, with the two most abundant species identified as *Trichoderma* sp. and *Pichia guilliermondi*. The diversity and abundance of endophytic fungi were both significantly higher in the organic field as compared with the conventional field (Xia et al., 2019). In this section, we have highlighted some of the endophytic studies that have adopted this method. The limitation of this sequencing approach is that it is designed majorly for fungi species.

2.4.5 Illumina amplicon sequencing

Recent NGS researches utilize Ion Torrent, Illumina and Pacific Biosciences sequencing technology (Mardis, 2013). But Illumina sequencing has dominated the sequencing industry, these industries have embraced sequencing by synthesis technique (Bentley et al., 2008), using reversible-terminator nucleotides labelled with fluorescent, on the clonally amplified DNA templates which are immobilized to an acrylamide coated surface of a glass flowcell. The latest Illumina genome analyzer, the HiSeq 2500 has been established as a standard for massively parallel high throughput sequencing. However, in 2011 Illumina developed a machine that can produce a lower throughput with fast turnaround called the MiSeq. The MiSeq was designed purposely for the clinical diagnostic industries and smaller laboratories. Some of the differences between HiSeq and MiSeq is presented in Table 2.2. Illumina sequencing is important in endophytic research because it helps in detecting all the communities of microbes present in an environmental sample (Plant). However, Illumina HiSeq is most preferred in structural and functional analysis because it gives longer reads as compared with MiSeq and the function of each detected organism can be established. Although there are other sequencing platforms such as Ion Torrent and PACBIO, their application is limited because they are very expensive (Shokralla et al., 2012).

Illumina MiSeq was used by Mashiane et al. (2018) to study the diversity of bacteria from the cultivars of Bt maize and its isogenic parental line from South Africa at differential growth stages. Also, bacterial community consisting of seven strains (*Ochrobactrum pituitosum, Enterobacter cloacae, Stenotrophomonas maltophilia, Herbaspirillum frisingense, Ochrobactrum pituitosum, Pseudomonas putida, Chryseobacterium indologenes*, *Curtobacterium pusillum*, and *Chryseobacterium indologenes*) which represent three out of the four most dominant phyla present in maize roots were detected using Illumina MiSeq sequencing (Niu et al., 2017). As evident from literature, limited or no studies have been carried on endophytes from maize plant using Illumina Hiseq, thereby making this area a novel study focus for researchers.

Table 2.2: Applications, merits and demerits of Illumina NGS platforms (adapted from Shokralla et al. (2012)

Categories	**Required sequencing coverage > 1Gb**	
	Illumina HiSeq	**Illumina MiSeq**
Complete genome shotgun sequencing	+	-
Short amplicon sequencing (≤200 bp)	+	-
Transcriptome sequencing	+	-
Long amplicon sequencing (≥200 bp)	-	-
Multiplexing	+	-
Paired-end sequencing	+	+
Advantages	Expensive cost instrument and runs; Low cost per Mb for a small platform; speedy times for Illumina run and longest Illumina read lengths	Cheaper cost per Mb of data; can run high output (8 lanes) and rapid run (2 lanes) flow cells with several possible read-length configurations
Disadvantages	Relatively small reads and expensive cost per Mb compared to NextSEq or HiSeq	Expensive cost of the instrument; high cost per run; needs well-trained personnel; about 20% downtime; can not run one rapid run and one high output flow cell at the same time

2.5 Computational tools used in endophytic microbiome studies

Recent development in sequencing of environmental samples on a large scale platform have left researchers with many questions as regards the taxonomic composition (ITS amplicon data and SSU rRNA genes and ITS amplicon data), the functional potential (shotgun metagenomics) and the activities of microbial community (metatranscriptomics) which also include those associated with the stems, leaves, and roots of healthy and fresh plants. The challenge of combining these large datasets and deducing any meaningful biological information from them has led to the development of bioinformatics/computational tools and pipelines in recent years and still stands as an interesting study area for researchers.

2.5.1 Preprocessing of raw sequences

After obtaining sequenced metagenome data, pretreatment of raw sequenced data is very important to have reads with high quality for subsequent analysis. Numerous computational tools and platforms have been developed over the years for this function. Some of the software tools and platforms are Trimmomatic software (Elmagzob et al., 2019), RDP tools (Elmagzob et al., 2019), MG-RAST (Tian et al., 2015), UCHIME (Akinsanya et al., 2015b, Furtado et al., 2019). The importance of pretreatment for the sequenced data include (i) The removal of linkers and adapters (ii) elimination of replication and chimeras, (iii) demultiplexing of barcoded samples and quality control and (iv) quality control and barcoded samples demultiplexing. UCHIME is a frequently explored tools for check and removal of chimeras from raw sequenced data, while QIIME and MOTHUR are most common platforms developed of recent for denoising of metagenome data. After preprocessing, the sequencing reads are then grouped based on their distinct barcodes and primers are subsequently removed.

2.5.2 Marker gene amplicon data

Due to the popularity and wide acceptability of fungal ITS amplicon and bacterial 16S rRNA sequencing, bioinformatics tools specially designed for the analysis of marker gene data is very important in increasing our understanding of the diversity of endophytic microbes inhabiting the plants cultivated in natural environments. The most frequently used bioinformatics toolkits for amplicon data interpretation are MOTHUR (Schloss et al., 2009), BioMaS (Fosso et al., 2015) and QIIME (Caporaso et al., 2010), which provides an avenue for pre-processing of the sequenced data (error correction, de-noising, merging of paired-end reads, binning, demultiplexing, etc.), and also for the estimation of diversity. Marker gene data analysis most times require reference data with a wide collection for effective taxonomic classification of the illustrative sequences. Among the most commonly used databases are the Greengenes (DeSantis et al., 2006), the Ribosomal Database Project (Cole et al., 2008), SILVA, which encompasses 16S and 18S sequences for eukaryote (Pruesse et al., 2007) and the oomycete and fungal ITS UNITE database (Abarenkov et al., 2010a, Kõljalg et al., 2005). Many pipelines have been created to assist in the identification of specific fungal species from ITS data and also for the frequency calculation of each species, examples include Clotu (Kumar et al., 2011a), Plutof (Abarenkov et al., 2010b), CloVR-ITS (White et al., 2013), PIPITS (Gweon et al., 2015). Despite the fast growth and regular updates carried out on these databases, they still have ‘blind spot’ which makes them ineffective in the analysis of some taxonomic groups of microorganism, some of which include fungi, protists or viruses. However, result from the taxonomic classification of marker gene fragments at species or genus can really not be trusted.

One of the major steps to be taken during amplicon data processing is the grouping of sequences predicted to emanate from similar microbial species into Operational Taxonomic Units (OTUs), most times using a fixed approach of sequence similarity (for example 97% for archaea and bacteria). There are three main approaches for OTU inference: (i) de novo OTU clustering for those that do not rely on a reference database sequences, examples include UCLUST (Edgar, 2010) or UPARSE (Edgar, 2013). (ii) reference-based methods (Edgar, 2010) and (iii) hybrid approaches which generally carry out a first pass reference iteration, followed by de-novo clustering of the omitted sequences, example include SortMeRNA (Kopylova et al., 2012). The step of OTU clustering is important in a bid to reduce the number of sequences to be processed to reasonable quantity through the selection of representatives individual OTUs to give room for subsequent analyses of taxonomic classification and diversity (Garrido-Oter, 2018). However, this approach exposes amplicon-based studies to a lot of limitations, such as the resolution limit (generally 3% of sequence identity), the addition of data outputs (OTUs which exclusively consist of sequencing errors or PCR amplification) and the need to base future analyses on the belief that OTUs are ecologically and functionally homogeneous units, in spite of many contrasting findings, e.g when it has to do with plant-associated microbes (Bai et al., 2015). Interestingly**,** there is a new algorithm called DADA2 which now replaces the "OTU selection" step instead producing higher resolution tables of amplicon sequence variants (ASVs) (Callahan et al., 2016, Wagner et al., 2019, Kuźniar et al., 2020). Also, recent research reported the functional prediction of metagenome sequences using PICRUSt (Langille et al., 2013). Unlike the profiling of natural communities through culture-independent approach, where it is very difficult to identify erroneous and complete sequences, studies with synthetic communities have an easy setup that can greatly reduce the difficulties associated with the handling of raw data individually without necessarily clustering them into units taxonomically. Surprisingly, the present toolkits and bioinformatics pipelines are not designed to explore this experimental setup.

2.5.3 Diversity assessment and statistical analyses

Subsequent analyses of abundance in data generally involve the inference of ecological networks, which relies greatly on the co-existence of OTUs across the samples (Faust et al., 2012), alpha-diversity calculation within a sample e.g Chao, Shannon, or Phylogenetic Diversity (PD) indices and beta-diversity between samples such as Unifrac or Bray Curtis distances (Lozupone et al., 2011). A common approach used in diversity study involves carrying out a dimensionality reduction step in bid to compare groups of samples from different environments such as Non-Metric Multidimensional Scaling, PERMANOVA, Analysis of Principal Coordinates, ANOSIM among others, or the assessment of the role of each environmental factors through deconvolution of variance using Canonical Correspondence Analyses, and Linear Mixed Models (Garrido-Oter, 2018).

Another major step in the analysis involves the test for differentially abundant OTUs between different conditions, this has to do with the use of statistical tests specific for count data. A lot of libraries and tools have been designed for this purpose, examples include DESeq (Anders et al., 2013), vegan (Oksanen et al., 2010), or phyloseq (McMurdie and Holmes, 2013) and their effectiveness in testing hypothesis for different types of experiment has been unravelled through the use of simulated data (McMurdie and Holmes, 2014).

2.5.4 Shotgun metagenomics

Another novel substitute for sequencing marker genes in the study of taxonomic diversity of endophytic communities is the sequencing of the entire genomes present in an environmental sample, a method identified as shotgun metagenomics. Shotgun metagenomics, as opposed to marker gene metagenomics, has an edge in giving a better understanding of the functions alongside the taxonomic assemblage of the microbes (Enagbonma et al., 2019), thus providing a mechanistic view of the endophytic community rather than just a catalogue of the microbes present in an environmental sample (Garrido-Oter, 2018). Figure 2.3 presents the flow chart of events involved in shotgun metagenomics sequencing. However, the difficulty of the computational analyses needed for shotgun metagenome data is more than that of studies which are amplicon-based, particularly studies on different communities of microbes associated with plants (endophytes), where there is always difficulty with de novo metagenome assembly and where only a small percentage of the genes coding for protein can be accurately annotated (approximately 41% of predicted reading frames) (Bulgarelli et al., 2015, Ofek-Lalzar et al., 2014, Zarraonaindia et al., 2015).

There exist three major difficulties that must be overcome before data can be successfully interpreted, which are most times addressed by different computational tools: (a) shotgun metagenome assembly are carried out using established assemblers like SOAP (Li et al., 2010, Luo et al., 2012) or computational tools specifically created for environmental daSILVAta such as Ray Meta (Boisvert et al., 2012), MetaVelvet (Namiki et al., 2012), Snowball (Gregor et al., 2016), or Meta-IDBA (Peng et al., 2011), MetaSPAdes (Nurk et al., 2013), MEGAHIT (Li et al., 2015a). (b) binning of sequence fragments such as taxator-tk (Dröge et al., 2014), Kraken (Wood and Salzberg, 2014), MaxBin2 (Wu et al., 2014), MEGAN (Huson et al., 2007, Huson et al., 2016) , PhyloPytiaS+ (Gregor et al., 2016), and MetaBAT (Kang et al., 2015). (c) classification and annotation of metagenomes, which generally involves the prediction of open-reading frames using PRODIGAL (Hyatt et al., 2010) or MetaGeneMark (Zhu et al., 2010), which is then followed by homology searches against annotation databases, like SEED (Overbeek et al., 2005), KEGG (Kanehisa et al., 2013), eggNOG (Powell et al., 2014), COG (Galperin et al., 2014), PFAM (Garrido-Oter, 2018), and TIGRFAMs (Selengut et al., 2006).

Shotgun metagenomic data is very useful in the identification of a wide variety of species, but few studies exist on its fungi identification potentials (Donovan et al., 2018). Some available tools use both search algorithms and custom-built databases for fungi identification, and examples include USEARCH, GhostX, DIAMOND and BLAST (Edgar, 2010, Altschul et al., 1990, Suzuki et al., 2014, Buchfink et al., 2015), FindFungi (Donovan et al., 2018). These tools help in the identification of database sequence related to reads in the metagenome. On the other hand, algorithms like Kraken (Wood and Salzberg, 2014) and KAIJU (Menzel et al., 2016) allocate reads to the lowest common ancestor (LCA). KAIJU interprets reads and make a comparison between them and a references protein database, Kraken makes a comparison between the nucleotide queries and a nucleotide database. However, both Kraken and KAIJU gives a fast result because they utilize exact k-mer matches, contrary to other slower alignment-based methods. Other databases available for shotgun metagenomics apply their own distinct pipelines to analyze and host datasets at the same time (Donovan et al., 2018). MG-RAST gives a comprehensive analysis of the datasets uploaded by a user through continuously updated pipelines (Meyer et al., 2008).

Another way of estimating taxonomic abundances using shotgun metagenome data is the generation of marker genes that are cladespecific sets, a method that can possibly be used on synthetic communities to give room for strain level and intra-species resolution, given that a high ratio of microbes to plant reads will be achieved. However, as evident from literatures, no study exists on endophytic microbes from maize plant using shotgun metagenomics approach, hence, making this approach, a novel area which researchers can explore.

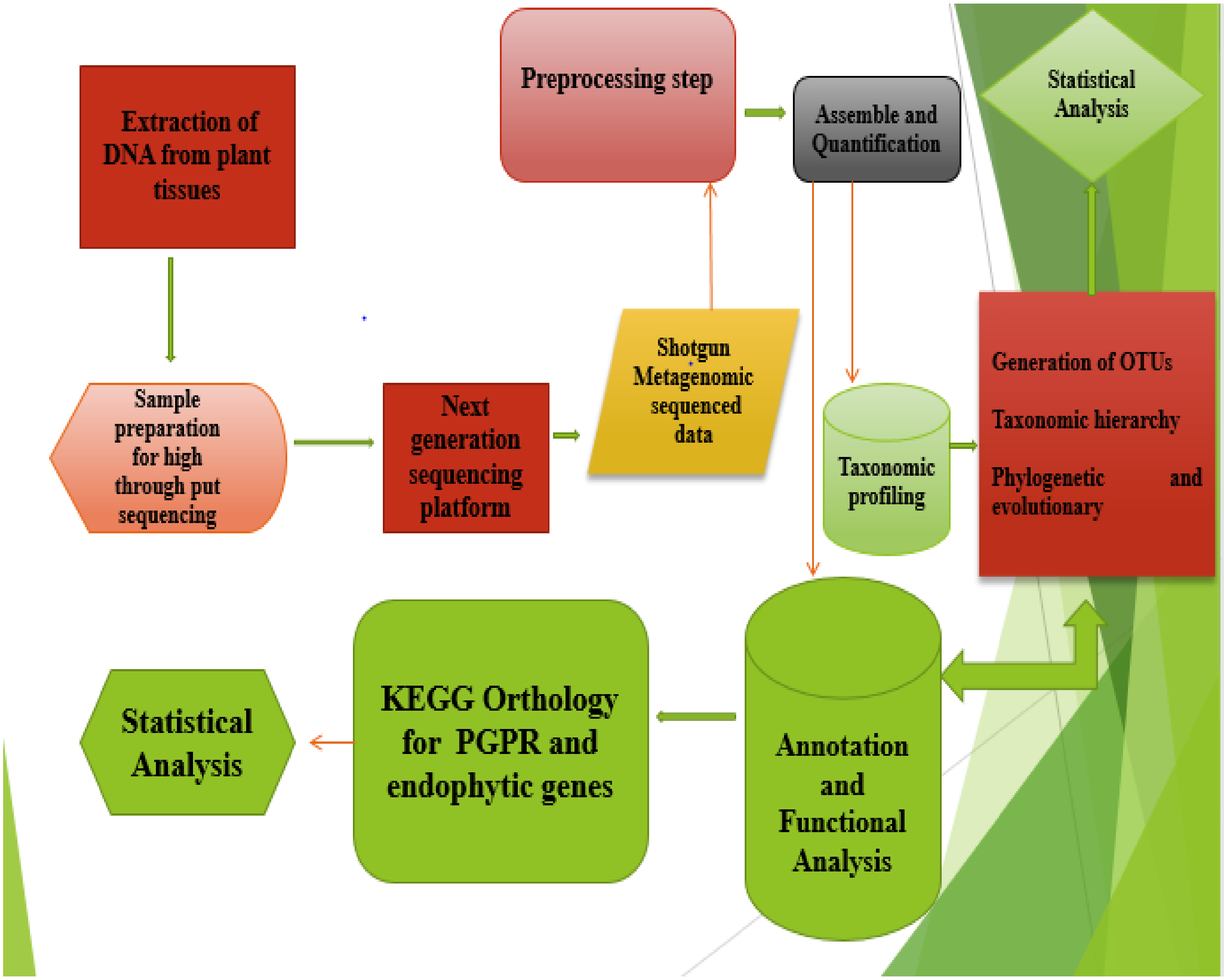


Figure 2.3: Work flow chart in shotgun metagenomics sequencing.

2.5.5 Phylogenomic analyses of endophytic microbes

Phylogenomics is the reorganization of relationships that exist between organisms depending on their genomic sequences and of studies involving genome evolution and gene function. Most times, the summary from a species tree or phylogeny is needed as the basis for many analyses, with the overall aim of gaining more insight into the biological process by the reconstruction of its evolutionary history. This strategy remains one of the best and frequently adopted computational techniques applied in sequence data analysis but has several limitations which affects its application. Firstly, some of the steps generally involves the comparisons of the pairwise sequence among all genomes, for instance in the determination of homology relationships amidst gene-coding genes (orthologous groups or gene families inference) (Sonnhammer and Östlund, 2014, Emms and Kelly, 2015), which produce very poor hierarchy (generally quadratic cost) with the number of organisms involved in the study. This places a limit on the quantity of dataset through which de novo inference of homology can utilize. Secondly, sampling bias (for example preferences for culture-independent organisms, clinical isolates or model organisms etc) often gives an unbalanced dataset which does not reflect the true diversity of microbes in a community and this may give misleading phylogenetic inferences (Kumar et al., 2011b). Finally, the largely adopted clonal method for microbial reproduction prevents mutation from being evident in many genetic backgrounds e.g. in plants, thereby making it extremely strenuous to pinpoint the effect of homogenous genetic background and that of mutation (Falush and Bowden, 2006, Chen et al., 2014).

Phylogenomic analysis of desired traits in complex population structure is difficult, it most times gives false-positive results and failure to identify the real causal genetic determinants (Earle et al., 2016). One possible way of overcoming this difficulty of relatedness is not to focus only on the genomic features available in the genome, but preferably on the changes observed during their evolutionary histories. In studies involving microbiome, it is important to analyze the evolutionary history of the community of microbes against the backdrop of the host phylogeny. Testing the hypothesis is not realistic when studying the microbial genomic and taxonomic diversity between different species of host, as the significant differences do not give reasonable differences between true co-evolution and niche adaptation. Furthermore, despite the fact that the correlation between host species tree and community member phylogenies can be used as a proof of co-diversification, it still doesn’t give a convincing understanding of the genetic basis of the interrelatedness. Some commonly used computational platforms and tools used in the metagenomic study of endophytic microbiomes are presented in Table 2.3.

Table 2.3: Commonly used computational platforms and tools used in the metagenomic study of endophytic microbiomes

Software/Platform	**Module/ Programme**	**Function**	**References**
Trimmomatic Software	-	Read trimming	Elmagzob et al. (2019)
HMMER	Hmm-Search Program	Read trimming and alignment	Chauhan et al. (2019)
ACE Pyrotag pipeline	-	Read trimming	Elmagzob et al. (2019)
MUSCLE	Multiple sequence alignment	Alignment of sequence reads	Abdelfattah et al. (2016)
MOTHUR	-	Determination of the OTUs or taxa, which were differentially represented between the samples	Schloss et al. (2009) Tian et al. (2015)
MG-RAST	-	Quality control	Tian et al. (2015)
	KEGG	Determination of the metabolic pathway	Tian et al. (2015)
	SEED	Annotation of Genomes	Tian et al. (2015)
	PcoA	Principal coordinate analysis	Tian et al. (2015)
	CCA	Canonical correspondence analysis	Furtado et al. (2019)
CLC Genomics workbench	de novo assembly	Assembly of Genome	Tian et al. (2015)
	RDP Classifier	Quality control	Elmagzob et al. (2019)
	Shannon, ACE, chao1 and Simpson index,	Abundance base coverage, richness and diversity estimation	Abdelfattah et al. (2016)
	UniFrac	Principal coordinate analysis	Lozupone et al. (2011)
	Against SILVA database	Sequences quality check and alignment	Akinsanya et al. (2015b)
FLASH	-	Merged paired-end reads	Elmagzob et al. (2019)
SILVA NGS Server	-	Taxonomic classification	Tian et al. (2015)
MEGA/MEGA5	Neighbour-Joining methods and Jukes-Canter Model	Constructions neighbour-joining trees and phylogenetic trees	Tamura et al. (2013)
MEGAN	Lower common ancestor (LCA)	Taxonomic assignment of genes, species	Zimmerman and Vitousek

	algorithm	richness analysis	(2012)
UCHIME	-	Removal and detection of Chimeras	Akinsanya et al. (2015b), Furtado et al. (2019)
UPARSE	-	Clustering of OTU’s	Akinsanya et al. (2015b)
UNITE	-	Taxonomical classification	Furtado et al. (2019)
STAMP	Two-Sided Welch’s t-test	Pairwise statistical comparison of taxonomy between samples	Furtado et al. (2019)
QIIME	-	Denoising of sequences	Elmagzob et al. (2019)
	Blast Taxon Assigner	Assigning taxonomy to the sequences	Caporaso et al. (2010)
USEARCH	Fast-filter command	Trimming of demultiplexed reads	Wemheuer et al. (2019)
	Cluster-OTUs command	Clustering of dereplicated reads	Wemheuer et al. (2019)
	Usearch-globus command	Assigning of OTUs to reads	Wemheuer et al. (2019)
Blast against RDP database	-	Phylogenetic classification	Abdelfattah et al. (2016)
Blast against COG database		Functional characterization	Galperin et al. (2014)
Blast 2X against KEGG database		Functional annotation of reads	Kanehisa et al. (2013)
PyNAST	Against Greengenes core reference set	Alignment of representative OTUs sequences	Mashiane et al. (2018)
R software Package	Shanon index, Pielou’s index	Richness, diversity and evenness estimation	Wemheuer et al. (2019) Cui et al. (2018)
PAST	NMDS	Visualization of Beta diversity metrics	Chauhan et al. (2019), Elmagzob et al. (2019)
	ANOSIM	Analysis of the strength and statistical significance of sample groupings	Carrell and Frank (2015)
	PERMANOVA	Analysis of the strength and statistical significance of sample groupings	Carrell and Frank (2015)
UNITE	-	Taxonomical classification	Abdelfattah et al. (2016)

FAST UniFrac	PcoA	Evaluation of differences in community structure and parameters.	Lozupone et al. (2011)
Deseq	-	Differential test among OTUs	Taudière et al. (2018), Gdanetz and Trail (2017)
RDP (RDP database Project)	Tree builder, RDP classifier and aligner	Sorting of sequences, trimming and alignment, biodiversity analysis, and taxonomic classification of sequences	Elmagzob et al. (2019), Dong et al. (2018)
Infernal aligner	-	Aligning of multiple clean sequences	Chimwamurombe et al. (2016)
Chimera slayer	-	Removal of chimera from sequences	Carrell and Frank (2015)
XLSTAT	Canonical correspondence analysis CCA	Determination of correlations between the microbial community.	Johnston-Monje and Raizada (2011)
CANOCO	Redundancy analysis (RDA)	Visualized correlations between community structure and parameter.	Geisen et al. (2017)
PICRUSt	-	Functional prediction	Huang (2019)
LefSe	-	Determination of the OTUs between 2 or more groups using relative abundances.	Sun et al. (2019c)
DADA2	-	Determination of the OTUs or taxa, which were differentially represented between the samples	Callahan et al. (2016),Wagner et al. (2019),Kuźniar et al. (2020)

2.6 General limitations and prospects of metagenomics and computational analyses on the plant-associated microbial community.

There are many technical issues that must be addressed before metagenomics and computational analysis of a plant-associated microbial community can be fully embraced. Some of them are discussed below:

1. The fast advances experienced in sequencing techniques have produced an increase in depth of sequencing, thus making the storage of metagenomics data a major concern. For example, the 454 pyrosequencing techniques can generate approximately 1,000,000 reads per sequencing run (approximately 0.7 GB), while Illumina HiSeq. 2500 can produce greater reads (greater than 600GB per run) (Oulas et al., 2015, Dröge and McHardy, 2012). Subsequent analysis of raw sequence will increase the amount of data by 10-20 times for each sequencing run (Dröge and McHardy, 2012). Such a large amount of sequenced data is now a major concern for computational analysis (Jünemann et al., 2017). However, next generation sequencing-based metagenome studies on plant-associated microbial communities especially endophytes are still novel. Profiling of more newly sequenced microbial metagenomes is still needed to statistically support the results obtained in prior studies, which necessitates the need for larger space for data storage. Therefore, a standard data storage platform should be created in the study of metadata from plant-associated microbial communities in future.
2. Most of the computational tools and techniques employed in the processing of data were adopted from the fields of artificial intelligence, data mining and statistical methods (Tabish et al., 2013). However, the characteristics of the microbial data of interest may be different from the original data for which the tools were initially developed (Tabish et al., 2013). Although computational methods have been effectively used in analysis involving

genomic data, some difficulty still exists. Presently, binning and assemble of large data sequences is still a major challenge (Wei et al., 2015), which can be ascribed to inadequate computational capacity. Also, there is still inconsistency between the speed of data processing and accuracy of the results arising from the fact that taxonomic assembly and binning takes several hours to many days (Dröge and McHardy, 2012). Thus, to overcome this challenge, there is urgent need to intensify efforts towards the development of new and powerful computational methods which can successfully integrate supercomputing techniques into the present tools to enhance the speed at which analysis of microbial genomic data will be completed.

3. The comparatively high cost of sequencing has limited many metagenomic studies, in that they are performed without replicates (Ju and Zhang, 2015), which could affect the reproducibility of data, thereby making it difficult to ascertain whether the observed differences were significant. Therefore, sequencing with replicates is advocated for a more dependable data analysis. Also, up till now, most computational analysis rest greatly on reference databases. It is important to state that present databases for computational analysis can be difficult considering the chance of having incomplete databases. Therefore, regular updating of databases with data of high quality in order to enhance the analysis of plant-associated microbial metagenomics information is urgently needed.
4. Metagenomics methods give an intensive understanding of microbial communities, but most of the studies have been majorly descriptive and explanatory in a bid to proffer an answer to the initial question of ‘who is present?’ (Jünemann et al., 2017). The difficulty that exists between laboratory and field-scale data and extrapolation still persist. As a

matter of fact, there still is a knowledge gap between what can be observed from the community of microbes through bioinformatics methods and the actual species and gene functions that can be manipulated. This is because the microbial ecosystem is a very complex network which involves interactions that are dynamic spatio-temporal among microorganisms between the ambient environment and microorganisms (Widder et al., 2016, Zhang et al., 2019).

2.7 Conclusion

Plants are colonized by diverse microorganisms which are collectively called microbiota, of which endophytes are the most populous among the beneficial ones. These microorganisms make up a complex community and establish an intimate relationship with the host which researchers are just getting to understand in recent years. This review assessed the importance of metagenomics and computational methods in the analysis of sequenced data gotten from environmental samples of microbes associated with the plant (endophytes). Several metagenomic techniques were studied and their limitations presented especially in the study of endophytic microbes from maize plant. It can therefore be concluded that shotgun metagenomics still stands out as the best techniques to be employed in the study of endophytes as it has longer reads. It can also give us more insights into the structural, functional and metabolic pathways of the microbes identified from the sequenced data.

Computational analyses do help in answering questions such as the composition of endophytes in terms of evolutionary relationship, taxonomic composition, functions and what controls the underlying mechanisms of their association. It is important to note that some of the challenges with amplicon data are sequencing errors and PCR outputs limit the resolution that can be obtained, approximately 97% of the sequence identity in hundreds of basepairs with conserved markers like 16S rRNA. When wide genomic and phenotypic variation is obtained within a similar taxonomic unit, the poor resolution makes it very difficult to interpret the amplicon data. However, removal of chimeras and de-noising methods are ususally carried out before endophytic studies of the community data, which might reduce the complexities. Furthermore, the use of phylogenomics for error correction within the 3% sequence identity threshold can be done i.e. by categorizing the new sequences in a references tree and getting rid of errors that deviate completely from what a model DNA evolution would project and it also has the capacity to enhance resolution and accuracy. Another promising avenue researchers should look into, is the integration of large quantity data into large scale meta-analyses. Also, there a need to explore more computational frameworks that will move beyond the absence and presence of inferred orthologous groups to using more advanced genomic features such as like k-mers of random size, which do not depend on annotation and can represent other sequence alternatives. The major difficulty confronting methodological update is how matrices of features will be handled, a task which may be computationally difficult. However, this study advocates for considerable synergy among researchers from different disciplines such as environmental sciences, environmental engineering, and biological sciences in order to overcome the current limitations of metagenomics and computational methods.

CHAPTER THREE

3.0 EXPLORING THE POTENTIALITIES OF BENEFICIAL ENDOPHYTES FOR IMPROVED PLANT GROWTH

Abstract

Pathogens affect crop plant growth, host health and productivity. Endophytes, presumed to live inside the plant tissues, might be helpful in sustaining the future of agriculture. Although recent studies have proven that endophytes can be pathogenic, commensal, non-pathogenic, and/or beneficial, this review will focus on the beneficial category only. Beneficial endophytes produce a number of compounds which are useful for protecting plants from environmental conditions, enhancing plant growth and sustainability, while living conveniently inside the hosts. The population of endophytes is majorly controlled by location, and climatic conditions where the host plant grows. Often the most frequently isolated endophytes from the tissues of the plant are fungi, but sometimes greater numbers of bacteria are isolated. Endophytes stand a chance to replace the synthetic chemicals currently being used for plant growth promotion if carefully explored by researchers and embraced by policymakers. However, the roles of endophytes in plant growth improvement and their behavior in the host plant have not been fully understood. This review presents the current development of research into beneficial endophytes and their effect in improving plant growth.

Keywords: Environmental condition, Food availability, Plant productivity, Rhizosphere, Tissue

This chapter has been published in *Saudi Journal of Biological Sciences* 2020; 27:3622-3633. https://doi.org/10.1016/j.sjbs.2020.08.002.

3.1 Introduction

It has been reported that the world’s population is likely to increase up to 9.1 billion by 2050 (Liu et al., 2017a). For this reason, governments at all levels are trying their best to ensure a continuous increase in agricultural productivity. However, ways to meet up with this target are becoming difficult. Climate change, urban sprawl, poor land management and over-dependence on synthetic fertilizers are some of the factors posing threats to agricultural development (Smith et al., 2016). The adoption of plant growth-promoting (PGP) microorganisms (beneficial endophytes) as biofertilizers in agriculture has shown great promise in providing an effective and eco-friendly approach in ensuring food security (Glick, 2014). Endophytes are examples of microorganisms with these prospects.

Endophytic microbes are microorganisms that successfully colonize the tissue of vascular plants and have been reported to be isolated in most plants in this group (Fadiji and Babalola, 2020a, Brader et al., 2017b). They were initially known not to be harmless to the host plants and their association with plants can be obligate or facultative (Nair and Padmavathy, 2014). A recent study by Brader et al. (2017b) showed that endophytes can also be defined in terms of their ecological niche and not only the function they perform in the host. The study further revealed that some species of endophytes can be either pathogenic or beneficial. The majority of these endophytes do not show any harmful effects on a few plant species, however, when tested on other plants, they may be pathogenic. The pathogenicity attribute of endophytes can be based on a number of biotic interactions and environmental factors. For example, fluorescent Pseudomonads, known to be beneficial to most plants, can be pathogenic to the leatherleaf plant under special conditions (Kloepper et al., 2013).

Nevertheless, endophytes have been observed to be active in biological control of phytopathogens, plant growth enhancement, and in the production of compounds or metabolites of biotechnological or pharmaceutical importance (Sharma et al., 2017). Growth of endophytes is generally strongly restricted by plants, and in order to overcome this hindrance, endophytes make use of numerous mechanisms of action in adapting to new living environments (Dudeja et al., 2012).

Endophytic bacteria are classified as those bacteria that live inside or on the surface of disinfected plant tissues and coexist symbiotically (Patle et al., 2018). Endophytic fungi, on the other hand, are fungi that reside inside the tissues of a plant without having any harmful effect on the plant. Even though most of them are not host-specific, certain group of endophytic fungi possess a greater occurrence in some plants, indicating their preference for these plant families as their host (Fadiji and Babalola, 2020a). There exists a wide diversity of endophytes, mostly with a great improvement in their ecological roles alongside the production of numerous amazing chemical secondary metabolites. Endophytes were reported to be naturally resident in many host plants (Suryanarayanan, 2013). Different endophytes can be found in different parts of a plant mainly in the stem, leaves or root (Fürnkranz et al., 2012). Most endophytes that are found in vascular plants were discovered to employ a plant-fungus interaction. This type of interaction is symbiotic; most endophytes compliment nutrients got from the host plants and also contribute significant benefits to the host plants. These endophytes live harmlessly within the tissues of the host they have colonized, thereby facilitating an indirect defense against herbivores (Bamisile et al., 2018).

Endophytes receive nutrition as well as protection from the host, while encouraging uptake of nutrients and protecting the host from abiotic and biotic stresses and pests. It has also been reported that the availability of endophytes affect the health of the plant, developments, growth, and the different types of the plant community, ecosystem functioning and population dynamic (Hardoim et al., 2015). Many endophytic microbes have been reported to have developed gradually finding their ways into the plant, and as this association continues, they devise new ways to inhabit, evolve, establish and improve the association they have established with the host (Goyal et al., 2016).

Different endophytic microbes exist mainly in roots of plants and decrease from the stem to the leaves. Different endophytic microbe species can be present in numerous plants, while some of the same species can be in a single plant. Some endophytes present in the host remain as latent, while other interactions may be pathogenic or non-pathogenic (Arora and Ramawat, 2014). In a bid to ensure stable symbiosis, endophytes produce many compounds which help in promoting host plant growth and improving environmental adaptation (Das and Varma, 2009).

One of the recent problems, agriculturists battle with is over-dependence on synthetic fertilizers for improving the growth of plants, which has several side effects on human health and is not eco-friendly. Efforts towards the improvement of endophytic resources could give us numerous benefits, such as the discovery of effective and novel metabolic compounds that might not easily be synthesized through chemical means. As a result of this, an urgent need for a proper understanding of the benefits of beneficial endophytes, the biology of plants and the ecology of the microbes are required. A number of experiments have been carried out, trying to evaluate how endophytes colonize the host vegetative tissues alongside their impact on growth promotion and health. This study proposed to give an outlook of beneficial endophytes (bacterial and fungal) and their potentials in improving plant growth with an emphasis on current trends in endophytic research.

3.2 Distribution pattern of endophytes in plant tissue

Endophytic microorganisms can be grouped into three main categories based on the approach they adopt while living inside plants. Obligate endophytes are microorganisms that cannot reproduce outside the plant tissue and most times are transferred through seed instead of developing in rhizospheric soil (Hardoim et al., 2015). Facultative endophytes are microorganisms that live freely in soil but colonize the plant roots at the slightest opportunity, using a systematic approach (Hardoim et al., 2015). The endophytic microbes that are helpful in enhancing plant growth and health belong to this category (Hardoim et al., 2015). Passive endophytes are microorganisms that do not originally intend to colonize the plant tissues but end up colonizing them due to events, such as wounds on the root hairs. Passive life may affect endophytes by making them less active, since the technical know-how required for cellular colonization of a plant is lacking (Hardoim et al., 2011), thus making them less appropriate as promoters of plant growth. However, a recent study showed that endophytes associate with plants in many forms, including fungi and bacteria (Mycoplasma or actinomycetes) that colonize plant tissues (Gouda et al., 2016).

The distribution of endophytes living inside plants depends strongly on a combination of the allocation of plant resources and the ability to colonize. Endophytes in the roots of plants often penetrate the site at which lateral roots emerge and help in colonizing the epidermis, in the root cracks and below the root hair zone (Zakria et al., 2007). Colonizers of this nature can effectively establish populations both intracellularly and intercellularly (Zakria et al., 2007). Once colonization is established, endophytes can relocate to other parts of the plant, other endophytes can relocate to some of the plant parts through the vascular tissues from where they begin to proliferate systemically (Johnston-Monje and Raizada, 2011). Johnston-Monje and Raizada (2011) demonstrated the transport of the endophytes using green-fluorescent-protein (GFP) labeling, into roots and tissues, the results showed that endophytes introduced into stems proceed into the roots and rhizosphere, thus suggesting that there may be a continuous distribution of endophytic organisms in the microbiome of the root.

The second factor affecting the distribution of endophytes is the way resources are allocated in the whole plant. Many unique endophytic microbe communities reside freely in the tissues of most plants (Johnston-Monje and Raizada, 2011). To buttress our point, studies by Garbeva et al. (2001) discovered that *Pseudomonas* sp. were more prominent in the stems of potatoes (*Solanum tuberosum*) than in the roots which enhanced crop growth, after its growth was considered for one month. Surette et al. (2003) suspected that higher presence of endophyte within crowns of carrot as compared with the metaxylem tissues might be attributed to a higher level of photosynthate present in the crown regions, which probably supply more resources for a larger community to increase. When plant tissues are effectively colonized, endophytes can be freely distributed in the host plant, thereby enhancing plant growth promotion. However, discovering the mechanism behind this distribution is still an important focus.

3.3 Root colonization behavior of endophytes

Endophytes have the capacity to colonize any part of the plant including the embryo of seeds. The endophytes increase as the seedling germinates and during its early growth (Shade et al., 2017). As the seedlings continue to grow, the interactions between soil and roots microbiome start. The first step involved in the colonization process, especially for endophytic bacterial cells. is called attachment or adhesion (Kandel et al., 2017). Most bacterial endophytes in the surroundings of plant roots move towards the roots through chemotactic affinities for the exudates released by the roots of the plants. This is often followed by attachment to the surface of the plant root, which is very important in penetrating the entry sites at the lateral root emergence region or through other areas, as a result of wounds or mechanical injuries. The exopolysaccharides produced by bacterial cells may also help in enhancing the attachment of bacterial endophytes to the root surface and this is very important for endophytic colonization at early stages. The EPS secreted by bacterial endophyte *Gluconacetobacter diazotrophicus* Pal5 was reported as a vital factor for surface attachment and colonization of rice root (Meneses et al., 2011). Some structures of the bacterial cells such as cell surface polysaccharides, fimbriae and flagella can also aid the attachment of bacterial cells to plant roots. However, in a study carried on maize endophytes, it was observed that lipopolysaccharide (LPS) produced by bacterial endophyte is important for its attachment and endophytic colonization of the maize roots (Balsanelli et al., 2010). Also, it had been reported that binding of N-acetyl glucosamine of LPS with the lectins of the maize root is needed to enhance the attachment and subsequent colonization of the plant roots by the bacterial endophyte (Balsanelli et al., 2013). Microbial interactions in the rhizosphere are triggered by plant exudates which enhances the entrance of endophytes in the root of the plant. Endophytes eventually propel tissue colonization of plant and later continue by moving in the stem, leaves and the entire plant endosphere (Kandel et al., 2017).

Endophytic microbes are ever-present in many species of plant, living actively or latently enhancing tissue colonization. Endophytic bacteria are numerous and they colonize many species of plant. The spread of endophytes starts from the root and decreases across the plant stem and leaves. Immunological labeling techniques with the aid of monospecific polyclonal antibodies were applied against two *Herbaspirillum frisingense* strains and green fluorescent protein (GFP)-fluorescence tagging, the result showed that *H. frisingense* successfully colonize the root of *Miscanthus sinensis* (Rothballer et al., 2008). The capability of endophytes to successfully colonize the inner tissues of the host plant has exposed their importance in agricultural practice. The differences among endophytes in the endosphere are governed by questionable events which influence colonization processes. Soil factors have a great influence on how differently the community of endophytes colonizes the plant. The initial steps which soil bacteria use in colonizing plant roots are still questionable, considering the fact that it depends solely on the interaction that exists between plant root and bacterium. Reports have it that the way plant roots are colonized rests greatly on the diversity, abundance, physiological status and distribution of the supposed endophytes in the soil (Van Overbeek and Van Elsas, 2008). Various factors determine the community structures of endosphere and endophytic colonization. The capability of soil bacteria to enter the root of the plant through induced chemotaxis movement and colonize it effectively through microcolony formation and attachment is the distinctive factor an organism must possess in order to become an endophyte. Endophytes show some signs of their interaction with the plants by colonization and the formation of structures which are similar to ectendomycorrhiza and ectomycorrhizal. Endophytes are present in the vascular tissues of the plants serving as hosts, making asymptomatic colonization intracellularly or intercellularly throughout the root. Genetically engineered derivatives and wild-type strain PsJN of *Burkholderia* sp. strain PsJN tagged with *gfp* (PsJN: *gfp*2x) or *gusA* (PsJN:*gusA*11) genes were inoculated in the rhizosphere of *Vitis vinifera* L. cv. Chardonnay plantlets. The results showed that *Burkholderia* sp. strain PsJN successfully colonized root surfaces, cell walls and the whole surface of some rhizodermal cells (Compant et al., 2005). An endophytic fungus identified as *Hypocrea lixii* isolate F3ST1 was able to colonize onion plants thereby propelling antixenotic repellence of *T. tabaci* (Muvea et al., 2015). Patel and Archana (2017) reported that *Acinetobacter* sp and *Achromobacter* sp. isolated from *Poaceae* family (maize, wheat pearl millet, sorghum, and rice) colonized the root of wheat and enhanced growth improvement. *Bacillus* sp from tomato plant improved the growth of the wheat by colonizing its root (Tian et al., 2017). Meneses et al. (2017) showed that *Gluconacetobacter diazotrophicus* isolated from sugarcane successfully colonized the root of the rice plant and enhanced its growth. *Herbaspirillum seropedicae* isolated from sorghum also colonized the root and leaf of maize plant changing the metabolic profile and nitrogen fixation (Brusamarello-Santos et al., 2017). Change in gene expression was reported when *Herbaspirillum seropedicae* colonized the root of wheat (Pankievicz et al., 2016). *Pseudomonas fluorescence* was able to colonize the tissues of the plant, thereby leading to growth enhancement, when exposed to phosphate deficient conditions (Otieno et al., 2015). A study by Patel and Archana (2017) showed that *Ralstonia* sp. isolated from the *Poaceae* family colonized the root of maize and enhanced its growth. Endophytic bacteria Consortium (*Pseudomonas* spp., *Paenibacillus* spp., and *Sphingomonas azotifigens*) was also able to colonize the root, stem, and leaf of Ryegrass and aid its growth promotion (Castanheira et al., 2017). Figure 3.1 shows the different microbiomes present in the root region of a plant. In summary, it is evident that endophytes can colonize the tissues of the plant both intracellularly and extracellularly. Despite the fact that endophytes can be found in almost all tissues of the plant, roots still have the closest contact with the soil and may function as the first channel through which endophyte penetrates the plant.

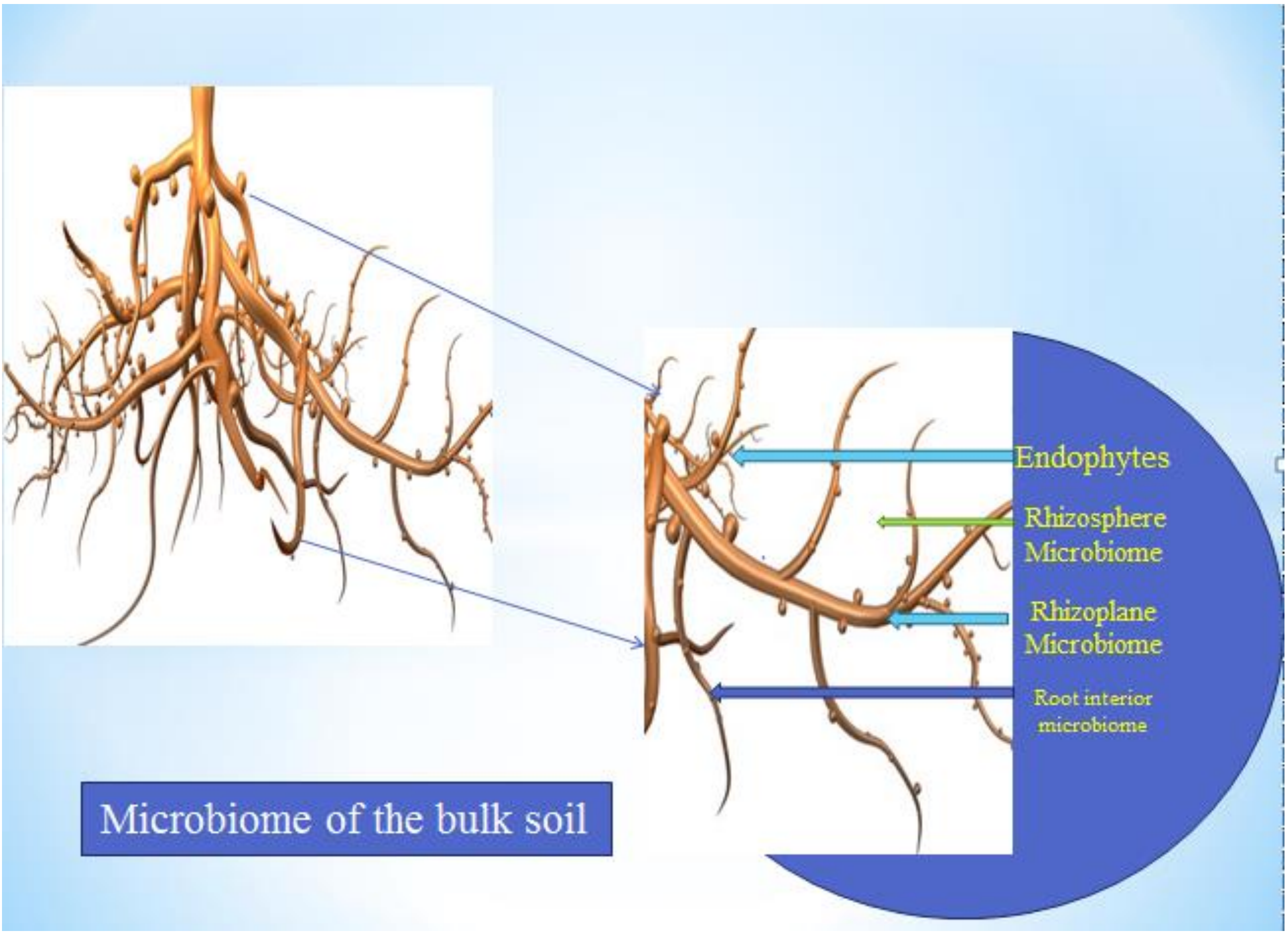


Figure 3.1. The model of the microbiomes present in the root

3.4 Contributions of endophytes to plant growth promotion

Endophytes have been reported to confer many types of protection to their host plant, viz. deterring herbivores by the production of alkaloids that are toxic to grasses, endurance to thrive in hot springs, and protection from pests in dicots (Arora and Ramawat, 2017). Endophytes are said to share close similarities with pathogens residing in the host plant. More evidence shows that the interaction between pathogen and endophytes occurs in different dimensions in the different hosts, and apparently, the physiology of the plant that has been disturbed may inhibit the pathogen's growth, modify the nutrient balance in a way that will favor the endophyte, or trigger the plant's defense mechanism (Busby et al., 2016). The plant's colonization by endophytic fungi proffers enhanced defense against some nematodes which affect plants. This is a complex occurrence, and the antagonistic mechanism exhibited by endophytes is yet to be fully understood (Busby et al., 2016). Thus, endophytes have great importance in the efficiency of the pathosystem and also in plant's diversity, survival, and conservation (Arora and Ramawat, 2017). Endophytes influence plant activities in many dimensions and the actual functions of endophytes have not been clearly defined, but host plants generally benefit from the presence of endophytic microorganisms in their tissues. Promotion of plant growth can be passively or actively achieved by endophytes using different mechanisms, even as metabolites from endophytes confer different health to host plants by triggering plant survival in abiotic and biotic conditions, and also plant growth enhancement. The summary of the applications of endophytes is presented in Figure 3.2.

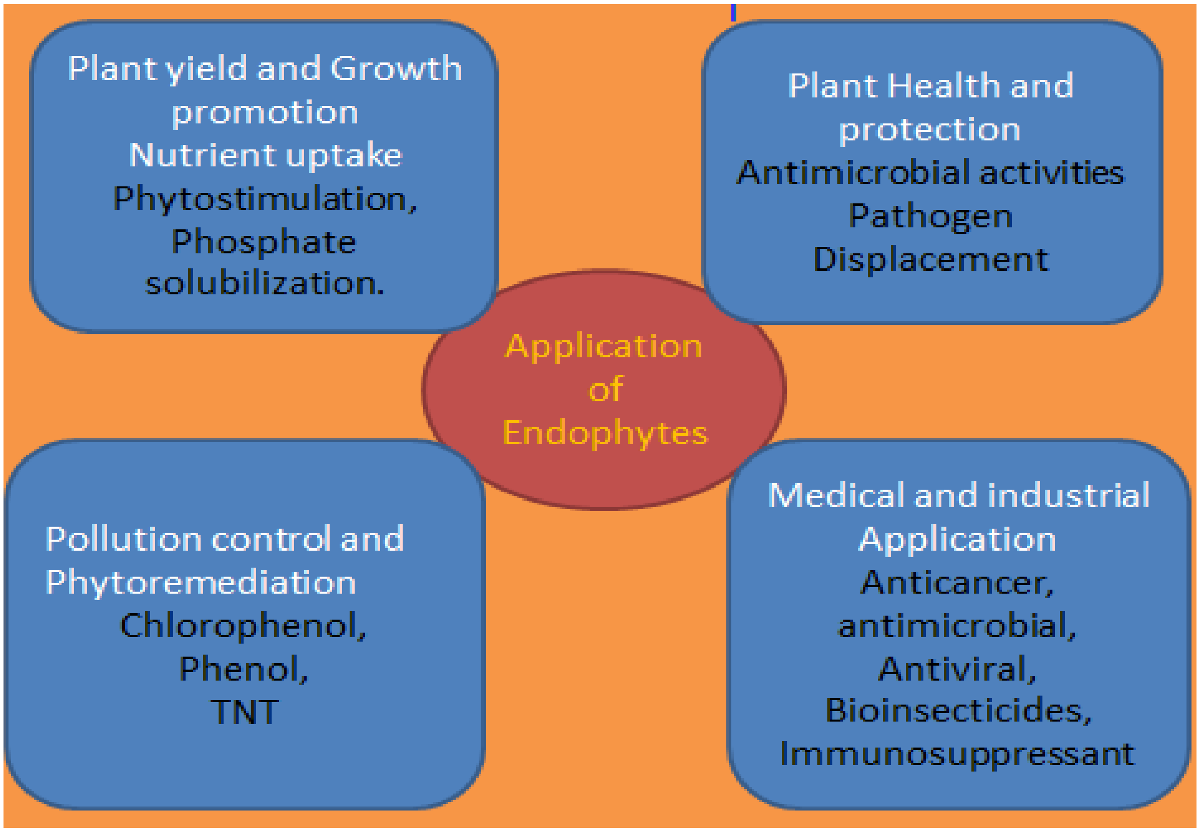


Figure 3.2: Applications of endophytic microbes in various research fields.

3.4.1 Phytostimulation or Biofertilization

The application of endophytes in agricultural sustainability has increased crop growth and yield (Li et al., 2016, Kumar et al., 2017). For years now, researches have established that endophytes possess the capacity to colonize plant tissues, thereby creating a strong symbiotic association with their hosts (Kumar et al., 2017). The interaction results in enhancement of plant growth and improvement in the plant's ability to survive under stress (Saravanakumar and Samiyappan, 2007). Biofertilizers are an eco-friendly, cheap, and renewable source of nutrients to plants which help in reducing our dependence on chemical fertilizers and play a significant role in increasing nutrient availability, thereby enhancing plant growth (Pal et al., 2015). Endophytes produce many phytohormones, some of which include cytokinins, auxins, and gibberellic acids. A study carried out on a wild cottonwood (*Populus trichocarpa*), shows that a diazotrophic endophytic bacterium, *Burkholderia vietnamiensis* was isolated, which supports plant growth promotion by secreting indole acetic acid (IAA) (Xin et al., 2009). The claim was established by comparing *B. vietnamiensis* inoculated plants with control plants, and it was found that more dry biomass weight and increased nitrogen content were gained by the inoculated plant. Increased amounts of bioactive compounds GA4, GA7, and GA4 were reported from a novel fungus strain, *Cladosporium sphaerospermum* which was discovered in *Glycine max* (L) Merr. roots, which helps in improving the growth of soybean and rice plants maximum (Hamayun et al., 2009). An endophytic fungus *Porostereum spadiceum* AGH786 was inoculated with Soya Bean seedling under NaCl stress in the greenhouse. The result showed that phytohormones such as GAs, JA and ABA, and isoflavones were secreted but GAs was secreted in higher quantity than in the control (Hamayun et al., 2017). Potshangbam et al. (2017) reported that some endophytic fungi such as *Fusarium*, *Sarocladium*, *Aspergillus*, and *Penicillium* isolated from maize and rice plants were determining factors in plant growth improvement. The organisms were observed to enhance disease suppression, stress tolerance and plant growth improvement. Some promising endophytic bacteria were isolated from *Echinacea purpurea* and *Lonicera japonica* in a study carried out by Gupta et al. (2016). The isolates were found effective in siderophores production, phosphate solubilization, hydrogen cyanide production, indole acetic acid production, and fixing of atmospheric nitrogen. Endophytic bacteria are becoming prominent in plant growth promotion because of their ability to increase the nitrogen present in the soil. Some endophytic bacteria such as *Rhizobium* spp. and non-nodulating strains such as *Brevibacillus choshinensis, Microbacterium trichothecenolyticum, Micromonospora* spp. *and Endobacter medicaginis* have been reported to be present in the root nodules of a plant (Igiehon and Babalola, 2018).

Fouda et al. (2015) studied endophytic fungi isolated from *Asclepias sinaica* and identified as *Penicillium chrysogenum* and *Alternaria alternata*. The results showed that the isolates enhanced root growth and root elongation, which was attributed to ammonia and IAA production. Abdallah et al. (2016) conducted research on endophytic bacteria isolated from *Withania somnifera* fruits to assess their ability to promote plant growth. The result showed that the most active isolate *Alcaligenes faecalis* was found to produce indole-3-acetic acid and enhance phosphate solubilization. Also, endophytic fungi associated with mangrove were assessed for their ability to promote the growth of *Oryza sativa* L. It was reported that all the endophytic fungi isolated enhanced the growth of *O. sativa* L. “Cempo Ireng” (Tumangger et al., 2018). Different phytohormones produced by endophytes will improve plant growth and reduce the dependence on synthetic fertilizers.

3.4.2 Antimicrobial activity

Many beneficial endophytes discovered in plants have been found to exhibit antimicrobial properties. They assist in the control of some pathogenic microorganisms in plants and/or animals. Most endophytes in medicinal plants exhibit broad-spectrum bioactivity towards pathogenic microorganisms (Devaraju and Satish, 2011). About 37 bacterial endophytes were isolated from *Samanea saman* Merr. and *Tectona grandis* L. plants, and results showed that eighteen isolates produced effective inhibitory compounds against *Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis,* and the growth of *Candida albicans* was inhibited by 3 isolates through *in vitro* method (Chareprasert et al., 2006). The antimicrobial potential of the endophytic fungi like *Alternaria* sp., *Chaetomium* sp., *Alternaria tenuissima, Colletotrichum truncatum, Dothideomycetes* sp., *Thielavia subthermophila*, and *Nigrospora oryzae*, discovered in a medicinal plant known as *Tylophora indica,* were tested against *Fusarium oxysporum* and *Sclerotinia sclerotiorum* and found to strongly inhibit their growth (Kumar et al., 2011c). A summary of other studies is presented in Table 3.1.

3.4.3 Source of bioactive compounds

Endophytes are able to synthesize some bioactive compounds that strengthen plant defense against pathogenic organisms, and some of these compounds have been used in the discovery of novel drugs. A recent report has it that many natural products originated from endophytes, some of which include terpenoids, flavonoids, alkaloids, and steroids. Antibiotics, anticancer, antidiabetic, immunosuppressants, antiviral and biological control agents, among others, are some of the characteristics attributed to bioactive metabolites present in endophytes (Joseph and Priya, 2011).

Geldanamycin and rifamycin are Maytansinoids, which belongs structurally to ansamycin family of polyketide macrolactams and most times are produced by three close families of the plant (*Rhamnaceae, Celastraceae,* and *Euphorbiaceae*) and some bacteria isolates such as

Actinosynnema pretiosum and mosses. It has been speculated that rhizospheric microbes might also take part in the plant's maytansinoids biosynthesis (Nair and Padmavathy, 2014).

Another group of biologically active compounds produced by endophytes is siderophores, which help in chelating microorganism iron ions for improved plant growth. They have been applied in the area of medicine and agriculture. They are also an important component of microorganisms which show a virulence trait, consequently affecting animals, people, and plants. Studies were conducted on five different strains of an endophytic fungus with dark septate identified as *Phialocephala fortinii*, and three siderophores were produced, namely ferrichrome C, ferricrocin, and ferrirubin, whose secretion depends greatly on the iron (III) concentration and pH of the growth medium (Nair and Padmavathy, 2014). However, *P. fortinii* shows promise for use in industrial manufacturing of siderophores. A plant *Taxus chinensis* produced an endophyte identified as *Metarhizium anisopliae*, which was discovered to be the source of taxol (Liu et al., 2009). Also, the leaves of a medicinal plant identified as *Justicia gendarussa* harbor an endophyte named *Colletotrichum gloeosporioides*, which is also notable for the production of taxol (Gangadevi and Muthumary, 2008). Table 3.1 gives a summary of some other studies where bioactive compounds were produced by endophytes.

Table 3.1: Summary of other studies on the antimicrobial activities and bioactive compounds produced by endophytes

Endophytes	**Type of Endophytes**	**Host Plant**	**Pathogen active against**	**Compounds secreted.**	**References**
Phomopsis sp.	Endophytic fungi	*Plumeria acutifolia*	*Pseudomonas* sp, *Escherichia coli, Klebsiella* sp, *Bacillus subtilis, Staphylococcus aureus.*	-	Nithya and Muthumary (2010)
Phomopsis sp.	Endophytic fungi	*Allanmands cathartica*	*Pseudomonas sp, E. coli, Klebsiella* sp., *B. subtilis, S. aureus*.	Terpene	Nithya and Muthumary (2011)
Fusarium solani	Endophytic fungi	*Taxus baccata*	*Staphylococcus. epidermidis, S. aureus*, *S. flexneri*, *B. subtilis*.	1-tetradecene, 8-pentadacanone, 8-octadecanone, 10-nonadecanone,octylcyclohexane	Tayung et al. (2011)
Xylaria cubensis, Cyanodermella sp., *Lasella* sp	Endophytic fungi	Citrus, Zanthoxylum of Rutaceae and Cinnamomum of Lauraceae	*Erwinia carotovora, Xanthomonas campestries, Ralstonia solanceae.*	-	Ho et al. (2012)
Alternaria sp, *C. gloeosporoides, Fusarium* sp., *Pestatiopsis* sp.	Endophytic fungi	*Biota orientalis, Pinus excels* and *Thuja occidentalis*	*Streptococcus faecalis, Salmonella typhi*	-	Subbulakshmi et al. (2012)
Botrytis sp	Endophytic fungi	*Ficus benghalensis*	*Klebsiella* sp, *E.coli*	-	Senthilmurugan et al. (2013)

Aspergillus sp	Endophytic fungi	*Bauhinia guianensis*	*E.coli*, *P.aurigonosa*, *S.aureus*, *B.subtilis*	Fumigaclavine C and Pseurtotin C	Pinheiro et al. (2013)
Pestalotiopsis mangiferae	Endophytic fungi	*Mangifera indica* Linn	*E.coli*, *B.subtilis*, *K. pneumonia*.	4-(2,4,7-trioxa-bicyclo[4,10]-heptan-3-yl	Subban et al. (2013)
Alternaria alternata, *A. citrimacularis*, *A.niger*	Endophytic fungi	*Aegle marmelos*	*S.typhi*, *Proteus mirabilis*, *S.epdermidis*, *S.aureus*, *Shigella* Sp, *Shigella* sp, *P. aeruginosa*, *E.coli*, *K.pneumoniae*	-	Mani et al. (2015)
Bacillus atrophaeus, *Bacillus mojavensis*	Endophytic bacteria	*Glycyrrhiza uralensis* (Licorice)	*F. oxysporim*, *Fulvia fulva*, *A. solani*, *C. goleosporoides*, *Verticillium dahlia*	1,2-bezenedicarboxyl acid, Methyl ester, Decanodioic acid, bis(2-ehtylhexyl)ester.	Mohamad et al. (2018)
Arthrinium sp MFLUCC16-1053	Endophytic fungi	*Zingiber cussumunar*	*Staphylococcus aureus*, *E.coli*	Laurenan-2-one, 3E-cembrene A, B-cyclocitral, sclareol, cembrene, farnesol, β-isocomene	Pansanit and Pripdeevech (2018)
Xylaria sp, *Penicillium* sp	Endophytic fungi	*Piper aduncum*, *Aliberta macrophylla*	*Cladosporium cladosporoides*, *C. Sphaerospernum*	Dihydroiso-1-caumarin, (3R,4R)-4,7-dihydrooxymellein, (R)-7-hydroxylmellein.	Oliveira et al. (2011)
Fusarium solani	Endophytic fungi	*Taxa baccata*	*Candida albicans*, *C. Tropicalis*	Octylcyclohexane, 8-octadecanone, 1-tetradecane, 8-pentadecanone, 10-nonadecanone	Tayung et al. (2011)
Alternaria sp, *C. gloesporoides*, *Fusarium* sp, *Pestalotropsis* sp	Endophytic fungi	*Biota orientalis*, *Pinus excels*, *Thuja occidentalis*	*C. albicans*	-	Subbulakshmi et al. (2012)
Phoma sp		*Cinnamomum mollissimum*	*Aspergillus niger*	5-hydroxyramulosin	Santiago et al. (2012)

Lasmenia sp, *Ophiceras tenuisporium*, *Xylaria cubensis*, *Cyanodermella* sp	Endophytic fungi	Citrus, Zanthoxylum of Ruatceae, Cinnamomum of Laureceae	*Alternaria solani, B.cinera, Colletotrichum gloesporiodes, C. higginsianum, C. lageniformis, Fusariun oxysporium, Monacha fruticola, Penicillium digitatnum, Puccinia sidii, Pythium aphanidermatum*	-	Ho et al. (2012)
Chaetomium globosum, *Myrothecium verrucaria*	Endophytic fungi	*Caloptropis procera*	*Alternaria alternata, Botrytis cinera, F. oxysporum, Pythium ultimum*	-	Gherbawy and Gashgari (2014)
Phomopsis sp	Endophytic fungi	*Aconitum carmichaeli*	Clinical Isolates	Gavodermside D and Clavasterols	Wu et al. (2013)
Pestalopsis mangiferae	Endophytic fungi	*Mangifera indica* Linn	*C. albicans*	-	Subban et al. (2013)
Meyerozima sp and *Chaetomium globosum*.		*Trattinnickia rhoifolia* (Burseraceae) and *Protium heptaphyllum*	*F. oxysporum*	Cladosporin, chaetoviridin A and Chaetoatrosin A	Fierro-Cruz et al. (2017)

3.4.4 Biocontrol activities

Endophytic microorganisms are often acknowledged as having some biocontrol activities, and are therefore a possible replacement for inorganic chemicals. Endophytes play a beneficial role not only for controlling conifers, but in insect herbivory too (Posada and Vega, 2006). A fungal endophyte identified as *Beauveria bassiana* has been reported to control pathogens of insects such as borer insects, which mostly attack seedlings of sorghum (Tefera and Vidal, 2009), and coffee (Posada and Vega, 2006). *Botrytis cinerea* is an organism that causes rot of tomato fruits and reduces their shelf life and postharvest quality. However, bacterial endophytes identified as *Bacillus subtilis*, which was found resident in the tissues of *Speranskia tuberculata*, gave a strong antagonistic effect through *in vitro* studies on *B. cinerea* (Wang et al., 2009). *Pinellia ternate* agglutinin (*PtA*) gene was expressed in *Chaetomium globosum* YY-11, an endophyte discovered in grape seedlings, alongside *Enterobacter* sp. and *Bacillus subtilis* which are endophytic bacteria got from the seedlings of maize (Zhao et al., 2010). These recombinant endophytic genes were active in controlling populations of pests such as in the seedlings of most crops. Also, in a related study, *Enterobacter cloacae* that harbors *PtA* gene was discovered as an active bio-insecticidal agent in controlling white-backed planthopper, *Sogatella furcifera* (Zhang et al., 2007). However, the application of recombinant endophytic organisms as biocontrol agents becomes essential, since they produce antipest proteins through a novel technique for controlling plant pests, these endophytes can successfully colonize crop plants. A summary of similar studies is presented in Table 3.2 below. This biocontrol activity by endophytes boosts plant resistance to diseases and reduces dependence on pesticides.

Table 3.2: Summary of major findings on the biocontrol activities of endophytes

Endophytes	**Type of endophytes**	**Plant Source**	**Pathogens**	**Major Findings**	**References**
Ulocladium, Penicillium, Cladosporium, Aspergillus, Fusarium Chaetomium, Alternaria, Paecilomyces, Bipolaris, Trichoderma, Diaporthe, Nigrospora and *Phoma.*	Endophytic fungi	Strawberry leaves	Third instar larvae of *D. fovealis*	The result showed that *Paecilomyces* isolates were found to induce the highest mortality rates on the pathogens	Amatuzzi et al. (2018)
Ochrobactrum sp (CB361-80) and *Pantoea* sp (CC372-83)	Endophytic fungi	(*Cucumis sativa* L.)	*Pseudomonas syringae* pv. Lachrymans	The result showed that the isolates were able to control angular leaf spot disease in cucumber	Akbaba and Ozaktan (2018)
Serratia (B17B), *Enterobacter* (E), and *Bacillus* (IMC8, Y, Ps, Psl, and Prt)	Endophytic bacteria	Papaya, snap bean and flowering dogwood	*Phytophthora capsici*	Phytophthora blight, caused by *Phytophthora capsici*, which is the most destructive disease of bell pepper in the United States was successfully reduced *invitro*	Irabor and Mmbaga (2017)
Leptosphaeria sp, *Penicillium simplicissimum, Acremonium* sp, and *Talaromyces flavus*	Endophytic fungi	Cotton	*Verticillium dahliae* strain Vd080	The Verticillium wilt of cotton was controlled and improvement in cottonseed yield in tested cotton fields was observed.	Yuan et al. (2017)
Endophyte A22F1	Endophytic fungi	Flowering dogwood (*Cornus florida*)	*Phytophthora capsici*	The result showed the control of root rot pathogens in pepper.	Mmbaga and Gurung (2018)

3.4.5 Nutrient cycling

One of the important processes of balancing existing nutrients and making present the nutrients for every component in an ecosystem is called the nutrient cycle. Biodegradation of biomasses that are dead is one of the numerous methods of bringing used minerals back into the ecosystem which consequently brings them to the level where they can be utilized by the organism. This then becomes a continuous chain process. Many saprophytic organisms perform an active role in the nutrient cycling process. Some studies have proved that endophytes showcase a vital function in the biodegradation of host plant litters (Promputtha et al., 2010). In plant litter biodegradation, endophytic microorganisms, first of all, colonize the plant and then trigger the saprophytic organisms to act on it through an antagonistic reaction, thereby giving an increase in the decomposition of litters (Nair and Padmavathy, 2014). He et al. (2012) reported that virtually all endophytes have the potential for organic matter decomposition, some of which include hemicellulose, lignin, and cellulose, which are desired in decomposing different groups of organic matters.

3.4.6 Biodegradation and Bioremediation

Most endophytic microbes have the capacity to decompose complex organic compounds. Bioremediation is a way of removing waste and pollutants present in the environment by the activities of a microorganism. It is a bioprocess that depends greatly on microorganisms in the breaking down of waste products. This is achievable because of the numerous microorganisms which are available in nature. The impact of endophytic microbes in exhibiting bioremediation by *Nicotiana tabaccum* was studied by Mastretta et al. (2009). The inoculation of *N. tabaccum* alongside endophytic microbes showed an increase in the biomass number when exposed to cadmium (Cd) stress, and the number of noninoculated plants was lower when compared to inoculated plants. This finding, however, showed the beneficial roles of endophytes from the seeds of plants on the accumulation and toxicity of metals. Some fungal endophytes were assessed for their ability to degrade the plastic polymer polyester polyurethane (PUR) (Russell et al., 2011). Many organisms showed their capacity for the degradation of PUR effectively in liquid and solid media; however, genus *Pestalotiopsis* gave the best result. Two isolates of *Pestalotiopsis microspora* successfully used PUR as their only carbon source when exposed to anaerobic and aerobic conditions. An enzyme serine hydrolase was predicted to be responsible for this attribute when molecular characterization was carried out, this enzyme can boost the stress tolerance potentials of the plant (Russell et al., 2011).

Endophytic bacteria aid phytoextraction of most heavy metals. Many studies on how endophytic bacteria can remove heavy metals have been carried out, indicating endophytes can help enhance the stress tolerance potential of the plant (Rajkumar et al., 2010). Endophytes are also found to be active in the degradation of polyaromatic hydrocarbon (PAH) (Radwan, 2009). Many types of microorganism nowadays can produce strong surface bioactive biomolecules of biosurfactants with varying molecular size and chemical properties. The bioremediation ability of an endophytic bacteria identified as *Pseudomonas fluorescence* RE1 (GenBank: MF102882.1) was assessed on heavy metals such as Cr, Cd, Ni, and Zn. The study revealed that the endophyte was able to withstand heavy metals at high concentration and can be used for survival by plants in environments contaminated with heavy metal (Karnwal, 2018). This biodegradation and bioremediation activities attributed to endophyte could be helpful for the survival of the plant in extreme condition.

3.4.7 Cold and Drought Stress Tolerance

Endophytes have been reported to enhance plant tolerance to cold stress. A study carried out by on Subramanian et al. (2015) on tomato plants showed that inoculation with the psychrotolerant endophytic bacteria, *Pseudomonas vancouverensis* OB155 and *P. frederiksbergensis* OS261 enhances survival under cold stress (10–12^{o}C). Reduced membrane damage and elevated antioxidant activities were recorded when compared with the control plant. However, genes for cold acclimation (LeCBF1 and LeCBF3) were produced by the endophyte inoculated plants (Subramanian et al., 2015). Also, an endophyte, *Burkholderia phytofirmans* strain PsJN induced growth and also strengthened the cell wall of Arabidopsis which resulted in increased resistance to cold stress (Su et al., 2015). Endophytes were also reported to boost plant tolerance to drought. Through the transcriptomics method, it was observed that endophytic *B. phytofirmans* PsJN showed diverse functions when inoculated in potato plants (Sheibani-Tezerji et al., 2015). Transcript used in cellular homeostasis, transcriptional regulation and ROS detoxification were improved in potato inoculated with *B. phytofirmans* PsJN in a drought stress area. This indicates that endophytes can detect physiological changes in plants and regulate gene expression for adaptation to that environment. Bacterial endophytes therefore have the prospect of being used as a protective agent in agricultural practices under severe climatic conditions and they can affect physiological responses of the plant to stresses.

3.4.8 Secretion of volatile organic compounds

An endophytic fungus known as *Hypoxylon* sp. which was found resident in the tissues of *Persea indica* gave an array of volatile organic compounds (VOCs) notable among them were 1,8- 1-methyl-1,4-cyclohexadiene, cineole, temporarily reported as alpha-methylene-alpha-fenchocamphorone, among others that are yet to be identified. It produced a strong VOC antimicrobial compound active in inhibiting *Phytophthora cinnamomi, Botrytis cinerea*, *Cercospora beticola*, *Sclerotinia sclerotiorum*, and *Cercospora beticola*. This may have a big impact in the interactions between the fungus and how it survives in the host tissue (Tomsheck et al., 2010). They undeniably showed that *Hypoxylon* sp. produced 1, 8-cineole (a monoterpene), which is a novel compound. This octane derivative can be used as a fuel additive just as many VOCs produced by *Hypoxylon* sp. The study suggests that sourcing for fungi that can produce VOCs like *Hypoxylon* sp. will increase their utilization in industries, medicine, and in the production of energy for improved agricultural practices.

Phomopsis sp. a fungal endophyte, which was unusually isolated from *Odontoglossum* sp., secreted a distinct number of VOCs which are benzene, ethanol, and 2- propanone, and a monoterpene having a peppery odor called sabinene (Singh et al., 2011). Gases from *Phomopsis* sp. have antifungal characteristics and mixtures of the VOCs have similar antibiotic activity against numerous plant pathogenic fungi. A natural thujospen was also revealed to be produced by *Penicillium decumbens* Thom C. (Polizzi et al., 2011). Suwannarach et al. (2013) showed that *Nodulisporium* sp. CMU-UPE34 was able to produce 31 VOCs. The GC-MS analysis of the results showed that numerous VOCs are produced, among which are acids, alcohols, esters, and monoterpenes. However, eucalyptol, also called 1, 8-cineole was the only volatile compound found to be produced in a large quantity. Many chemicals such as butyl, ethanol, and ethyl acetate which are VOCs spectrum have been reported to be produced naturally by *Ceratocystis fimbriata*, after thorough GC-MS analysis, and have biotechnological importance in plant growth promotion (Li et al., 2015b, Kaddes et al., 2019). More studies need to be carried out and channeled towards VOCs that have antimicrobial properties which will help in improving plant growth.

3.4.9 Combined roles performed by some endophytes

A number of endophytic microbes are known to possess the ability to carry out different activities within their hosts. Some Endophytes were discovered to have both antimicrobial and herbicidal properties (Li et al., 2012b). An endophytic bacterium, *Bacillus* sp. SLS18, common for plant growth-promotion, was also studied for its activity in biomass production when *Solanum nigrum* L. was exposed to manganese and cadmium l (Li et al., 2012b). Results showed that it displayed great resistance against antibiotics and heavy metals. The strain was also found to produce siderophores, indole-3-acetic acid, and 1-aminocyclopropane-1-carboxylic acid deaminase.

3.5 The influence of environmental conditions on endophytic microbe population

Endophytes are numerous and they survive in different environments, and some may even grow at extreme conditions (Compant et al., 2010). The population of endophytes varies from species to species and plant to plant. In the same species, the endophytic population may not only be unique from one region to another but may also differ with a change in climatic conditions in the same region. Some of the major factors affecting endophytes are temperature, elevation, latitude, and rainfall which can work together in influencing the composition of endophytes in plants. Climate change may result in an uncontrolled rainfall which could either occur in short supply or in excess (Enebe and Babalola, 2018). Excessive rainfall leads to flooding and erosion. These factors can sometimes affect the physiology of the plant, thus revamping plant and endophyte interactions. Chareprasert et al. (2006) studied temporal changes and the way they affects the total endophytic fungi population and observed that matured teak leaves (*Tectona grandis* L.) and the rain tree (*Samanea saman* Merr.) gave a greater number of species and genera, with higher frequency of colonization as compared to juvenile leaves, and their presence increased across the rainy season. Thongsandee et al. (2012) reported that the endophytic population and frequency in *Gingko biloba* L. shows considerable difference in sampling dates for all organs of plants studied, which are, petiole, young leaves, and twigs. They observed that *Phyllosticta* sp.

was present in both petioles and leaves initially examined starting from August with its peak in October in Japan. *Phomopsis* sp. was also detected in all twigs examined throughout the planting year. These results infer that the abundance of the two dominant endophytes differed with seasons and are also organ-specific.

Dry environments may be helpful in selection and discovery of drought-tolerant endophytes (Yandigeri et al., 2012); studies focusing on an area in Namibia characterized with a prolonged dry season showed that many endophytic microbe strains that are desiccation-resistant were detected in maize, pearl millet, and sorghum (Grönemeyer et al., 2012). Similarly, environments that are cold help in the selection of endophytes that are psychrophilic (Nissinen et al., 2012).

However, recent studies carried out on the endophere microbiome of plants using high-throughput sequencing have showed that genotype (Rodríguez-Blanco et al., 2015), host plant species (Ding and Melcher, 2016), growing season (e.g., of trees) (Shen and Fulthorpe, 2015, Ding and Melcher, 2016), developmental stage (e.g., seedling or mature plant) (Yu et al., 2015, Ren et al., 2015a), geographical location (field conditions) (Edwards et al., 2015), host plant nutrient status (Hameed et al., 2015), fertilization (Rodríguez-Blanco et al., 2015) and cultivation practice (Edwards et al., 2015) are some of the factors reported to have significant influence on the plant endopshere microbiome.

Studies comparing the diversity and abundance of endophytic bacteria between transgenic glyphosate-resistant cultivars and wild-type soybean plants observed a higher diversity and abundance in the culturable endophytes compared with the wild-type plants (de Almeida Lopes et al., 2016). They reported that the genotype of the plant influenced the functional diversity of bacterial endophytes and IAA-producing strains were isolated from one of the three genotypes of sweet potato studied.

Alongside host properties, variations in environmental temperature and CO_2 regulate bacterial endophyte communities. Understanding how bacterial endophytes respond to climate change, especially in the case of high temperature and CO_2, can help in terms of policies that involve environmental issues. A study by Ren et al. (2015b) showed that bacterial endophytes from the plant leaves are more influenced by climate than bacterial communities of the soil. The community structure of bacterial endophytes inhabiting leaves of rice was affected by high CO_2 levels at the filling and tillering stages, but not at maturity, and this effect can be linked to the level of N fertilization levels (Ren et al., 2015a). Also, Ren et al. (2015b) showed that endophytes community inhabiting leaves at different locations within the plant reacted differently to increase in CO_2. Available oxygen also affected bacterial endophytes community inhabiting rice, especially the diazotrophs.

3.6 Challenges and advances in isolation and identification of endophytes

Most endophytes have been found to be culturable, although some are still not culturable. This has widespread effects in measuring and identifying endophyte community structure and diversity. Recent studies have proved the existence of endophytes through various cultivation-independent experiments and fluorescence in situ hybridization-confocal laser scanning microscopy studies (Berg et al., 2014b). The use of modern molecular tools alongside complimenting culture-independent techniques is now widespread. These methods have their base as a polymerase chain reaction (PCR), useful for amplifying a DNA region, most times through16S rRNA, subsequently followed by purification methods for analyzing endophyte communities, some of which sometimes include community fingerprinting or cloning techniques (Gao and Tao, 2012). However the biased results attributed to PCR present one of the major challenges faced in identifying these endophytes (Lu et al., 2018). Currently, researchers are considering ways of combining both culture-independent and dependent approaches because each has bases inherent to it (Reinhold-Hurek and Hurek, 2011).

The merging of culture-independent methods and culture-dependent approaches has helped in discovering numerous endophytes that are uncultured in most plant species (Pereira et al., 2011). These species might show some important functional roles in the plant (Sessitsch et al., 2012). New techniques will help scientists to further explore the world of these organisms despite reports of being uncultivable (Stewart, 2012). The use of Ribosomal DNA (rDNA) ITS has been established to be a valuable source in resolving phylogenetic relationships among genera or species staring from lower levels (Nair and Padmavathy, 2014). It was also recorded that the identification of nonsporulating fungi using ITS sequences analysis was effective in reducing the effect of the biased report often associated with fungi identification. Furthermore, ITS data and the Large Subunit (LSU) are strong tools to end the difficulty often associated with the taxonomy of endophytic microbes from Basidiomycetes (Rungjindamai et al., 2008). In addition, more recently, genomic and metagenomic studies have gained a lot of attention in endophytic research, as the approach can help to identify different microbes (culturable and nonculturable) present in an environment (Fadiji and Babalola, 2020c). This approach will also help to predict the functions of endophytes as regards whether it is beneficial, pathogenic or nonpathogenic and also identification of many uncharacterized taxa (Brader et al., 2017b, Fadiji and Babalola, 2020c).

3.7 Limitations in the use of endophytes

There is a serious need to explore the world of endophytic microorganisms in a bid to identify competent ones that will perfect their function effectively under the influence of complex rhizospheric plant-microbe interactions, and different ecological situations. This is because numerous problems already exist which are associated with the applications of endophytes, some of which originate from microbial community-plant interaction complexity and exhibition of poor rhizospheric competence in the presence of endogenous microorganisms (Schulz et al., 2002). The population of endophytes is also disturbed by a persistent change in the condition of their environment and emerging soil biological, chemical and physical properties. Being affected with factors earlier mentioned, the effectiveness of an endophytic microbial population is not clear.

Apart from assessing the functionality of endophytes, marketing, proper formulation, and production methods are also some of the limitations in the use of these beneficial microbes for agricultural practices. Another concern with the use of endophytes from plants is that some of them are opportunistic pathogens for animal, plant, or human pathogens and the application of these microbes can cause mild to severe illness and sometimes outbreaks of disease.

3.8 Future outlook

Considering the importance of endophytes, it is strongly recommended that future studies should focus on the way the endophytes react with the plant host in order to ascertain the best way to make them effective for continuous crop production. Most endophytes known for their numerous functions were isolated through culture-dependent methods; there is still a need to explore culture-independent techniques such as genomics and metagenomics studies in order to be able to detect more novel functions and species. Also, the mechanisms of action of most endophytes are yet to be fully understood. Though some studies are ongoing in this regard, it is very important that the different underlying mechanisms of action of these endophytes should be urgently examined, especially in the way they interact with other microbes in the tissue of plants. Mechanisms backing up the ways of distribution are not clear because endophyte species differ from one plant to the other; they are still a novel field to be explored. A better understanding of functions encoded by endophytic genomes could help us to have insight to the mechanisms involved in plant-microbe interactions and establish genomic determinants of endophyte lifestyle. Experiments studying the transcriptome characterization dynamics of most endophytes and their host plants are promising methods in understanding some of the factors that drive plant–endophyte interactions. Further studies can also focus on the following:

i. Plant-microbe interaction for adaptation and stress tolerance.
ii. How host plant secondary metabolism is affected by symbiosis.
iii. How microbial secondary metabolism is affected by symbiosis.
iv. The use of metagenomics and bioinformatics tools for the determination of endophyte diversity, evolutionary relationship and prediction of the real functions of endophytes.

3.9 Conclusion

Attention has been shifted to the world of endophytes due to their ability to promote plant growth through different mechanisms and functions as shown in this study**.** Numerous species of endophytes isolated from many agricultural plants shows that they play a notable role in balancing plant physiology, restoration of available nutrients in the plant, and phytoremediation among others. The world of endophytes has attracted many researchers in the last couple of years, as shown by the over 32,000 articles published about their important attributes as seen on Google Scholar, in both review and research papers. It is a known fact that sustainable agriculture needs self-contained functioning and inputs that are cheap and ecofriendly. To combat the emerging increase in food demand, the use of biological dependent techniques is needed, of which this study has presented endophytes as a possible option. Still, the limitations facing endophytes are some of the hurdles affecting their usage in agriculture.

CHAPTER FOUR

4.0 ELUCIDATING MECHANISMS OF ENDOPHYTES USED IN PLANT PROTECTION AND OTHER BIOACTIVITIES WITH MULTIFUNCTIONAL PROSPECTS

Abstract

Endophytes are abundant in plants and studies are continuously emanating on their ability to protect plants from pathogens that cause diseases especially in the field of agriculture. The advantage that endophytes have over other biocontrol agents is the ability to colonize plant's internal tissues. Despite this attributes, a deep understanding of the mechanism employed by endophytes in protecting the plant from diseases is still required for both effectiveness and commercialization. Also, there are increasing cases of antibiotics resistance among most causative agents of diseases in human beings, which calls for an alternative drug discovery using natural sources. Endophytes present themselves as a storehouse of many bioactive metabolites such as phenolic acids, alkaloids, quinones, steroids, saponins, tannins, and terpenoids which makes them a promising candidate for anticancer, antimalarial, antituberculosis, antiviral, antidiabetic, anti-inflammatory, antiarthritis, and immunosuppressive properties among many others, even though the primary function of bioactive compounds from endophytes is to make the host plants resistant to both abiotic and biotic stresses. Endophytes still present themselves as a peculiar source of possible drugs. This study elucidates the mechanisms employed by endophytes in protecting the plant from diseases and different bioactivities of importance to humans with a focus on endophytic bacteria and fungi.

Keywords: Antibiotics, Commercialization, Drug, Metabolites, Pathogen.

This chapter has been published in *Frontiers in Bioengineering and Biotechnology* 2020; 8:467. https://doi.org/10.3389/fbioe.2020.00467

4.1 Introduction

Endophytic microorganisms are referred to as the microbes that inhabit the internal parts of a plant. They gain entrance into the seed, leaf, stem, and root of a plant and they are not harmful to the host plant (Yadav, 2018). Endophytes improve plant growth by secreting phytohormones and consequently help in nutrition improvement using bidirectional nutrient transfer and enhancement of the health of plants by protecting them against phytopathogens (Andreozzi et al., 2019, Shen et al., 2019). Plant-endophyte interaction triggers the protection of plants against harmful conditions of the environment such as heavy metal presence and drought (Kushwaha et al., 2019, Khan et al., 2019). Endophytes are numerous and studies have it that they are present in many plants; they became important due to their capacity to produce many bioactive metabolites and biotechnologically relevant enzymes (Khan et al., 2014, Rajamanikyam et al., 2017). Most times when endophytes are inoculated in the plant, they produce significant biomass increment and also help in boosting commercial agriculture (Shen et al., 2019, Santoyo et al., 2016). Endophytes are gaining biotechnological and industrial relevance as a result of their ability to secrete secondary metabolites, serve as biocontrol agents, antimicrobial agents, antitumor agents and immunosuppressants, and to secrete antiviral compounds and develop natural antioxidants, antidiabetic agents, antibiotics, and insecticidal products (Yadav, 2018, Gouda et al., 2016).

In the last 20 years, endophytes isolated from most plants have shown themselves to be a rich source of natural products for industrial and agricultural use, amongst several other applications. Enzymes can be used to replace poisonous chemicals. They thrive best under normal temperatures and neutral pH. As the years progress, researchers are beginning to see prospects in microbial enzyme production. There are many reports currently that microorganisms isolated from extreme environments have great biotechnological applications in medicine, agriculture and industry (Archna et al., 2015, Yadav et al., 2015, Sahay et al., 2017, Singh et al., 2016). This review aimed to present the various mechanisms of action used by endophytes in protecting a plant and report some bioactivities of importance to people with special emphasis on endophytic bacteria and fungi.

4.2 An overview of endophytes

The word endophyte connotes ‘‘in the plant’’, and studies have established that endophytes emanate from the phyllosphere and rhizosphere (Verma et al., 2017). Endophytes are generally isolated from the internal tissues of plants after surface sterilization. Plant association with microorganisms may be classified in many forms such as mycorrhizal, pathogenic, epiphytic, saprotrophic, and endophytic based on the type of colonization and their roles (Brader et al., 2017c). Only a few microorganisms such as endophytic microbes and mycorrhizal fungi can be exceptional and find their way into the inner tissues of a plant. Endophytic microorganisms such as bacteria, fungi, eukarya, and archaea inhabit plant tissues (De Tender, 2017), they are known not to cause any harm to the host plant. They exhibit a symbiotic association with tissues of most plants and sometimes can be slightly pathogenic. These endophytic microbes have been identified in many varieties of plants some of which are Rice, Wheat, Tomato, Cowpea, Maize, Strawberry, Chickpea, Mustard, Sugarcane, Chilli, Citrus, Soybean, Cotton, Pearl millet and Sunflower (Verma et al., 2017, Yadav et al., 2018).

The advent of microbial biotechnology has helped in establishing the fact that microorganisms play significant roles in industry, agriculture and medicine (Gouda et al., 2016, Rajamanikyam et al., 2017). Having a better understanding of the diverse roles microorganisms play in the ecosystem will enhance the ways they can be applied in the field of agriculture most importantly for plant growth and crop yield (Nair and Padmavathy, 2014). The world of endophytes has attracted the interest of many researchers due to their significant roles in promoting growth and in enhancing the survival of plants under extreme conditions (Shen et al., 2019). Bioactive metabolites secreted by endophytic microorganisms are useful in industries, agriculture and the field of medicine. Plants perform a major function of determining the type of microorganism that can be associated with it by the makeup of its root exudates (Andreozzi et al., 2019). Thus, the interaction between endophytic microorganisms and plants greatly depends on the capacity of these microbes to use the exudates produced by the plant roots as their energy source (Kandel et al., 2017). Endophytes can efficiently enhance growth promotion using different modes of operations and increasing the resistance of plants to extreme conditions (Yadav, 2018). Notably, endophytic microbes have been used in the mass production of industrially relevant products such as antibiotics, enzymes, and riboflavin among others (Latz et al., 2018). The resistance to antibiotics is on the increase especially among organisms that cause disease and this has great public health implications if proper care is not taken (Adegboye et al., 2012).

Microbial biotechnology has gone beyond the production of only metabolites such as ethanol and butanol, now biotransformation of many chemicals has been incorporated to reduce the impact of environmental pollution using different strategies such as bioremediation, waste management, and composting. For some decades now, attention has been shifting to the use of microorganisms, animals and plants for the production of new drugs (Gouda et al., 2016, Latz et al., 2018). These products, mainly from natural sources, are less toxic and cheaper. Endophytic fungi have a great prospect for the secretion of numerous bioactive metabolites. Some of these phytonutrients or metabolites like polyphenol and anthocyanin can reduce diseases such as cancer and heart diseases.

4.3 General mechanisms employed by endophytic bacteria and fungi in plant protection

Endophytic microorganisms help in boosting plant fitness through several mechanisms of action. The generally mechanisms employed by endophytic bacteria and fungi are discussed in this section. The modes of action include direct and indirect mechanisms as illustrated in Figure 4.1. These mechanisms are discussed in detail below.

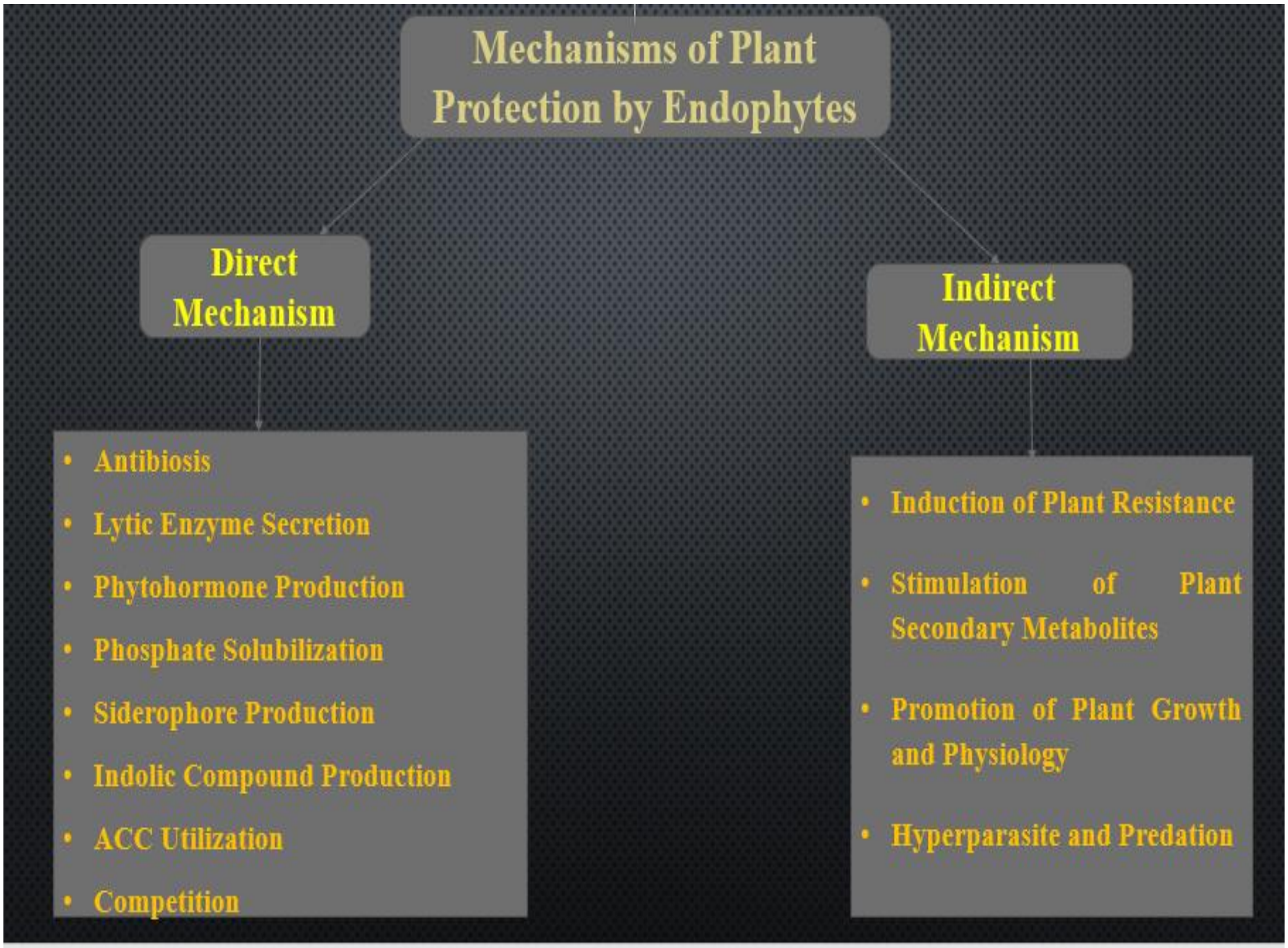


Figure 4.1: Mechanisms employed by endophytes for plant protection.

4.3.1 Direct mechanisms of plant protection from pathogens

Recent studies carried out on endophytes have established their capacity to enhance host defense against diseases and reduce the damages attributed to pathogenic microorganism (Ganley et al., 2008, Mejía et al., 2008). The most common strategy employed by these researchers is in vitro direct plate antagonistic reaction against pathogens or by comparing the rate of survival of plant inoculated with control. Although some studies have presented new mechanisms used by endophyte in reducing the effects of pathogens, current knowledge about endophytes, pathogen and plant regulations still is not fully understood (Ganley et al., 2008). In this section, we shall be discussing a direct mechanism (endophytes-pathogens interactions) and indirect mechanism (enhanced plant defense). In the direct mechanism, endophytes directly produce antibiotics which help in suppressing pathogens. However, direct endophyte-pathogen interactions are compounded and responsive to species-specific antagonism (Arnold et al., 2003). Some examples of direct mechanisms used by endophytes are discussed below.

4.3.1.1 Antibiotics produced by endophytes

Most endophytes have been reported to produce some secondary metabolites and some of them exhibit antibacterial and antifungal properties which help in inhibiting the growth of phytopathogenic microorganisms (Gunatilaka, 2006). Many types of research are still ongoing in a bid to identify endophyte metabolites for possible commercial use. Different bioactive compounds have been studied for their ability to inhibit many phytopathogens (Suryanarayanan, 2013, Daguerre et al., 2017). Also, many metabolites with antimicrobial properties have been discovered from endophytes, some recently reviewed ones are flavonoids, peptides, quinones, alkaloids, phenols, steroids, terpenoids and polyketides (Mousa and Raizada, 2013, Lugtenberg et al., 2016). When many microbial species are present in the same plant, the association propels the secretion of metabolites by the endophytes or the host to inhibit the growth of microbes that are harmful (Kusari et al., 2012). In some instances, the endophytes and the host plant use some distinct pathways in enhancing the production of metabolites, some use induced metabolism which helps in metabolizing the product of the other (Kusari et al., 2012, Ludwig-Müller, 2015). It was later concluded that many endophytic strains cannot produce the compounds independently (Heinig et al., 2013).

An endophyte isolated from *Cassia spectabilis*, named *Phomopis cassia* was able to synthesize five substances similar to 3,11,12-trihydroxycadalene and cadinane sesquiterpenes in which one of the five derivatives produced the most active antifungal metabolite against *Cladosporium cladsporioides* and *Cladosporium sphaerospermum* (Silva et al., 2006). Alkaloids were reported to have strong potential in inhibiting the proliferation of microbes, for instance, altersetin, a novel alkaloid which was isolated from the endophyte Alternaria spp., exhibited a strong antibacterial effect against many gram-positive bacteria that are pathogenic (Hellwig et al., 2002). Another metabolite which exhibited antibiosis is a volatile oil. An endophytic fungus from the tropical trees known as *Muscodor albus* produced many volatile organic compounds, including aciphyllene, 2-butanone and 2-methyl furan which were reported to produce antibiotic properties (Atmosukarto et al., 2005). Also, fungal endophytes isolated invitro from *Artemisia annua* can suppress the growth of most phytopathogenic organisms by the production of antifungal compounds such as n-butanol and ethylacetate (Liu et al., 2001). *Tian et al. (2017)* assessed the role of anti-fungal protein produced by *Epichloë festucae in controlling Sclerotinia homoeocarpa* in *Festuca rubra.* The result presented this attribute by fescues as one of the unique ones. The mechanism of plant protection used by Paraconiothyrium strain SSM001 linked with the production of taxol from yew tree (*Taxus* spp.) against dangerous wood-decaying fungi was investigated by Rafiqi et al. (2013) and Soliman et al. (2015). A summary of related studies on the antimicrobial properties of endophytes is presented in Table 4.1.

Table 4.1: Summary of studies on the antimicrobial activities of endophytes

Endophytes	**Plant Host**	**Activity**	**Compounds**	**Class of compound**	**References**
			Endophytic bacteria		
B. subtilis	-	Antifungal	Bacilysocin	Phospholipid	Tamehiro et al. (2002)
B. subtilis	*Allanmands cathartica*	Antifungal	Terpene	Terpenoids	Nithya and Muthumary (2011)
B. subtilis	-	Antibacterial	Subtilin	Peptides	Singh et al. (2017)
Bacillus atrophaeus, *Bacillus mojavensis*	*Glycyrrhiza uralensis* (Licorice)	Antifungal	1,2-bezenedicarboxyl acid, Methyl ester, Decanodioic acid, bis(2-ehtylhexyl) ester.	Polyketides	Mohamad et al. (2018)
Lysinibacillus, *Staphylococcus*, *Enterobacter*, *Pseudomonas* and *Bacillus species*	*Combretum molle*	Antibacterial	-		Diale et al. (2018)
B. licheniformis, *B. subtilis subsp. Inaquosorum* and *B. Pumilus*	*Moringa peregrina*	Antibacterial and Antifungal	-		Aljuraifani et al. (2019)
			Endophytic Fungi		
Phoma sp	*Cinnamomum mollissimum*	Antifungal	5-hydroxyramulsin	Polyketides	Santiago et al. (2012)
Geotrichum candidum, *Cylindrocladium* sp. *Fusarium* sp. *Cladosporium cladosporioides* sp., *Mucor pusillus*, *Rhizopus* sp. and *Alternaria alternata*	*Phyllanthus reticulatus* Poir	Antibacterial and Antifungal	-		Pai and Chandra (2018)
Phompsis sp	*Aconitum*	Antifungal	Gavodermside and Clavasterols	Steroids	Wu et al. (2013)

	carmichaeli				
Xylaria sp F0010	*Abies holophylla*	Antifungal	Griseofulvin	-	Park et al. (2005)
Chaetomium globosum	*Ginkgo biloba*	Antifungal	Chaetomugilin A and D	Azaphilone derivative	Qin et al. (2009)
Pestalotiopsis mangiferae	*Mangifera indica* Linn	Antibacterial	4-(2,4,7-trioxa-bicyclo[4,10]-heptan-3-yl	Phenols	Subban et al. (2013)
Aspergillus sp	*Bauhinia guianensis*	Antibacterial	Fumigaclavine C and Pseurtotin C	Alkaloids	Pinheiro et al. (2013)
Phomopsis sp., *Botryosphaeria* sp	*Garcinia sp*	Antibacterial and Antifungal	-	-	Phongpaichit et al. (2006)
Nigrospora sphaerica (URM-6060) and *Pestalotiopsis maculans* (URM-6061)	*Indigofera suffruticosa Miller*	Antibacterial	-	-	Santos et al. (2015)
MR1B and MRB.2	*Catharanthus roseus and Euphorbia hirta*	Antibacterial and Antifungal	Citreoisocoumarin, paxilline, nigricinol, fatty acid, sceptrin, cladosporin	Isocoumarin derivative	Akpotu et al. (2017)
			Endophytic actinomycetes		
Streptomyces noursei	-	Antifungal	Nystatin	Steroids	Fjærvik and Zotchev (2005)
Streptomyces sp	-	Antibacterial	Harmaomycin	Peptide derivatives	Bae et al. (2015)
Streptomyces remosus	-	Antifungal	Tetracyclin	Steroids	Nelson (2001)
Streptomyces sp	*Grevillea pteridifolia*	Antibacterial	Kakadumycin A Echinodermycin	Peptides	Castillo et al. (2003)
Streptomyces sp TP-A0595	*Allium tuberosum*	Antifungal	6-Prenylindole	Alkaloids	Singh and Dubey (2018)
Aeromicrobium ponti	*Vochysia divergens*	Antibacterial	1-Acetyl-β-carboline, Indole-3-	Alkaloids	Gos et al. (2017)

			carbaldehyde, 3-(Hydroxyacetyl)-Indole, Brevianamide F, and Cyclo-(L-Pro-L-Phe)		
Streptomyces sp. neau-D50	*Glycine max*	Antifungal	3-Acetonylidene-7-Prenylindolin-2-one and 7-Isoprenylindole-3-carboxylic acid	Alkaloids	Zhang et al. (2014a)
Actinosynnema pretiosum	*Maytenus serrata*	Antibacterial	Ansamitocin	Polyketides	Siyu-Mao et al. (2013)
Streptomyces sp. TP-A0456	*Aucuba japonica*	Antibacterial	Cedarmycin A and B	Terpenes and Terpenoids	Sasaki et al. (2001)
Streptomyces aureofaciens CMUAc130	*Zingiber officinale*	Antifungal	5,7-Dimethoxy-4-pmethoxylphenylcoumarin; 5,7-Dimethoxy-4-phenylcoumarin	Coumarins	Taechowisan et al. (2007)
Streptomyces sp. BT01	*Boesenbergia rotunda* (L.)	Antibacterial	7-Methoxy-3, 3′,4′,6-tetrahydroxyflavone and 2′,7-Dihydroxy-4′,5′-Dimethoxyisoflavone, Fisetin, Naringenin, 3′-Hydroxydaidzein, Xenognosin	Flavonoids	Taechowisan et al. (2014)
Streptomyces sp.DSM 1175	*Alnus glutinosa*	Antibacterial	Alnumycin	Quinones	Singh and Dubey (2018)
Dactylosporangium sp. strain SANK 61299	*Cucubalus* sp	Antifungal	Streptol	Tannins	Singh and Dubey (2018)
Verrucosispora maris AB-18-032	*Sonchus oleraceus*	Antibacterial	Proximicin	Peptides	Fiedler et al. (2008)

4.3.1.2 Lytic enzymes secretion

Most microorganisms secrete lytic enzymes for the hydrolysis of polymers (Gao et al., 2010). About 1350 compounds can be secreted; among them are cellulose, hemicellulose, proteins, DNA and chitin (Tripathi et al., 2008). For endophytes to colonize the surface of plants, they produce numerous enzymes which successively aid the hydrolysis of the plant cell wall. These enzymes help in reducing phytopathogens indirectly and also aid the fungi cell wall degradation. There are numerous types of enzymes some of which are chitinases, cellulases, hemicellulases and 1, 3-glucanases. Application of mutagenesis to the genes of 1, 3-glucanase present in a strain of *Lysobacter enzymogenes* reduced the biocontrol activity towards the damping-off disease of sugar beet caused by Pythium and tall fescue leafspot disease (Gao et al., 2010). The lytic enzymes produced by Streptomyces have a strong effect on antagonizing cacao witches broom disease (Macagnan et al., 2008). Even though enzymes may not be solely effective as an antagonizing agent, they may enhance antagonistic activities when combined with other mechanisms. Pectinase was also reported to aid the reduction of pathogenesis in a plant (Babalola, 2007).

4.3.1.3 Production of phytohormone

Endophytes produce phytohormone which enhances plant growth promotion and changes the morphology and structure of the plant. As a result of this attribute, endophytes have gained ground in the area of agricultural sustainability (Sturz et al., 2000). The mechanism adopted by endophytes in the production of phytohormones in the host plant is related to the mechanism used by rhizobacteria in plant growth promotion. They help in growth promotion and protection of non-leguminous plants by the secretion of gibberellic acid (Khan et al., 2014), auxins (Dutta et al., 2014), indole acetic acid (Patel and Patel, 2014, Khan et al., 2014) and ethylene (Kang et al., 2012, Babalola, 2010).

Indole acetic acid (IAA) triggers plant cell division, differentiation and extension; stimulates of seed and tuber germination; increases the rate at which root and xylem develop, enhances lateral initiation, controls the rate of vegetative growth and the formation of adventitious root formation; as well as the formation of pigments and biosynthesis of metabolites, controls responses to gravity, light, and fluorescence, affects photosynthesis and resistance to extreme conditions (Gao et al., 2010). IAA secreted by plant growth-promoting bacteria sometimes slows down the physiological processes listed above by affecting the level of auxin secretion by the plant. Also, the IAA produced by endophytic bacteria has the capacity to increase the root length and surface area, thereby giving room for the plant to have better access to nutrients from the soil. Additionally, IAA production expands bacteria cell walls and increases the secretion of exudates alongside providing more nutrients for growth enhancement of other beneficial bacteria present in the rhizosphere. Therefore, the IAA produced by endophytic bacteria is recognized as the major effector molecule in phytostimulation, pathogenesis, and plant-microbe interaction (Gao and Tao, 2012). Several have studies demonstrated that endophytic actinomycetes also produce plant growth-promoting compounds such as IAA which have been reported to enhance the formation and elongation of plant adventitious roots in a plant (de Oliveira et al., 2010, Shimizu, 2011).

4.3.1.4 Phosphate solubilization

The third most important nutrient for plant growth is potassium (K) and endophytes are capable of solubilizing forms of potassium that are insoluble. Most soil-related microorganisms are capable of solubilizing insoluble phosphate to enhance the production of P, thus making it available for plant use (Alori et al., 2017). The most common mechanism used for inorganic phosphate solubilization is the dissolution of mineral compounds such as organic acids, protons, siderophores, carbon dioxide (CO_2) and hydroxyl ions (Olanrewaju et al., 2017). The existence of microorganisms that solubilizes potassium might have opened our eyes to an alternative means of making potassium available for plant uptake (Rogers et al., 1998). Endophytes also introduce organic acids into the soil which help to solubilize the phosphate complexes and change them into ortho-phosphates for plant absorption and usage. Numerous bacteria species namely *Bacillus mucilaginosus*, *B. circulans*, *Pseudomonas* sp, *Burkholderia, Paenibacillus* sp., *Acidothiobacillus ferrooxidans,* and *Bacillus edaphicus* were identified in the release of the accessible form of potassium from potassium-bearing minerals in soils (Yadav, 2018). As abundant as phosphorus is in the soil, unfortunately, many of its remains do not exist in an insoluble form (Miller et al., 2010b). Many studies have shown the role of endophytic microorganisms as a biofertilizer and biocontrol agent. For example, endophytes isolated from the root nodule for peanut, identified as *Pantoea* spp was reported to have strong solubilizing activity (Yadav et al., 2018). Similarly, endophytic actinomycetes have been reported to perform an important role in phosphate solubilization and also enhances its availability to plants through chelation, acidification, and mineralization and redox changes of organic phosphorus (Singh and Dubey, 2018). Solubilization of phosphate alongside secretion of phytase was demonstrated by an endophytic actinomycete, *Streptomyces* sp., which significantly improve plant growth (Jog et al., 2014).

4.3.1.5 Siderophore production

Siderophores are small molecular compounds which are capable of chelating iron which can be produced by endophytes and can make iron available for plant use while starving pathogens of iron (Yadav, 2018). Some of the siderophores known to be produced by endophytes can confer biocontrol activities such as hydroxymate, phenolate and/or catecholate types (Rajkumar et al., 2010). Also, the iron-deficient plant is enhanced by siderophores which help in the fixing of nitrogen since diazotrophic organisms require Fe^2+ and Mo factors for the functioning and synthesis of nitrogenase (Kraepiel et al., 2009). There are many literature evidences to support the insecticidal properties of endophytes (Azevedo et al., 2000). Some endophytes reduce pest penetration of the stele by thickening the endodermal cell wall (Gao et al., 2010). Others destroy insects by producing secondary metabolites. Though some toxic metabolites are traceable to endophytes some of these metabolites are pyrrolizidine, alkaloids, pyrrolopyrazine alkaloid, peramine ergot alkaloid, and ergovaline (Wilkinson et al., 2000).

In the case of plant growth-promoting bacteria, Fe^{2+} is oxidized to Fe^{3+}-siderophore complex in the bacterial membrane, which is later introduced into the cell by endophytes through a gating mechanism (Gao et al., 2010). The concentration of soluble metals increases when siderophores bind to the metal surface (Rajkumar et al., 2010). Once the level of heavy metal contaminants is removed, different mechanisms are employed by plants to ingest iron from bacterial siderophores, for example, iron chelates aid the direct absorption of siderophore-Fe complexes, or ligand exchange (Schmidt, 1999). A siderophore-producing endophyte, *Pseudomonas* strain GRP3 was tested on *Vigna radiate* for iron nutrition and the result showed that after 45 days, the plants showed a reduction in iron and chlorotic symptoms, while there was an increase in the content of chlorophyll a and chlorophyll b when the plant was inoculated with strain GRP3 as compared to the control (Sharma et al., 2003). Some endophytic actinomyces such as Streptomyces sp. GMKU 3100, *Streptomyces* sp. mhcr0816, *Streptomyces* sp. UKCW/B and *Nocardia* sp have been reported to produce siderophores (Singh and Dubey, 2018). Similarly, S. acidiscabies *E13* was also reported as a superb producer of siderophore which enhances the growth of *Vigna unguiculata* under nickel stress conditions (Sessitsch et al., 2013).

4.3.1.6 1-Aminocyclopropane-1-carboxylate (ACC) utilization

Generally, ethylene is an essential metabolite for the normal growth and development of plants (Khalid et al., 2006). This important hormone known for enhancing plant growth is secreted by almost all plants and is affected by different abiotic and biotic activities in the soil which improve physiological changes in most plants. The occurrence of extreme conditions such as pathogenicity, drought, salinity and heavy metals increases the level of ethylene which has side effects on the growth of the plant; this may result in alteration of the cellular processes and defoliation which affects the yield of the crop (Bhattacharyya and Jha, 2012). Many endophytic bacterial species that can produce ACC deaminase have been discovered in genera like *Achromobacter, Agrobacterium, Acinetobacter, Bacillus, Enterobacter, Pseudomonas, Serratia, Ralstonia, Rhizobium, Alcaligenes, Burkholderia* etc (Kang et al., 2012). Most of the bacterial endophytes trap the ethylene precursor of ACC and change it into ammonia and 2-oxobutanoate (Arshad et al., 2007). Lugtenberg and Kamilova (2009) reported that some stresses like radiation, heavy metals, flooding resistance due to stress coming from polyaromatic hydrocarbons, high light intensity, wounds, high salt concentration, insect predation, draft, and extreme temperature can be overcome by plants that can produce ACC deaminase.

4.3.1.7 Competition with pathogens

Competition is a strong mechanism used by endophytes in preventing pathogens from colonizing the host tissue (Martinuz et al., 2012). Endophytes possess the ability to colonize many plant tissues systemically or locally (Latz et al., 2018). For example, they act through colonization and the lurking of nutrients that are available and by occupying the position that is available for pathogens to carry out their activities (Rodriguez et al., 2009). This can be further buttressed using a study by Mohandoss and Suryanarayanan (2009), who discovered that destruction of endophytes in mango leaves by the application of fungicides in its treatment allows other fungi to inhabit the niche, especially pathogenic fungi.

The mechanism used for competition by most endophytes usually takes place in combination with other mechanisms, instead of acting independently. Since the control method employed by endophytes is often local, they will, however, need to systematically colonize the part of the host where most pathogens may attack. The colonization of the root of oilseed rape with endophyte *Heteroconium chaetospira* could not successfully prevent clubroot symptoms (Lahlali et al., 2014)**.** The result, therefore, indicates the limitations that may be encountered with competition as a biocontrol method, as it may be inactive when there is a high presence of microorganisms causing disease. The symptoms of *Phytophthora* sp were successfully reduced when treated through a foliar application with mixtures of endophytes from leaves of cacao tree leaves, thus showing competition as one mechanism of disease suppression in a plant. However, some of the strains were also observed to produce other active metabolites which is an indication that, competition might not be the only mechanism used in controlling the disease (Arnold et al., 2003).

4.3.2 Indirect mechanisms of plant protection from pathogens

Plants employ several mechanisms to survive in extreme conditions such as drought, salt stress, and cold. Some of the rapid noticeable biochemical and morphological changes observed include the hypersensitive response, cellular necrosis and phytoalexin production. In long term evolution, non-specific (general) resistance and specific resistance are examples of innate resistance developed for pathogen resistance (Kiraly et al., 2007). Those that possess specific resistance can resist infection from one or a few pathogens while the non-specific resistance is active against many pathogens. Endophytes increase the plant defense mechanism through the production of secondary metabolites and enhanced resistance.

4.3.2.1 Induction of plant resistance

For over twenty years now, many studies have concentrated on the way plants respond to attack from parasites and pathogens using various categories. Induced systemic resistance (ISR) and Systemic acquired resistance (SAR) are the two resistance patterns which have attracted the most attention of researchers. ISR, which is induced by some nonpathogenic rhizobacteria, is moderated by ethylene or jasmonic acid which cannot be linked with the building up of pathogenesis-related (PR) proteins. SAR, which is caused by infections from pathogens is mediated by salicylic acid and linked with the building up of PR proteins (Tripathi et al., 2008). These PR proteins have many enzymes, such as 1, 3-glucanases and chitinases which help in the direct lysing of invading cells, and strengthening of cell wall boundaries to build resistance against infection and cell death (Gao et al., 2010). ISR produced by endophytes can also be linked with the enhancement of genes that are expressed in pathogenesis. The root of tomato harbours important endophytes called *Fusarium solani* which prompt ISR against *Septoria lycopersici*, the causative agent of tomato foliar pathogens and activate PR genes, PR7and PR5 activities in the roots (Kavroulakis et al., 2007). Redman et al. (1999) reported that the inoculation of a nonpathogenic mutant strain of *Colletotrichum magna* on *Cucumis sativus* and *Citrullus lanatus* produced a high amount of peroxidase, lignin deposition and phenylalanine ammonialyase which help in protecting the plant against diseases which are caused by *Fusarium oxysporum* and *Colletotrichum orbiculare*. Reduction in the lesions on leaves was observed when *Neotyphodium lolii* engaged against four different pathogens, which could be attributed to enhanced peroxidase and superoxide dismutase activities of the host plant (Tian et al., 2008).

4.3.2.2 Plant secondary metabolites stimulation

Secondary metabolites from plants are compounds which have limited functions in the life cycle of the plant but are of great importance in its adaption to different environments (Bourgaud et al., 2001). Notable among all the secondary compounds produced by a plant is an antimicrobial molecule with a low molecular weight called phytoalexins (Gao et al., 2010). It has many substances in it, some of which are terpenoids and flavonoids among many others. *Orchis morio* and *Loroglossum hircinum* were the first to produce phytoalexins in response to a fungal attack initiated by a French botanist called Noel Bernard, outcomes of other studies showed that phytoalexins can now be produced through some abiotic stress factors such as heavy metals ion, salt stress and UV light (Gao et al., 2010). Some studies have concentrated on the production of phytoalexins when triggered by pathogens (Pedras et al., 2008). The production of plant secondary metabolism moderated by endophytes is still a new research area. Findings revealed that the elicitors of *Fusarium* E5 could propel triterpene and dipertene production in cell suspensions of *E. pekinensis*. Li and Tao (2009) reported a similar result in *Taxus cuspidate* culture suspensions, in which culture supernatants of endophytes resulted in increased production of paclitaxel when compared with the control. It is suspected that the co-culturing with elicitor endophytes is a likely way of increasing plant secondary metabolites and boosting plant resistance. Endophytic colonization induced the production of hydrolase for plant cells to reduce the growth of fungi, therefore making endophytes act as elicitors through hydroxylation production (Gao et al., 2010). Some elicitors like glycoprotein, polysaccharides and lipopolysaccharides trigger plant defense mechanisms and increase secretion of plant secondary metabolites which effectively reduce attack by pathogens. However, there is limited information as regards the way in which endophytes survive in the host plant when producing large quantities of secondary metabolites are produced (Gao et al., 2010).

4.3.2.3 Promotion of plant growth and physiology

Endophytes sometimes support the host plant defense mechanism against plant pathogenic microorganisms by taking over the plant physiology (Gimenez et al., 2007). As the growth of the plant increases, it develops vigor and resistance to different stresses both abiotic and biotic, this is considered as one of the strategies used by the plant for defense against pathogens (Kuldau and Bacon, 2008). Several studies have shown that plants inoculated with endophytes recorded an increase in growth, drought resistance (Gao et al., 2010) and tolerance to any type of soil

(Malinowski et al., 2004). Plant growth can be enhanced by several compounds, an endophyte, *Colletotrichum* sp, isolated from *A. annua* produces a substance called indole acetic acid (IAA) which helps in regulating plant physiology (Lu et al., 2000). Dai et al. (2008) reported that extracts from *Fusarium* sp. E5 produced auxin. Another mechanism adopted by endophytes can be said to be the release of phytohormones (Dai et al., 2008). We can, therefore, believe that plant growth promotion when triggered by endophytes will indirectly protect the plant against pathogens.

4.3.2.4 Hyperparasites and predation

Hyperparasites is another mechanism endophyte use to protect their host ecologically. In this mechanism, endophytes directly attack identified pathogens or their propagules (Tripathi et al., 2008). Endophytic fungi capture the pathogens by twisting and penetrating their hyphae and by the production of lyase which destroys the cell wall of the pathogen. For instance, *Trichoderma* sp. was able to capture and penetrate the hyphae of *Rhizoctonia solani*, a known plant pathogen; the observation was linked to biocontrol activities (Grosch et al., 2006). Another mechanism is microbial predation; this entails a general way of reducing pathogens of plants. Most endophytes exhibit their predatory characteristics in nutrient-deficient conditions. As an example, a variety of enzymes attacking the cell wall of fungal pathogens directly are produced by *Trichoderma* sp (Gao et al., 2010).

4.4 Endophytic bacteria and fungi as producers of bioactive compounds and bioactivities of importance to humans

Several reports have noted that bioactive metabolites secreted by endophytes are great sources of drugs for the treatment of different types of ailments and their potential applications in food, agriculture, medicine and cosmetic industries cannot be underestimated (Godstime et al., 2014, Shukla et al., 2014). The metabolites secreted by endophytes are classified into different functional groups such as alkaloids, terpenoids, flavonoids, benzopyranones, tannins, phenolic acids, quinones, steroids, tetralones and chinones (Figure 4.2) (Joseph and Priya, 2011, Godstime et al., 2014). Many factors have been reported to affect the way metabolites are extracted from endophytes, some of them are the climatic condition, the season of sample collection and geographical location (Shukla et al., 2014). With the recent developments in the synthetic process, extraction of metabolites from a natural source is becoming efficient and promising (Hussain et al., 2012). It has been linked with the development of microorganisms which may have integrated genetic information from higher plants, thereby ensuring better adaption to their host and they may perform some functions such as protection from insects, pathogens and animals (Gouda et al., 2016).

Infectious and parasitic diseases are responsible for almost half of the death rate all over the world (Menpara and Chanda, 2013). Endophytes have been reported as the source of many bioactive compounds and several secondary metabolites available commercially today (Singh and Dubey, 2015). Endophytic microorganisms are a depot of new metabolites that can be used as antimicrobial, antiarthritic, anticancer, immunosuppressant and anti-insect drugs (Jalgaonwala et al., 2011, Godstime et al., 2014). As at present, just a few plants have been studied for endophytes diversity and ability to produce bioactive secondary metabolites. Recent studies have reported that novel bioactive compounds produced by most endophytic microorganisms are important in overcoming the problem of antibiotic resistance by most pathogenic microorganisms (Godstime et al., 2014). Numerous bioactive compounds like vinblastine, amptothecin, hypericin, podophyllotoxin, camptothecin among others produced by endophytes have already been commercialized and have been found useful in agriculture and pharmacology (Joseph and Priya, 2011, Zhao et al., 2011).

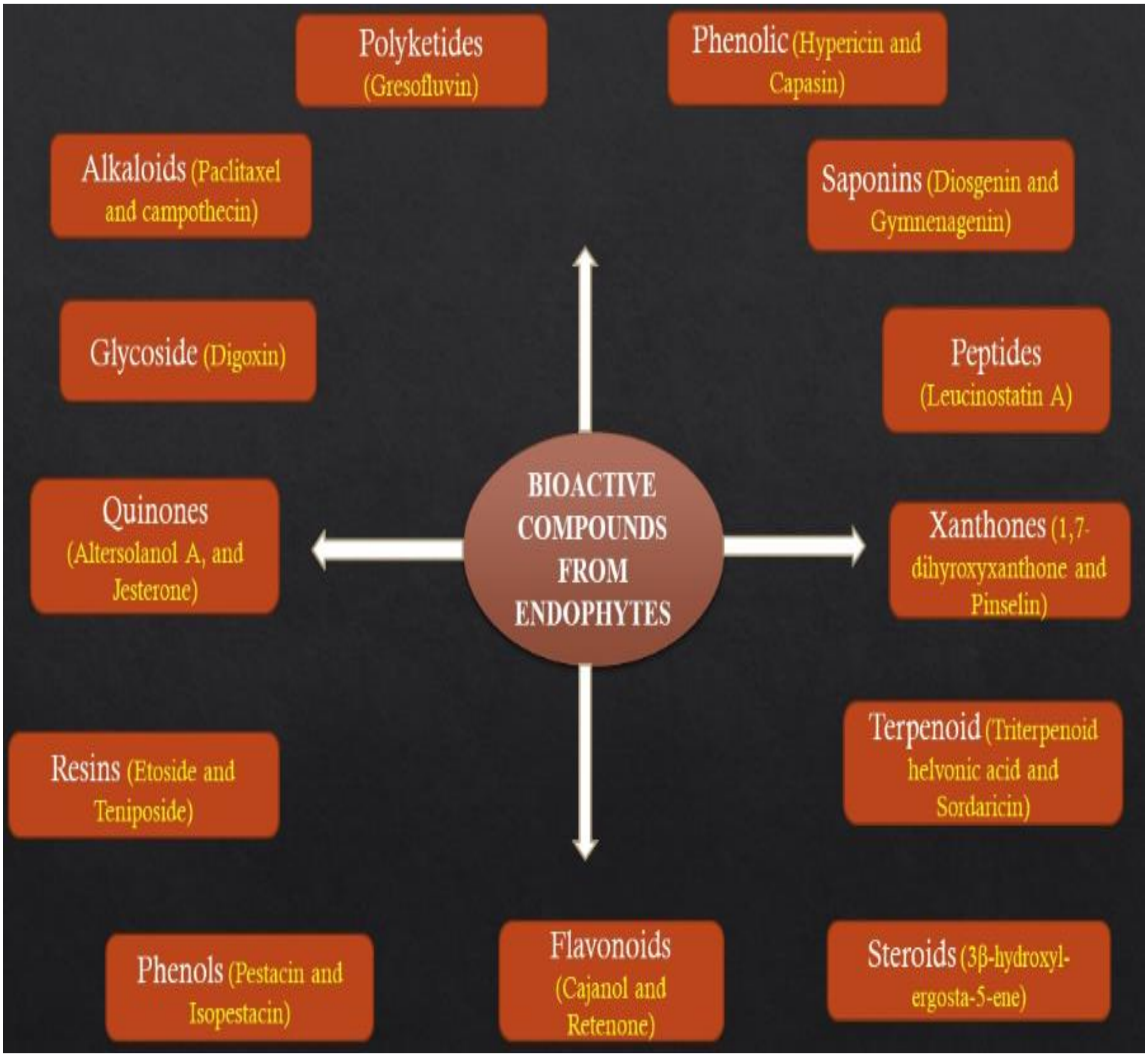


Figure 4.2: Bioactive compounds produced by endophytes

4.4.1 Anticancer activity and compounds

Cancer is a disease identified by the uncontrolled multiplication of abnormal cells which results in death in human beings when not controlled. Globally cancer prevalence is said to have increased to 9.6 million deaths and 18.1million cases in the year 2018 (Toghueo, 2019). All over the world, those who survive cancer disease within 5 years of its detection are approximated as 43.8 million (Toghueo et al., 2019). In 2004 cancer was said to be responsible for about 13% (estimated to be 7.4 million) of the world death (Gouda et al., 2016). The drugs used in the treatment of cancer show nonspecific toxicity for the multiplying normal cells have many side effects and many are still not active in the treatment of some cancer forms (Pasut and Veronese, 2009). The discovery of metabolites with cytotoxic properties has given insights in anticancer therapy for some decades (Pimentel et al., 2011). Endophytes have been reported to have the ability to produce novel metabolites that can serve as anticancer agents (Rajamanikyam et al., 2017). A summary of related studies on the anticancer properties of endophytes is presented in Table 4.2.

Table 4.2: Summary of studies on the anticancer prospects of endophytes

Endophytes	**Compound secreted**	**Class of compound**	**Activity**	**Cell active against**	**References**
		Endophytic fungi			
Fusarium oxysporium	Vincristine	Alkaloids	Anticancer	-	Zhang et al. (2000)
Mycellia sterilia	Vincristine	Alkaloids	Anticancer	-	Yang et al. (2004)
Enthrophospora infrequens	Camptothecin	Quinolone alkaloid	Anticancer	-	Puri et al. (2005)
Phomopsis cassiae	3,12-dihydroxydalene 3,12-dihydroxycalamenene 3,11,12-trihydroxycadalene	Terpenoids	Anti-proliferative	HeLa cervical cells	Silva et al. (2006)
Periconia atropurpurea	EtOAc extract	-	Cytotoxicity	-	Teles et al. (2006)
Garcinia sp	EtOAc extract	-	Antiproliferative and cytotoxicity	Vero cell lines	Phongpaichit et al. (2007)
Collentotrichum gloesporiodes	Taxol	Alkaloids	Cytotoxicity	Human cancer cells lines BT220, int 407, H116, HLK 210, HL251.	Gangadevi and Muthumary (2008)
Aspergillus fumigatus	Cytotoxic alkaloids	Alkaloids	Cytotoxicity	Leukaemia cancer cell line.	Konecny et al. (2009)

C. gloesporiodes	Taxol	Alkaloids	Anticancer		Nithya and Muthumary (2009)
Alternaria alternata	EtOAc extract	-	Antitumor and cytotoxicity	HeLa cells	Fernandes et al. (2009)
Alternaria sp	Xanalteric acids	Phenols	cytotoxicity		Kjer et al. (2009)
Fusarium solani	Camptothecin	Quinolone alkaloid	Anticancer	-	Shweta et al. (2010)
Lasidiplodia theobromae	Taxol	Alkaloids	Anticancer	MCF-7	Pandi et al. (2011)
Cephalotheca faveolata	Sclerotiorin	Polyketides	Anticancer	Colon cancer (HCT-116)	Giridharan et al. (2012)
Phoma sp	5-hydroxyramulosin	Polyketides	Anticancer	-	Santiago et al. (2012)
Penicillium sp	Arisugacin	Terpenoid derivatives	Anticancer	HeLa, HL-60, and K562 cell lines	Sun et al. (2014)
A. flavus	Solamargine	Steriods	Cytotoxicity	-	El-Hawary et al. (2016)
Taxomyces andreanae	Paclitaxel	Alkaloids	Anticancer	-	Alurappa et al. (2018)
Chaetomium sp, *Alternaria* sp and *Collentotrichum* sp	EtOAc extract	-	Cytotoxicity	HeLa and MCF-7 cells	Dhayanithy et al. (2019)
		Endophytic Actinomycetes			
Streptomyces thermoviolaceus TP-	Anicemycin	Alkaloids	Antitumor	-	Igarashi (2004)

A0648					
Streptomyces sp. SUC1	Lansai A-D	Alkaloids	Anticancer	-	Tuntiwachwuttikul et al. (2008)
Actinosynnema pretiosum	Ansamitocin	Polyketides	Antitumor	-	Siyu-Mao et al. (2013)
Micromonospora lupini Lupac 08	Lupinacidin C	Quinones	Antitumor	Murine colon carcinoma cells	Igarashi et al. (2011)
Streptomyces sp. CS	Naphtomycin A	Quinones	Antitumor	P388 and A-549 cell lines	Lu and Shen (2007)
Streptomyces laceyi MS53	6-alkalysalicilic acids, salaceyins A and B	Fatty acid derivatives	Anticancer	-	Singh and Dubey (2018)
		Endophytic bacteria			
Acinetobacter guillouiae	EtOAc extract	-	Anticancer	U87MG glioblastoma and A549 lung carcinoma cells	Sebola et al. (2019)
Bacillus subtilis PXJ-5, *Bacillus* sp. CPC3, *Bacillus cereus* ChST	Camptothecine	Alkaloids	Anticancer	-	(Shweta et al., 2013)
-	EtOAc extract	-	Cytotoxic	A549 lung cancer cell lines	Swarnalatha et al. (2016)
Pantoea sp	EtOAc extract	-	Anticancer	A549 lung carcinoma and UMG87 glioblastoma cell lines	Uche-Okereafor et al. (2019)

4.4.2 Antioxidant activity and compounds

The major significance of antioxidant compounds is the fact that they are very active in controlling diseases linked to the presence of oxygen-derived free radicals and ROSs, which may be responsible for the degeneration of cells, DNA damages, and carcinogenesis (Mishra et al., 2014). Antioxidants are now considered as promising alternatives in the treatment and prevention of diseases linked with ROS such as Diabetes mellitus, cancer, hypertension, Alzheimer's disease, parkinson's disease, ischemia and atherosclerosis. Most antioxidants have antiatherosclerotic, anti-carcinogenic, anti-inflammatory, antitumor, and antimutagenic activities both in small and large quantities (Hood and Shew, 1996, Mishra et al., 2014).

A phenolic metabolite identified as Graphislactone A, produced by *Cephalosporium* species, also, IFB-E001 found inside *Trachelospermum jasminoides* was found to have the ability to search for free radical and it exhibited stronger antioxidant properties than ascorbic acid and butylated hydroxytoluene (BHT) coassayed in the research (Suryanarayanan et al., 2009). Shoeb et al. (2014) also reported that an endophytic fungus *Penicillium thiomii* produced an antioxidant identified as terminatone. The crude extracts of *Rhodiola* spp. were also reported to scavenge DPPH, O_2^-, and OH radicals, and also in chelating Fe^{2+} (Cui et al., 2015). EtOAc extract of *Diaporthe* spp. was found to produce a strong antioxidant (Toghueo, 2019).

A novel compound called sesquiterpene isolated from *Acremonium* sp also showed strong antioxidant activity (Elfita et al., 2012). Ethyl acetate extract of endophytic fungus *Fennellia nivea* had a notable quantity of total phenolics which might be responsible for its high antioxidant activity (Saraswaty et al., 2013). *Aspergillus fumigatus* SG-17 was found to secrete a compound called (Z)-N-(4-hydroxystyryl) formamide (NFA), an equivalent of coumarin which functions as an antioxidant both in vitro and in vivo. Subsequent analysis through MS and NMR further established the claim (Qin et al., 2019). A summary of related studies on the antioxidant properties of endophytes is presented in Table 4.3.

Table 4.3: Summary of studies of antioxidant properties of endophytes

Endophytes	**Host plant**	**Compound secreted**	**Class of compounds**	**References**
		Endophytic fungi		
Strain AcapF3	*Tabernaemontana divaricate* (L), *Rauvolfia verticillata* (Lour.)	-	Phenol	Huang et al. (2007)
Aspergillus sp	*Calotropis procera, Catharanthus roseus, Euphorbia prostrate, Vernonia amygdalina* and *Trigonella foenum-graecum*	Gallic acid	Phenol	Khiralla et al. (2015)
Aspergillus minisclerotigens AKF1 and *Aspergillus oryzae* DK7	*Mangifera casturi* Kosterm	dihydropyran and 4H-Pyran-4-one,5-hydroxy-2-(hydroxymethyl-(CAS) Kojic acid	-	Nuraini et al. (2019)
Rhodiola spp	Alpine Plants	salidrosides, p-tyrosol, and rosavins	phenolic and flavonoid	Cui et al. (2015)
Phoma sp., *Colletotrichum spiralis*, *Chaetomium* sp	-	MeOH extract	Phenol	Singla (2019)
Penicillium citrinum CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *Cryptendoxyla*	*Tragia involucrata* Linn	L-ascorbic acid	-	Danagoudar et al. (2018)

hypophloia CGJ-D2				
Aspergillus niger, A. flavus, Alternaria alternata	*Lannea coromendalica*	EtOAc extract	Phenolic compound	Premjanu and Jaynthy (2014)
Chaetomium globosum	*Adiantum capillus*	EtOAc extract	Phenolic compound	Selim et al. (2018)
Phyllosticta sp	*Guazuma tomentosa*	EtOH extract	Phenol	Srinivasan et al. (2010)
		Endophytic bacteria		
Methylobacterium radiotolerans MAMP 4754	*Combretum erythrophyllum*	EtOAc extract, Chloroform extract	Alkaloids, flavonoids, Phenol and steroids	Photolo et al. (2020)
Lactobacillus sp	*Adhathoda beddomei*	EtOAc extract	phenolic compounds	Swarnalatha et al. (2015)
Pseudomonas hibiscicola, Macrococcus caseolyticus, Enterobacter ludwigii, Bacillus anthracis	*Aloe vera*	EtOAc extract	Alkaloids and flavonoids	Akinsanya et al. (2015a)
Pseudocercospora sp. ESL 02	*Elaeocarpus sylvestris*	Terreic acid (1) and 6-methylsalicylic acid	-	Prihantini and Tachibana (2017)
EC3	*Carica papaya* L.	Gallic acid	phenolic compounds	Sarjono et al. (2019)
		Endophytic actinomycetes		
Streptomyces aureofaciens CMUAc130	*Zingiber officinale*	5,7-Dimethoxy-4-pmethoxylphenylcoumarin; 5,7-Dimethoxy-4-phenylcoumarin	Coumarins (Alpha Benzopyrones)	Taechowisan et al. (2007)
Streptomyces sp. Tc052	*Alpinia galanga*	Kaempferol, Isoscutellarin,Umbelliferone and Cichoriin	Flavoniods	Singh and Dubey (2018)
Micromonospora sp. PC1052	*Puereria candollei*	S-adenosyl-Nacetylhomocysteine	Peptides	Boonsnongcheep et al. (2017)
Streptomyces sp. MS1/7	-	2-Allyloxyphenol	Phenol	Singh and Dubey (2015)

4.4.3 Antidiabetic activity

Nature has given us many natural resources which can be used for medicinal purposes. The hypolipidemic and antidiabetic prospects of endophytic fungi extracts from *Salvadora oleoides* in Wistar albino rats induced with diabetes when loaded with glucose and alloxan was examined (Dhankhar et al., 2013). Glucose tolerance test showed that extracts from endophytic fungi such as *Phoma* sp and *Aspergillus* sp successfully reduced the glucose level in the blood of the rats. Akshatha et al. (2014) assessed antidiabetic prospects of endophyte extracts from the tissue of *Rauwolfia densiflora* and *Leucas ciliate,* two of the most prominent medicinal plants used in treating diabetes. The result showed that α-amylase inhibitor slows down the glucose from dietary complex carbohydrate and retards the rate at which glucose is absorbed. Also, Kaur (2018) screened endophytic fungi for their ability to act as for alpha-glucosidase inhibitors. It was reported for the first time that extracts from *Fusarium sp* and *Alternaria* sp act as alpha-glucosidase inhibitors, the study establishes endophytic fungi as sources of pharmaceutically important molecules.

Xylariaceae sp. also secreted a coumarone compound purified as 8-hydroxy-6,7-dimethoxy-3-methylisocoumarine which was reported to have been active against α-glycosidase (Indrianingsih and Tachibana, 2017). Pujiyanto et al. (2012) revealed that the crude extracts an endophytic bacterium identified as *Streptomyces olivochromogenes* which showed potential antidiabetic activity. Three compounds (*S*)-(+)-2-*cis*-4-*trans*-abscisic acid,7'-hydroxy-abscisic acid and 4-des-hydroxyl altersolanol A secreted by *Nigrospora oryzae* reported to be active against α-glycosidase (Uzor et al., 2017). GancidinW (GW) secreted by *Streptomyces paradoxus* VITALK03 was also reported to be active against α-glycosidase (Ravi et al., 2017).

4.4.4 Immunosuppressive activity

There have been ongoing studies on how to identify an effective agent for the suppression of immunological disorders especially autoimmune diseases and graft rejection (Rajamanikyam et al., 2017). *Fusarium subglutinans* an endophytic fungus was found to secrete subglutinol A and B which act as an immunosuppressive agent. The drug produced from it is used to avert the problem of allograft rejection in patients who undergo a transplant and it is promising in the treatment of autoimmune diseases like insulin-dependent diabetes and rheumatoid arthritis (Padhi et al., 2013). An antifungal peptide compound called Pseudomycins which was reported to be active against human pathogen *Candida albicans* found to contain special amino acids like L-chlorothreonine, L-diaminobutyric acid, and L-hydroxyl aspartic acid (Castillo et al., 2003).

Ambuic acid which is a cyclohexenone belongs to the family of pseudomycins which was secreted by *Pestalotiopsis microspore* and found to be active against human pathogens. A bioactive agent from Streptomyces species identified as ambuic acid was effective against both gram-negative and gram-positive bacteria (Suryanarayanan et al., 2003). Crude extracts of fungi endophyte, *Penicillium* sp. ZJ-SY$_2$, showed strong immunosuppressive activity when structural elucidation was done using 1D, MS, 2D and NMR data. Compounds 1, 3, 5, and 7 showed strong immunosuppressive activity using IC$_{50}$ values ranging from 5.9 to 9.3 μg/mL (Liu et al., 2016). Three novel derivatives of xanthone, including two earlier reported to contain sulfur as natural products: sydoxanthone A (1) and sydoxanthone B (2)**,** and 13-*O*-acetylsydowinin B (3) were found to be secreted by an endophytic fungus, *Aspergillus sydowii.* Structural elucidation was done by, UV, MS and NMR data to establish the data. In *vitro* suppression assay carried out on LPS-induced and Con A proliferation of splenic lymphocytes of a mouse showed that the compounds have moderate immunosuppressive activities (Song et al., 2013).

Chloroform (CEEI) and methanolic extracts produced by *Entrophospora infrequens* exhibit delayed-type hypersensitivity (DTH) reactions (Pur et al., 2007). Three compounds isolated from *Pestalotiopsis leucothës* were found to be effective on T and B-cells and monocytes (Kumar et al., 2005). Madagundi et al. (2013) isolated endophytic fungi from *Ocimum sanctum* Linn. and assessed their extracts *in vitro* for immunomodulatory properties on human polymorphonuclear (PMN) cells such as phagocytosis. The immunosuppressive assay of Curtachalasin secreted by an endophytic fungus *Xylaria* cf. curta against cell proliferation of concanavalin A (ConA) induced T lymphocyte cell and lipopolysaccharide (LPS) induced B lymphocyte cell proliferation was reported by Wang et al. (2019). *Brevibacterium* sp. YXT131 an endophytic actinobacterium modulated the immune response by reducing the proinflammatory cytokines interleukin (IL)-12/IL-23 p40 in the serum of mice (Wei et al., 2018). The use of bioagents in immune modulation of some diseases is a current and novel research area.

4.4.5 Antiviral activity

The discovery of promising antiviral compounds for endophytes is still novel. There are still limited numbers of compounds that have been attributed to endophytes. The limiting factor in the production of antiviral compounds by endophytes is the fact that no antiviral screening systems exist. Most antibiotic products from endophytic fungi are known to strongly inhibit viral growth. The elucidation using mass spectrometry and NMR methods showed that two cytomegalovirus protease inhibitors in human and cytonic acids A and B were effective against the growth of viruses (Harper et al., 2001). Some metabolites secreted by endophytes from desert plants serve as a promising source in identifying potent inhibitors in the replication of HIV-1 (Wellensiek et al., 2013).

Alternaria tenuissima QUE1Se was reported to produce an antiviral compound called Altertoxins which was found to be effective against HIV-1 virus (Bashyal et al., 2014). Also, many extracts from endophytic fungi were tested against the replication of HIV-1 virus in T-lymphocytes, four extracts were found not to be toxic but inhibited the virus with differences ranging from 75% to 99%. Three of the extracts were fractionated and the DB-2 fraction was observed to completely inhibit the replication of HIV-1 (Wellensiek et al., 2013). Compound extracted from *Emericella* sp.(HK-ZJ), namely aspernidine (A, B), dehydroaustin, emeriphenolicins (A,D), austinol, emerimidine (A, B), austin, and acetoxy dehydroaustin were reported to confer antiviral activity against the influenza A virus (H_1N_1) *(Zhang et al., 2009b)*. Extracts from endophytic fungi species *Aspergillus, Pestalotiopsis, Fusicoccum, Phomopsis, Guignardia, Penicillium,* and *Muscodor* were also assessed for their antiviral activity against Herpes simplex virus type 1 (HSV-1 ATCC VR-260), many of the fungi species showed weak to moderate antiviral activity (Phongpaichit et al., 2007). Also, crude extracts from 81 endophytic fungi isolated from many medicinal plants showed antiviral activity (Rajamanikyam et al., 2017). Recently, some endophytic actinomycetes were reported to possess antiviral properties, for example, *Streptomyces* sp. GT2002/1503 exhibited antiviral activity against R5 tropic HIV infection (Ding et al., 2010). *Jishengella endophytica* 161111 also secreted an antiviral compound, 2-(furan-2-yl)-6-(2S,3S,4- trihydroxybutyl) pyrazine which was active against influenza A virus subtype H1N1 (Wang et al., 2014).

4.4.6 Antiarthritis and anti-inflammatory Activities

The immune system of an individual plays an active role in certain deadly diseases, a hyperactive immune system may result in diseases such as arthritis. Rheumatoid arthritis (RA) is an inflammatory and autoimmune disease that is systemic with symptoms such as swelling, pain, bone and cartilage destruction which can later lead to permanent disability. Surprisingly, the exact causative agent of the disease is not known. Most researchers are currently looking for more medicinal agents from microbes because the present synthetic drugs are very costly and have many side effects (Choudhary et al., 2015). An endophytic fungus, *Talaromyces wortmannii,* isolated from *Aloe vera* secreted some bioactive metabolites which showed active anti-inflammatory activity. This ability is because a metabolite produced by the organism inhibit the release of IL-8 by blocking the activation of AP-1 and NF-$_{k}$B (Pretsch et al., 2014). Methanolic extracts from *Lepidosphaeria* sp, an endophytic fungus, also showed anti-inflammatory activity and it is promising as a drug which might be adopted for the treatment of inflammatory diseases like rheumatoid arthritis (Shah et al., 2015). The main reason for screening endophytes is to establish new inhibitors for pro-inflammatory cytokines which are encountered in many immunological pathways. Extracts of endophytic fungi isolated from *Mimusops elengi* (bakul), an important medicinal plant in India also showed strong anti-inflammatory activity (Deshmukh et al., 2009). Methanolic extract of Chaetomiun globosum was observed to be responsible for improved arthritis and mobility scores, and was concluded to possess a strong inhibitory effect on the morphological features of rheumatoid arthritis in an adjuvant-induced rat model (Abdel-Azeem et al., 2016).

4.4.7 Antimalarial activity

Malaria is still one of the major causes of mortality and morbidity in the world with over 3.3 billion people living with the ongoing risk of transmission (Ateba et al., 2018). In 2016, about 91 countries reported approximately 216 million new cases of malaria and 445,000 mortalities. The people most affected by malaria are those people that live in the subtropical and tropical regions of the world, people from Southeast Asia and sub-Saharan Africa where approximately 80% of cases of malaria recorded are traceable to *Plasmodium falciparum* (Ateba et al., 2018). The recent widespread of anti-drug resistant malaria parasites makes the search for alternative and new malarial treatment drugs urgent (D'Alessandro, 2009).

Munumbicins E-4 and E-5 produced by endophytic fungi showed antimalarial activity, which was found to have double the effect of chloroquine (Suryanarayanan et al., 2003). An endophytes *Diaporthe miriciae* was found to produce a secondary metabolite called epoxycytochalasin H which expresses strong antimalarial inhibition against a strain of *Plasmodium falciparum* that is resistant to chloroquine (Ferreira et al., 2017). A report by Ateba et al. (2018) showed that endophyte species *Paecilomyces lilcinus* and *Penicillium Janthinellum* are storehouse of novel metabolites that are active against *Plasmodium falciparum* and promising in the cure of malaria. Endophytic fungi, *Fusarium* sp and *Nigrospora* sp were also reported to secrete bioactive metabolites which showed antiplasmodial activity against *Plasmodium falciparum* (Kaushik et al., 2014).

4.4.8 Antituberculosis activity

Tuberculosis (TB) is a globally recognized communicable disease with the etiological agent as *Mycobacterium tuberculosis* which often affects the lungs. It has been one of the major disease troubling human beings for centuries. Death rate as a result of TB infection is estimated at two million annually with almost nine million new cases emerging every year (Tsara et al., 2009). Tuberculosis is responsible for more deaths of otherwise healthy people than diseases that are infectious such as malaria and AIDS (Corbett et al., 2003). The challenge with TB is that there is no effective treatment method for the disease. However, with the advent of Multi-drug resistance strains of *M. tuberculosis*, the disease has established itself as a major source of concern to humans (Khunjamayum et al., 2017).

Endophytes are capable of secreting some bioactive compounds that can successfully inhibit the prevalence of TB caused by *M. tuberculosis*. Endophytic fungi species, *F. solani* and *C.*

gleosporoides isolated from *G. glabra* showed strong inhibition against *Mycobacterium tuberculosis* strain H37Rv with MIC of 18.5 and 75 µg/ml, respectively (Shah et al., 2016). The crude extracts of endophytic bacteria, *Streptomyces* sp. and *Bulkholderia fungorum* were reported to show great inhibition against the pathogenic strain of *Mycobacterium tuberculosis* and the IC50 values recorded for them were less than 100 µg/ml (Khunjamayum et al., 2017). The diverse biological activities of endophytes are presented in Figure 4.3 below.

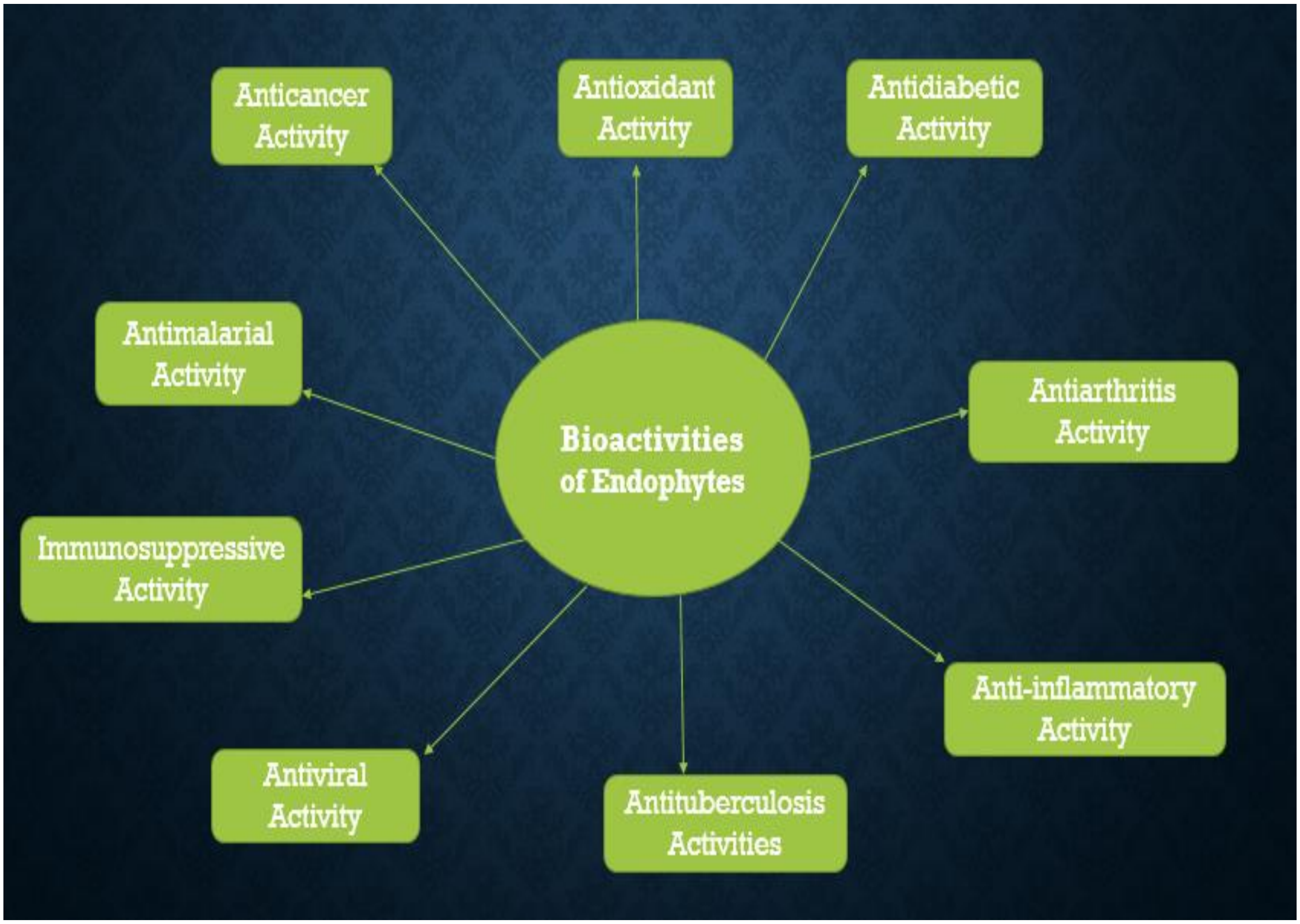


Figure 4.3: Biological activities of importance to humans present in endophyte's metabolites

4.5 Future prospects

Concise studies on a specific population of endophytes active in a host are required before bulk production can be carried out which often requires research with advanced technology. Studies are also needed as regards getting plant-specific inoculum doses of endophytes, this will help in reducing bulk production and also enhance productivity by reducing our dependence on synthetic fungicides, pesticides and fertilizers. Development of endophytes that can be sprayed just like most chemical pesticides will help in the acceptance of endophytes in integrated pest management.

Future studies will need to take into account the development of genomic tools and metabolomics tools for further studies on how endophytes colonize the plant and plant-microbe interaction. There is still a need to study the compounds produced by endophytes and their activities in reducing diseases. This will help in developing efficient markers for some important and distinct biocontrol agents and assessing the effects of plant genotypes, innate microbe community and most importantly the environment. This structured approach will also help in discovering new endophytes with important traits.

Molecular study of these endophytes is important in order to improve drug research. Also, metagenomics study will be very important in order to showcase the diversity of endophytes and the functions they are capable of performing through a detailed analysis of their genes. Molecular biology techniques can be used for the isolation and identification of the different types of genes present in the biosynthetic pathways and this will further open our eyes to new bioactive compounds at a commercial level as well as in the laboratory. Future studies should focus on the biosynthetic pathways which might be responsible for the secretion of numerous important bioactive compounds by endophytes.

Also, future studies can look into the development of endophytic nanoparticles which will help in improving the plant growth. Transfer of genes can also be employed in order to detect more efficient species. The idea of manipulating genes can help the host plants in developing new traits like phytoremediation and herbicide resistance, among others, which could more suitably regulate metabolism. There is no microbial technology that can be considered successful until it has been commercialized. The specificity of endophytes within a plant is one of the limitations in its large scale production.

4.6 Conclusion

The study attempts to appreciate the diverse mechanisms used by endophytes in protecting plants from diseases for sustainable agriculture. Endophytic microbes support the plant and accelerate plant growth by employing different mechanisms of action, both direct and indirect. The major benefit of embracing such beneficial microorganisms in the field of agriculture is to bring about reduction in the use of different agrochemicals such as pesticides, chemical fertilizers, other artificial chemicals etc and this would make agriculture more productive and sustainable. Endophytes can still be very useful in the biomedical field because endophytes can synthesize and secrete chemicals which may be used for the development of antibiotics of importance for human use. Many studies are still ongoing towards assessing the ability of endophytes to secrete novel bioactive compounds which will be of great importance in the treatment of human diseases.

Besides the numerous applications of endophytes in medicine, therapeutics and mining, some novel metabolites may be useful in sustainable agriculture and in enhancing plant growth. These metabolites can also confer insecticidal, and pest control activities, alongside enhancing plant nutrient uptake under extreme conditions such as drought, salinity and waterlogging. Taken as a whole, novel bioactive compounds secreted by endophytes especially endophytic actinomycetes, could offer immense contributions in addressing the present and future challenges in agriculture, environment and medicine. Finally, the application of metagenomics combined with next-generation sequencing technologies is expected to open up the numerous unexplored pool of antimicrobials secreted by yet uncultivated endophytic microbes.

CHAPTER FIVE

5.0 METAGENOMIC PROFILING OF THE COMMUNITY STRUCTURE, DIVERSITY, AND NUTRIENT PATHWAYS OF BACTERIAL ENDOPHYTES IN MAIZE PLANT

Abstract

This study investigated the diversity, structure and nutrient pathways of the root-associated bacterial endophytes of maize plant cultivated using different fertilizers to verify the claim that inorganic fertilizers have some toxic effects on the plant microbiome and are not ecofriendly. Whole DNA was extracted from the roots of maize plants cultivated with organic fertilizer, inorganic fertilizer and maize planted without any fertilizer at different planting sites in an experimental field and sequenced using shotgun metagenomics. Our results using the Subsystem database revealed a total of 28 phyla and different nutrient pathways in all the samples. The major phyla observed were *Firmicutes, Bacteroidetes, Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Tenericutes*, *Planctomycetes*, *Cyanobacteria*, and *Chlorobi*. *Bacteroidetes* dominated maize from organic fertilizer sites, *Firmicutes* dominated the no fertilizers site while *Proteobacteria* dominated Inorganic fertilizer. The diversity analysis showed that the abundance of endophytic bacteria in all the sites is in the order organic fertilizer (FK) > no fertilizer (CK) > inorganic fertilizer (NK). Furthermore, the major nutrient cycling pathways identified are linked with nitrogen and phosphorus metabolism which were higher in FK samples. Going by the results obtained, this study suggests that organic fertilizer could be a boost to sustainable agricultural practices and should be encouraged. Also, a lot of novel endophytic bacteria groups were identified in maize. Mapping out strategies to isolate and purify this novel endophytic bacteria could help in promoting sustainable agriculture alongside biotechnological applications in future.

Keywords: Endophytic microbes, Fertilizer application, Plant associated microbes, Shotgun metagenomics, *Zea mays*

This chapter has been published in *Antonie van Leeuwenhoek* 2020; 113:1559-1571. https://doi.org/10.1007/s10482-020-01463-w.

5.1 Introduction

The diversity of microorganisms is still yet to be fully understood, even though about 1.5 million species of bacteria and fungi have been estimated to perform important roles on earth such as symbionts, decomposers and ecosystem pathogens (Akinsanya et al., 2015b). Many microbial communities reside in plant tissues and these microbes, especially bacteria, perform important functions in plant health and growth, notable among them is the endophytic microbial community (Fouda et al., 2015, Tumangger et al., 2018).

Endophytes are a group of microbes which inhabit the tissues of plants without exhibiting any harmful effect on their host (Fadiji and Babalola, 2020c, Omomowo and Babalola, 2019). Endophytes perform many kinds of beneficial activities, such as plant yield and growth promotion, biocontrol activities, and phytoremediation improvement, among others (Staniek et al., 2008, Yang et al., 2017, Kumar et al., 2017). The endophytic microbes are more abundant in the roots than the stem and leaves (Dudeja et al., 2012). To ensure stable symbiosis, endophytic microbes secrete numerous bioactive compounds which enhance the growth of plants and also help them for better adaptation to the environment (Das and Varma, 2009). Endophytes also contribute to the metabolic and physiological activities in the host plants, some of which include nutrients acquisition, nitrogen fixation, stress tolerance and control of plant pathogens (Marag et al., 2018, Fadiji and Babalola, 2020a). Therefore, it is pertinent to have a better understanding of the diversity and structure of bacterial endophytes inhabiting plant tissues and their nutrient pathways. This could be a pointer towards identifying novel organisms and roles of endophytic bacteria in plant growth promotion (Yang et al., 2017).

Maize is one of the important cereal crops cultivated in different parts of the world (Alori et al., 2019). It is widely used as grain and fodder and it is widely consumed by animals and humans. Maize is also used in the production of oil, syrup, alcohol, acetic acid, lactic acid, glucose, gum, edible starch, laundry starch, adhesives, cornmeal, methanol and flakes (Du-Plessis, 2003). Maize being an important cereal crop, its protection against devastating pests alongside growth and yield enhancement is crucial. Therefore, sourcing for fertilizers of natural origins which are ecofriendly and without any negative effect is important. Thus, microbes such as bacterial endophytes which inhabit the tissues of plants need to be critically investigated as a potential source of bioactive metabolites for the inhibition of plant pathogens and growth promotion (Rai et al., 2007).

Since time immemorial, organic fertilizer has been the most patronized fertilizer in enhancing crop yield and boosting soil nutrient (Yan and Gong, 2010, Lin et al., 2019). However, the advent of inorganic fertilizers in the late 1970s has led to a decrease in patronage and acceptance of organic fertilizers for agricultural purpose (Zhu and Chen, 2002). In a bid to make food available for the ever-increasing human population, most farming practices have adopted the use of inorganic fertilizers in large quantities over the years (Lin et al., 2019). This intensive application of inorganic fertilizers has resulted in soil compaction, soil degradation, reduction in organic matter, nitrogen leaching among others currently affecting sustainable agriculture. Also, reports have it that the efficiency of inorganic fertilizers is depreciating over time (Sun et al., 2015, Nkoa, 2014).

Most studies on maize endophytes were carried out using culture-dependent methods (Celador-Lera et al., 2016, Menéndez et al., 2016, Sandhya et al., 2017) and culture-independent approaches (Pereira et al., 2011, Correa-Galeote et al., 2016, Liu et al., 2017b, Correa-Galeote et al., 2018). Most studies to date prefer the use of culture-dependent methods for the study of endophytic bacteria. However, only small groups of the entire microbial community are culturable (1–2%), while numerous groups are non-culturable (Hong et al., 2019).

The diversity of bacterial endophytes inhabiting maize plant have also been studied considering factors such as agricultural practices (Seghers et al., 2004), sampling site (Ding et al., 2013), genetic modification (Mashiane et al., 2018), soil cultivation history (Correa-Galeote et al., 2018) among others. However, few studies exist on the effect of different fertilizer applications on the diversity of bacterial endophytes in a plant through metagenomics (Xia et al., 2019). In recent years, techniques that promote sustainable agriculture have been embraced as a promising source for the discovery of novel information in food systems and agriculture (Wezel et al., 2009).

The overall genetic information of communities of microbes in a certain environment is referred to as metagenome; while metagenomics analyzes the genetic information. Recent developments in next-generation sequencing (NGS) technology have been employed in the analyses of soil, rhizospheres, and endophytic microbes using 454 pyrosequencing, 16S rRNA amplicon sequencing and ITS (Akinsanya et al., 2015b, Campisano et al., 2014, Correa-Galeote et al., 2018, Sengupta et al., 2017, Mashiane et al., 2018). This technology makes it possible to analyze bacterial endophyte metagenome and discard sequences data of plant origin from the whole metagenome dataset (Fadiji and Babalola, 2020c). Metagenome analysis can be employed in profiling the diversity and structure of bacterial endophytes in plant tissues and their metabolic pathways. However, to the best of our understanding, no study exists on the influence of inorganic and organic fertilizers on the diversity and nutrient pathways of bacterial endophytes from the maize plant using shotgun metagenomics. Shotgun metagenomics is advantageous over other similar techniques e.g. amplicon sequencing because the sequencing of the whole metagenome gives an extensive diversity and structural study of microbial communities in an environment (De Tender, 2017). In this study, we present the first metagenome analysis of bacterial endophytes in maize plant cultivated with organic fertilizer, inorganic fertilizers and without any fertilizer using shotgun metagenomics. The study is aimed at investigating the diversity, community structure and nutrient pathways of the root-associated bacterial endophytes in maize plant cultivated with different fertilizers using shotgun metagenomics.

5.2 Materials and Methods

5.2.1 Seed collection

The WEMA (WE 3127) maize seed used in this experiment was collected from North-West University School Farm, Molelwane, Mafikeng, North West Province, South Africa.

5.2.2 Experimental design and site description.

The long-existing (15 years) organic and inorganic experimental fields were located in North-West University School farm, Molelwane, Mafikeng, North West Province (S25^{o}47'25.24056", E25^{o}37'8.17464"), South Africa. This province is characterized by shrubs and trees. The mean temperatures experienced in the province ranges from 3 to 21^{o}C in winter and 17 to 31^{o}C in summer. The rainfall of the province is estimated at 360 mm per annum. The major plant cultivated in this experimental site had been the rotation of sorghum**,** maize and soybean for a long time (sorghum-maize-soybean), with soybean planted in 2018. The soil samples were analyzed for pH and other soil chemical parameters. The soil samples from the experimental sites had similar chemical and physical properties (22% Sand, 66% Silt, 12% clay, pH 6; 0.48% organic C, 0.15% total N, 101.5 ppm P, 0.962ppm K) (Supplementary Table S1).

Two fertilization regimes were used in this study namely inorganic fertilization (NK), and Organic fertilization (FK) which had been in existence for over 15 years, alongside the no fertilization as (CK). Dosage of inorganic fertilizer which had been in use is 150 N, 75 P_2O_5 and 75 K_2O all in kg ha^{-1} for the inorganic site while the organic fertilizer site has been experiencing the application of cattle manure with a dosage of 10,625 kg ha^{-1} for over 15 years following standard regulations (USDA, 2014), while the third site had never experienced fertilizer application. The maize seeds (WE 3127**)** were planted on the three different sites, each site with 10 m x 4 m in dimension. The planting was carried out in the summer of 2019. Irrigation was provided at all the sites as required to prevent drought stress. Weed management was done manually.

5.2.3 Root sampling

Due to the triangular shape of the entire farmland, each site was divided into three different regions for root sampling purposes. Each biological replicate for sequencing came from the roots of 10 randomly selected fresh plants in each region of the sites, making up 30 plants for each sites. The plants were collected at the fruiting stage of the plant (Xia et al., 2015). In total, 90 plant samples were evaluated; the three regions represent three replicates for each sampling site. The plant samples were kept with ice and transported to the laboratory the same day where they were processed immediately.

5.2.4 Surface sterilization of maize roots

Surface sterilization was carried out on the fresh roots using the method described by Liu et al. (2017b) after soil particles that come with the roots from the field have been removed through sieving. The roots were then submerged in 70% ethanol for 3 min, rinsed with 2.5% sodium hypochlorite solution for 5 min, washed again with 70% ethanol for 30 s and lastly washed with distilled water that has been sterilized. To ensure that the process of sterilization was perfectly carried out and epiphytes successfully removed, small parts of the sterilized roots were cut and plated on yeast extract-mannitol medium (Vincent, 1970). After 72hours incubation at 30^0C, the

Petri dishes were observed for bacterial growth. Maize roots from Petri dishes without contamination were chosen for DNA extraction (Correa-Galeote et al., 2018, Marag et al., 2018).

5.2.5 Extraction of DNA and shotgun sequencing

The roots were cut into 1 cm using a sterile scalpel and instantly macerated using a Qiagen TissueLyser. Total metagenome DNA was immediately extracted from the root tissue samples using the Qiagen DNeasy Plant Mini Kit (USA). Shotgun metagenomic sequencing was done at the Molecular Research LP, Texas, USA. Library preparation was done with Nextera DNA Flex kit (Illumina) following standard procedure. The actual DNA concentration in all the samples was evaluated using the Life Technologies Qubit® dsDNA HS Assay Kit. Fifty (50) ng of the DNA was used for library preparation. After the library has been prepared, the final concentration of the libraries was measured using the Qubit® dsDNA HS Assay Kit (Life Technologies), and the Agilent 2100 Bioanalyzer was used to ascertain the size of the library. The library size varies from 683 to 877 bp with an average of 731 bp pooling of libraries were done using 0.6nM ratios, and the paired-end sequencing was done with 300 cycles via the Illumina NovaSeq 6000 system.

5.2.6 Data analysis

The obtained sequences of each metagenome were transferred to metagenomics rapid annotation online server (MG-RAST) (Hong et al., 2019), where quality control of the raw data was carried out. This included, removal of adapter and low quality reads from the sequenced data using the Trimmomatic v 0.33 program with default settings (Bolger et al., 2014). The quality control process also includes the removal of artificial sequences, filtering of ambiguous bases, specification of minimum read size, and length filtering. After quality control analysis, annotation of the processed sequences was carried out using BLAT (Kent, 2002), against M5NR database (Wilke et al., 2012) which allows nonredundant integration of several databases.

Taxonomic profiling of endophytic bacterial was performed with the SEED database with specified parameters such as a 10^{-5} e-value cut-off and minimum 60% sequence similarity to a subsystem. Also, the SEED Subsystems level 3 databases were used to reveal the metabolic pathways of the bacterial endophytes. Sequences that failed annotation were discarded. However, since our concentration is on bacteria endophytes, which accounts for about 85% of the whole sequences (Supplementary Figure S1), we, therefore, discarded sequences for eukaryotes, archaea, viruses and maize plant. To suppress the influence of experimental error/noise, data normalization option was selected on the MG-RAST. The obtained endophytic bacterial table was aggregated according to each taxon and unclassified sequence reads were kept for statistical analysis. Furthermore, the relative abundance of the taxa in percentages was calculated, after the independent analysis of the 9 sequences using MG-RAST. The average values of the relative abundance of the 3 replicates for each sampling sites (CK, FK and NK) were used for statistical analysis. These standard sequences can be found on NCBI SRA dataset with the accession number PRJNA607664.

5.2.7 Statistical analyses

After the dataset was normalized, the Shinyheatmap was used for plotting relative abundance graph of endophytic bacteria communities at the phylum level (Khomtchouk et al., 2017). PAST version 3.20 (Carrell and Frank, 2015) was used to assess the Shannon diversity and Pielou Evenness indices for each of the sampling sites and the indices between the sites were examined via Kruskal–Wallis test. The beta diversity was described using the principal coordinate analysis based on an Euclidean distance matrix and the one-way analysis of similarities (ANOSIM) was used in assessing the differences in the community structure among the sites (Carrell and Frank, 2015). The principal component analysis using Euclidean matrix was employed to present how obtained endophytic bacteria phyla were dispersed among the maize plant fields.

5.3 Results

5.3.1 General analyses of sequenced data of the maize samples from different sites.

The average number of uploaded sequences were 1613298509 (CK), 1493819190 (FK) and 2651625071 (NK) sequences. After quality control assessment (QC) was executed in MG-RAST, the quantity of retained mean sequences was 334259767 (CK), 415505341 (FK) and 817699487 (NK) (Supplementary Table S2).

5.3.2 Phylum level distribution from maize samples across the different sites.

Metagenomic analysis using the Subsystem database revealed a total of 28 phyla in all the samples, but they were more abundant in samples in the order organic fertilizer (FK) > no fertilizer (CK) > inorganic fertilizer (NK). The major phyla observed were: *Firmicutes, Bacteroidetes, Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Chloroflexi, Verrucomicrobia, Tenericutes, Planctomycetes, Cyanobacteria*, and *Chlorobi*. The other phyla were also represented in one or more samples but at <1% of the bacteria. Of the 28 bacteria phyla observed, the abundance of the bacterial endophytes was higher in samples from organic site (FK) as compared to CK and NK sites (Figure 5.1), but the difference was not significant (P > 0.05) across other farming sites (Supplementary Table S3). PCA was used to show the distribution of the identified bacterial phyla between the farming/ fertilization sites with FK sites having the highest distribution (Figure 5.2).

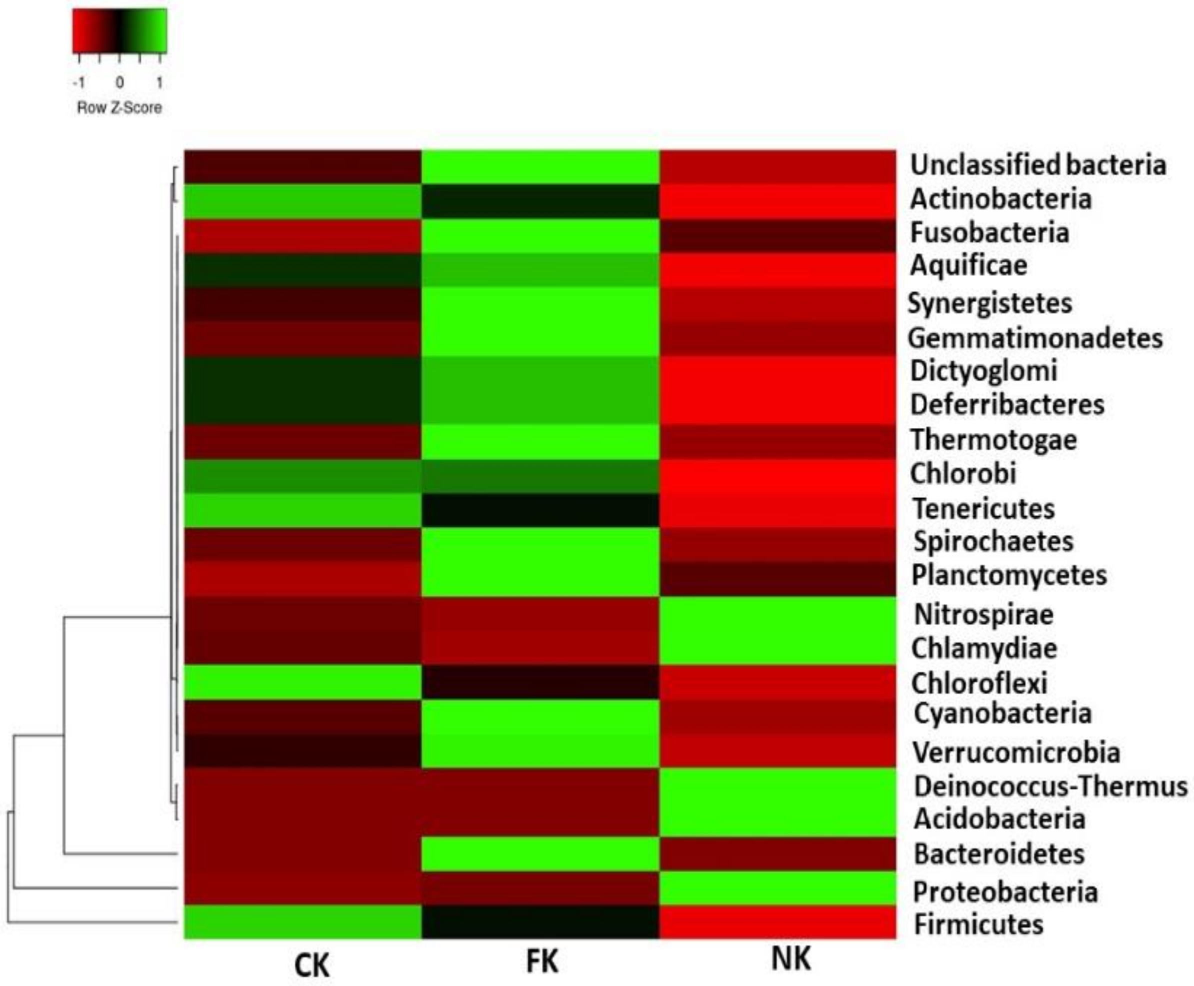


Figure 5.1: Relative abundance of bacterial phylum in Organic, inorganic and no fertilization maize sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

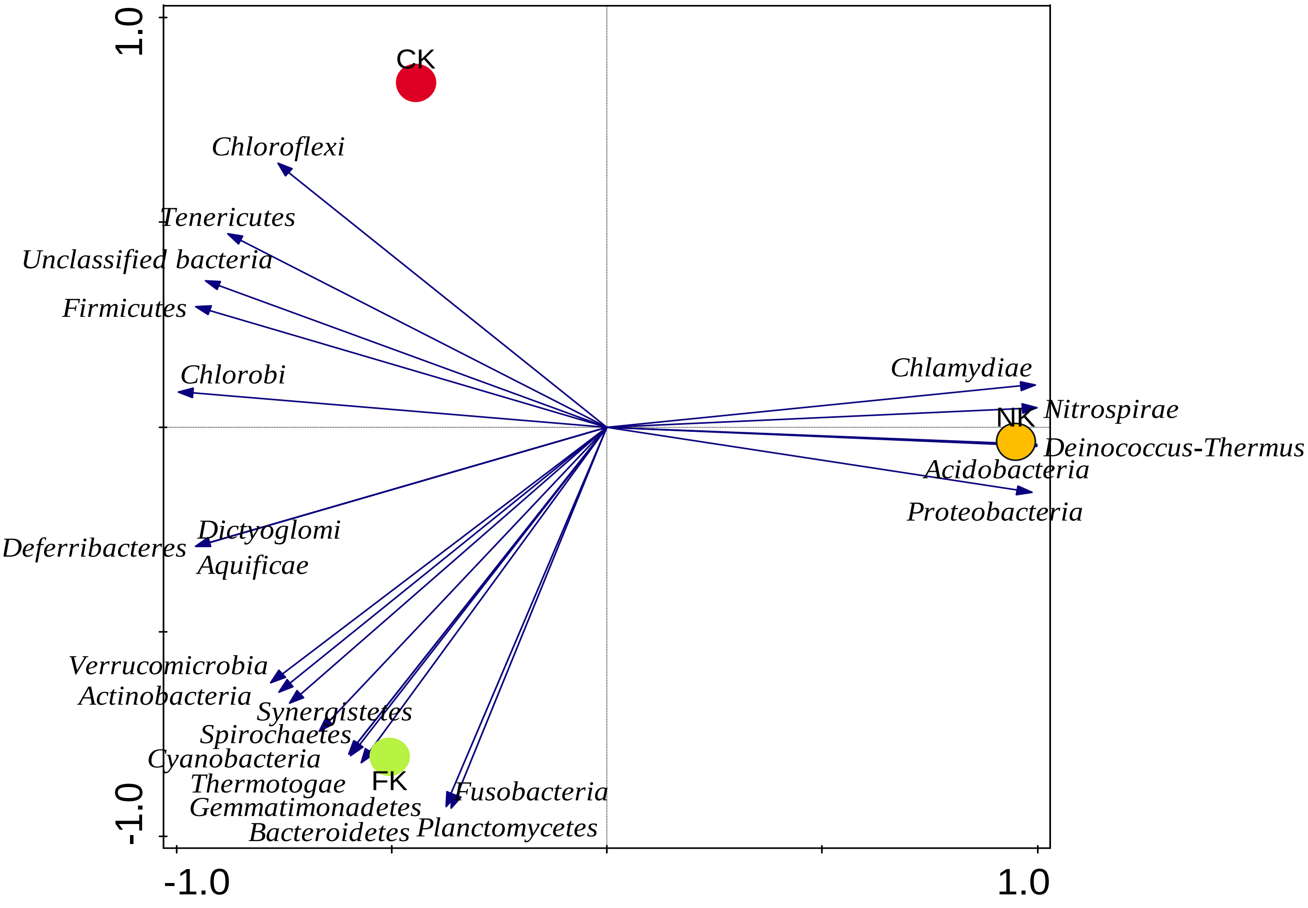
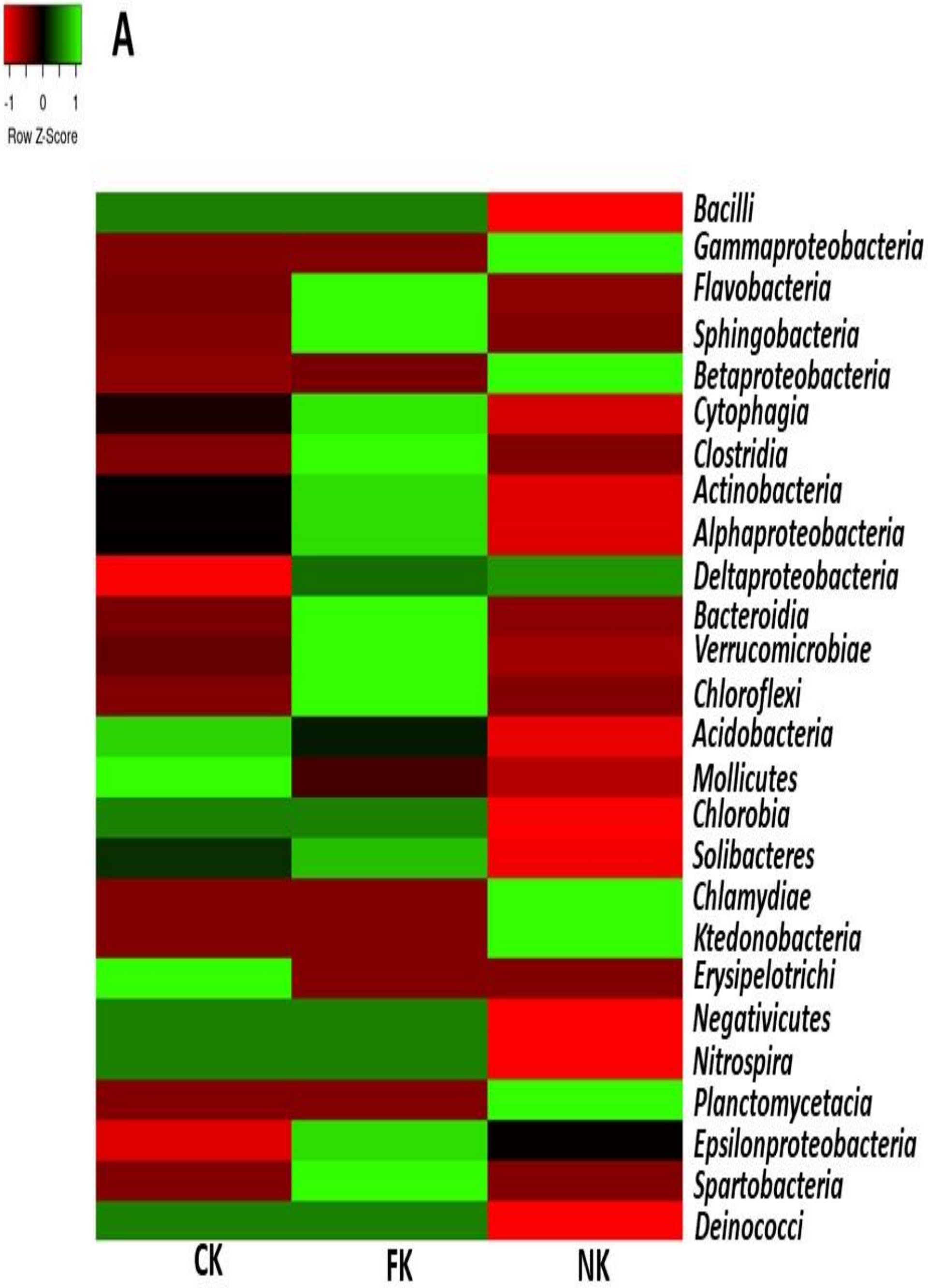


Figure 5.2: PCA graph of average endophytic bacteria metagenomes. The vector arrow represents the influence of bacterial endophyte metagenomes. Axis 1 (81.2%) and Axis 2(18.8%) explained the variations based on Euclidean dissimilarity matrix.

5.3.3 Structural composition of endophytic bacteria community from the farming sites.

Organic fertilizer (FK) sites had the most abundant phyla with dominant phyla as *Bacteriodetes*, *Cytophaga*, *Actinobacteria*, *Cyanobacteria* among others. *Firmicutes* dominated the no fertilizers site (CK=74%), followed by organic fertilizer (FK=37.44%) and were smallest in inorganic fertilizer (NK=10%) sites, although the difference that exists between them was not significant (P > 0.05). *Bacteroidetes* dominated organic fertilizer (FK=46%) sites followed by no fertilizers site (CK=11%) and lowest in Organic fertilizer (FK=9%). *Proteobacteria* dominated Inorganic fertilizer (NK=72.85%) sites followed by organic fertilizer (FK=37.44%) and lowest in no fertilizers site (CK=74%) (Figure 5.1; Supplementary Table S3). At the class level, the relative abundance of *Bacilli* was higher in FK and CK than NK. *Gammaproteobacteria* and *Betaproteobacteria* were more abundant in NK than other sites, while, *Flavobacteria*, *Sphingobacteria*, *Cytophagia*, *Clostridium* and *Actinobacteria* were most abundant in FK as compared to other sites (Figure 5.3A). Furthermore, at the order level, the relative abundance of *Bacillales*, *Flavobacteriales*, *Shingobacteriales*, *Cytophagales*, *Clostridales*, was much higher at FK than other sites. The relative abundance of *Pseudomonadales*, *Burkholderiales* and *Enterobacteriales* were dominant in NK than in CK and FK (Figure 5.3B).

At the family level, *Bacillaceae, Sphingobacteriaceae, Cytophagaceae, Staphylococcaceae, Paenibacillaceae, Pseudomonadaceae, Flavobacteriaceae, Bacteroidaceae, Burkholderiaceae, Enterobacteriaceae*, *Bdellovibrionaceae*, *Flammeovirgaceae*, and *Shewanellaceae* were most dominant in all samples (Figure 5.3C)**.** At the genus level, *Bacillus, Chitinophaga, Pedobacter, Chryseobacterium, Flavobacterium, Dyadobacter, Paenibacillus, Pseudomonas, Corynebacterium, Brevibacillus*, and *Acinetobacter* genera were predominantly identified in all the fertilization sites (Figure 5.3D)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Verrucomicrobiae
Chloroflexi
Acidobacteria
Mollicutes
Chlorobia
Solibacteres
Chlamydiae
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Negativicutes
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Planctomycetacia
Epsilonproteobacteria
Spartobacteria
Deinococci
CK
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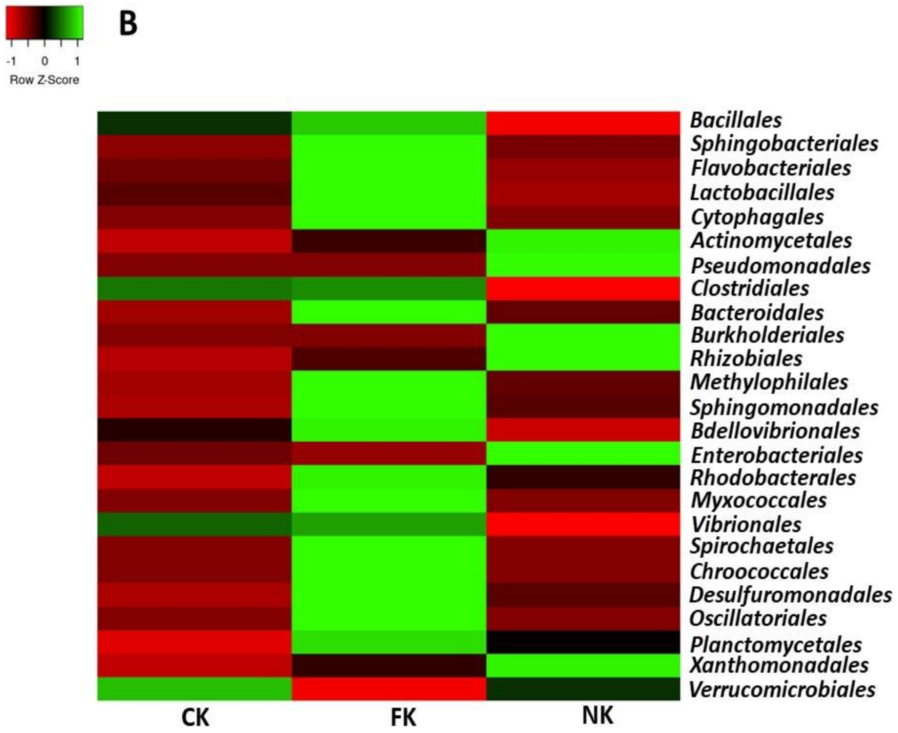
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Rhizobiales
Methylophilales
Sphingomonadales
Bdellovibrionales
Enterobacteriales
Rhodobacterales
Myxococcales
Vibrionales
Spirochaetales
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Planctomycetales
Xanthomonadales
Verrucomicrobiales
CK
FK
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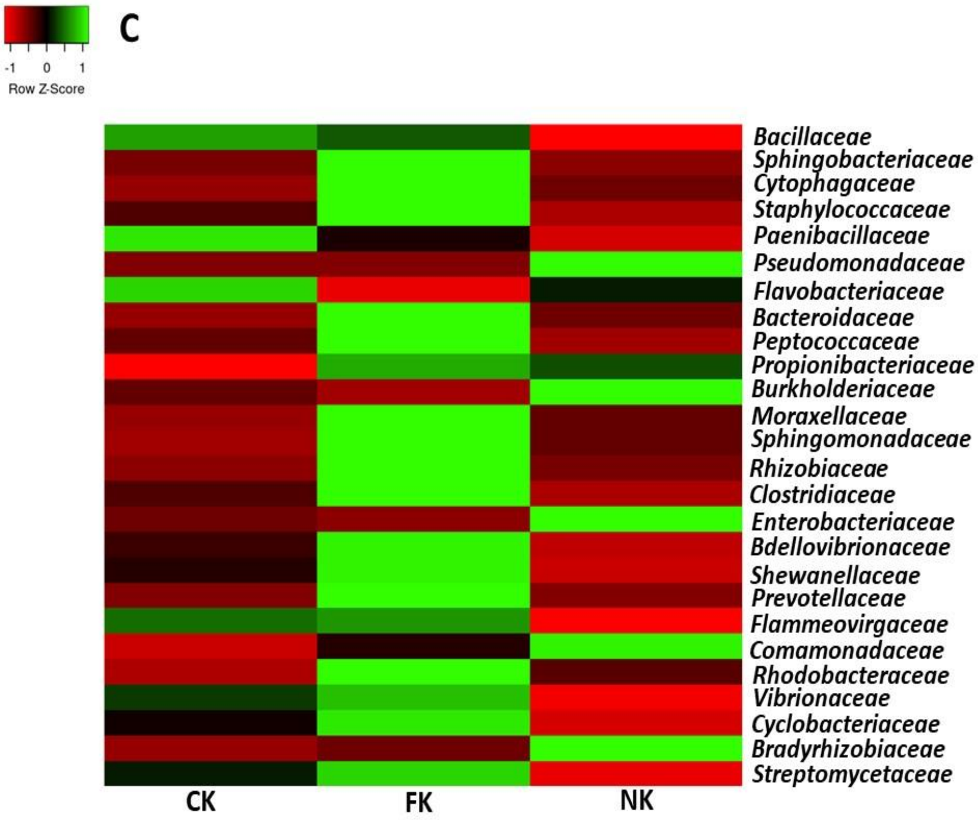
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Propionibacteriaceae
Burkholderiaceae
Moraxellaceae
Sphingomonadaceae
Rhizobiaceae
Clostridiaceae
Enterobacteriaceae
Bdellovibrionaceae
Shewanellaceae
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Flammeovirgaceae
Comamonadaceae
Rhodobacteraceae
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Cyclobacteriaceae
Bradyrhizobiaceae
Streptomycetaceae
CK
FK
NK

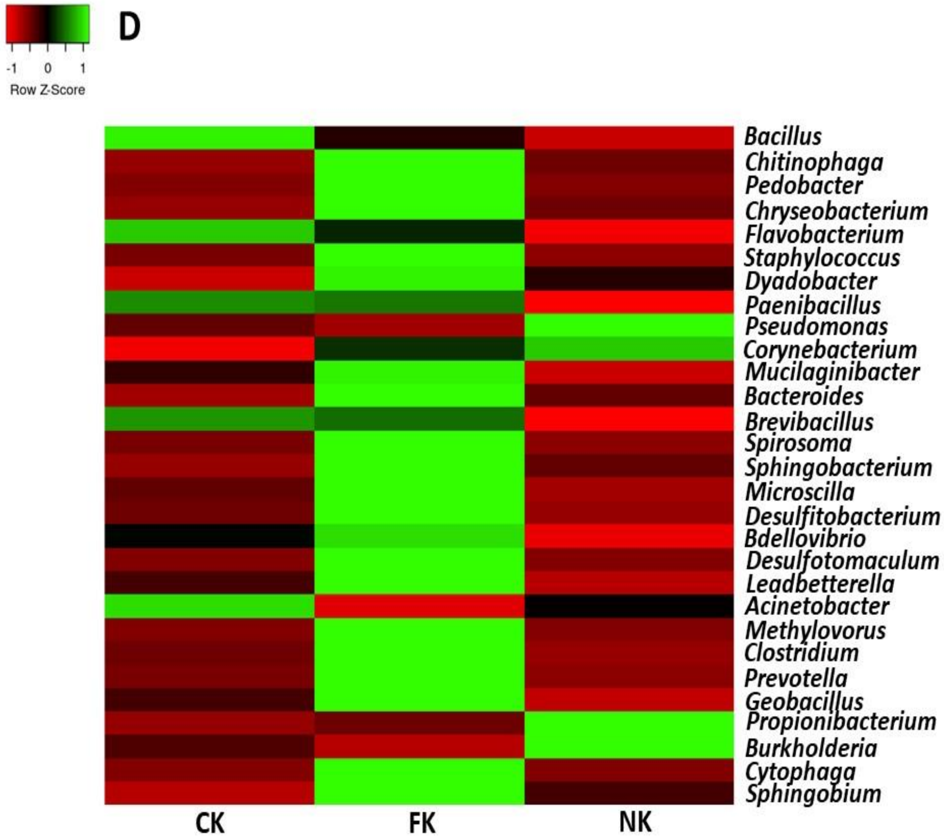


Figure 5.3: Heatmap of bacterial endophyte communities (A) class (B) order (C) family (D) genus. The scale bar represents the colour saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

5.3.4 Diversity estimation of endophytic bacteria obtained

Shannon and Evenness index calculated for all bacterial levels significantly differ ($P < 0.05$) within the family and genus levels, but at phylum, class and order levels were observed not to differ significantly ($P > 0.05$) (Table 5.1). The PCoA using Euclidean distance matrix was employed in analyzing the bacterial community composition (Figure 5.4). The PCoA plot showed that the FK samples differ significantly (ANOSIM, P = 0.01, R = 0.67) from CK and NK (Figure 5.4).

Table 5.1: Estimation of bacterial endophytes diversity and evenness among the sampling sites

	Indices	**CK**	**FK**	**NK**	**p-value**
Phylum	Shannon_H	0.92±0.16	1.21±0.16	1.00±0.16	0.638
	Evenness_e^H/S	0.11±0.19	0.15±0.21	0.12±0.16	
Class	Shannon_H	1.243±0.30	1.678±0.25	1.478±0.24	0.766
	Evenness_e^H/S	0.133±0.15	0.206±0.14	0.169±0.14	
Order	Shannon_H	1.195±0.40	1.455±0.32	1.993±0.36	0.978
	Evenness_e^H/S	0.144±0.14	0.18±0.12	0.3191±0.14	
Family	Shannon_H	1.405±0.38	1.932±0.36	1.866±0.4	0.020
	Evenness_e^H/S	0.1568±0.15	0.266±0.15	0.249±0.17	
Genus	Shannon_H	0.10±0.14	2.06±0.33	1.72±0.43	0.009
	Evenness_e^H/S	0.09±0.32	0.27±0.13	0.20±0.17	

Mean ± standard error (n= 3). Mean ± standard error (n= 3). p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

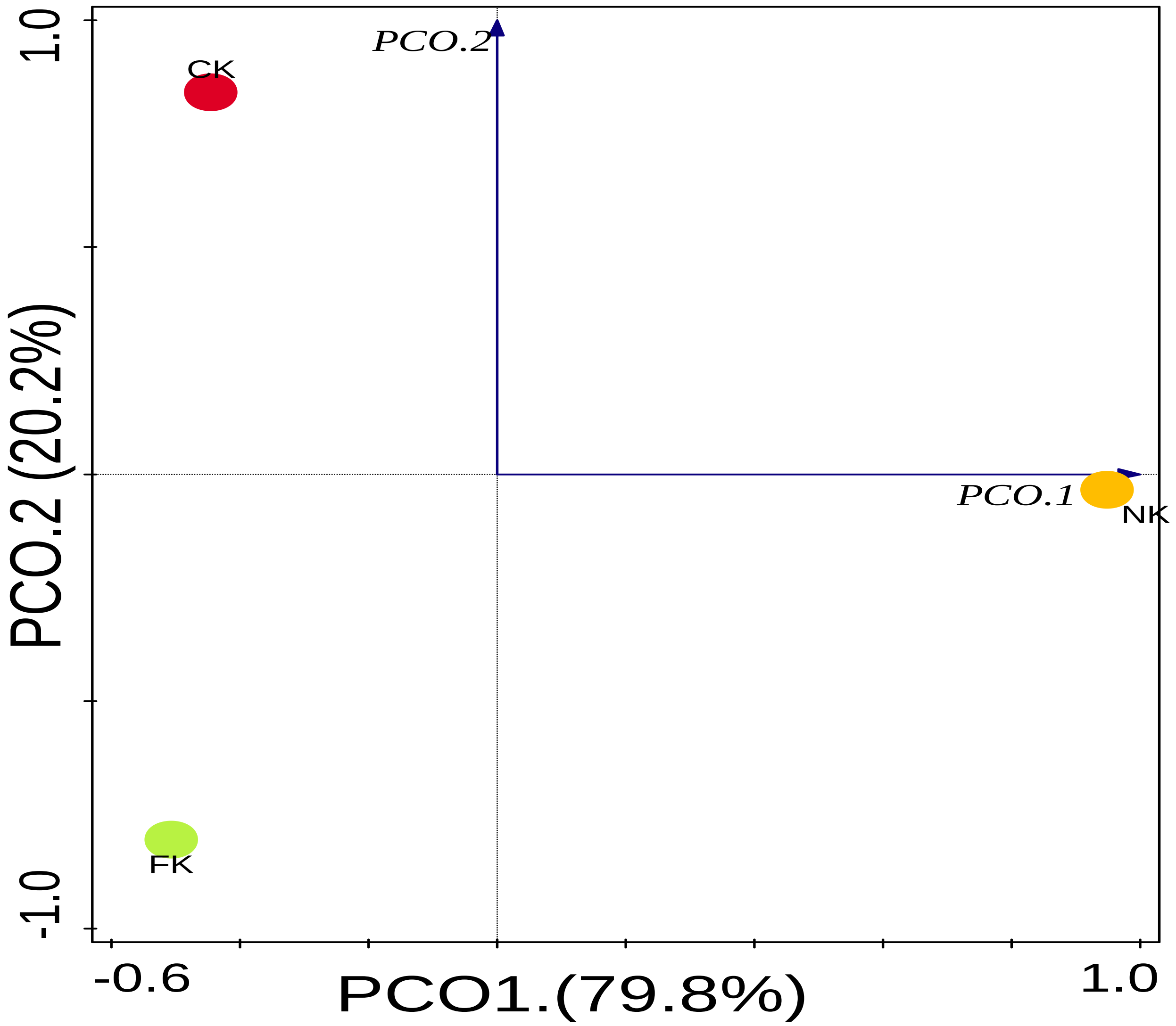


Figure 5.4: Principal coordinate analysis (PCoA) plot of endophytic bacterial community composition in the maize sites based on Euclidean dissimilarities. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

5.3.5 Pathways involved in nutrient cycling obtained from endophytic bacteria samples across the sites.

Sequences linked with nitrogen pathways, phosphorus pathways and secondary and iron metabolic pathways which are associated with nutrient cycling were observed in the endophytic bacteria across the sampling sites (Figure 5.5A-C). The relative abundance of the nitrogen and phosphorus pathways were higher in FK samples as compared to other sites, while the relative abundance of the secondary and iron metabolic pathways was higher in NK sites as compared to other sites, the differences were not significant (p>0.05) (Supplementary Table S4).

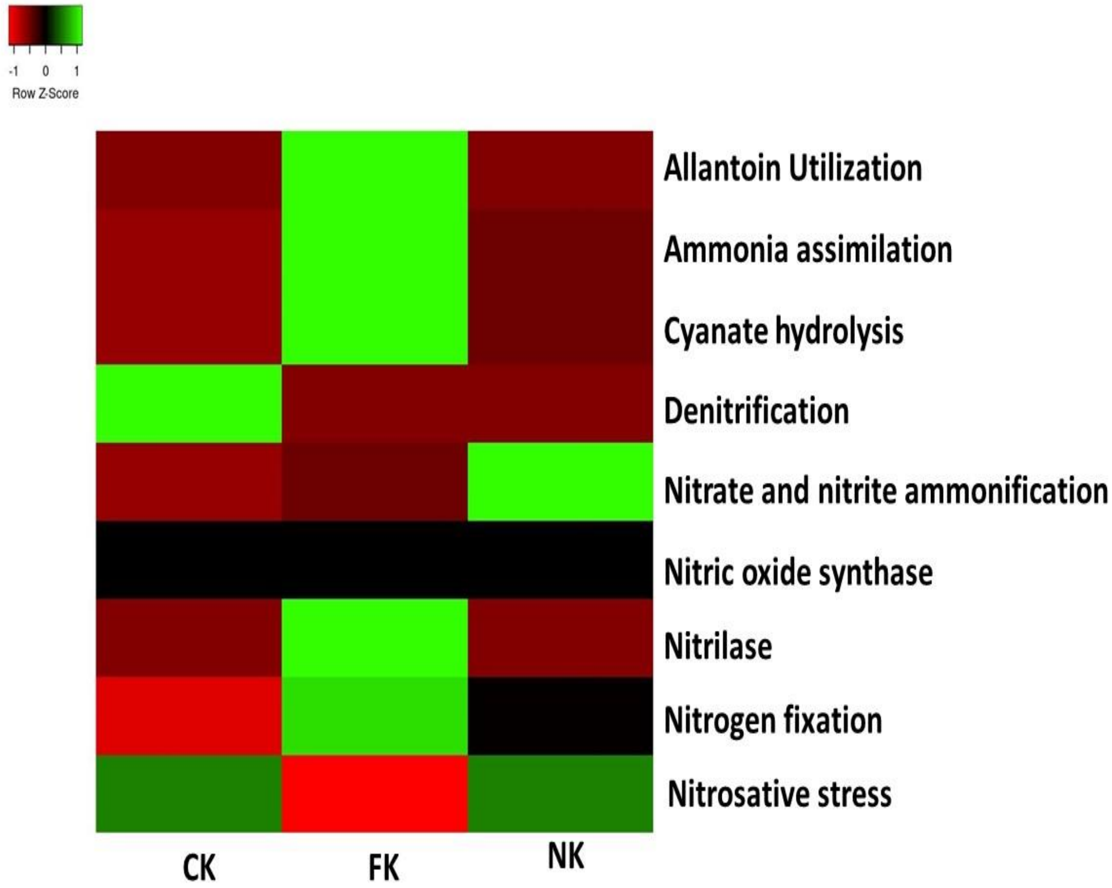


Figure 5.5A: Heat map showing the relative abundance of pathways involved in nitrogen metabolism in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

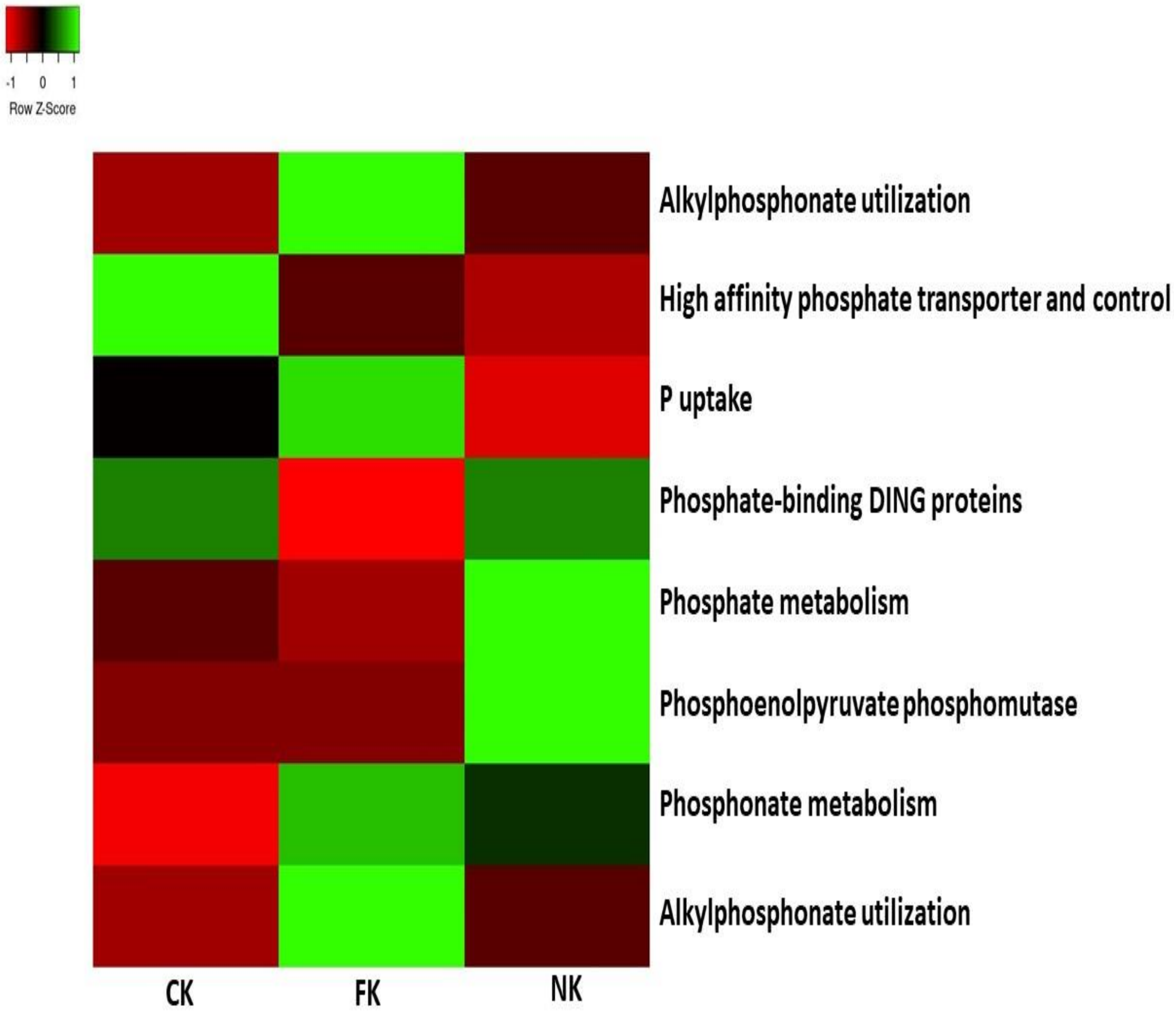


Figure 5.5B: Heat map showing the relative abundance of pathways involved in phosphorus metabolism in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

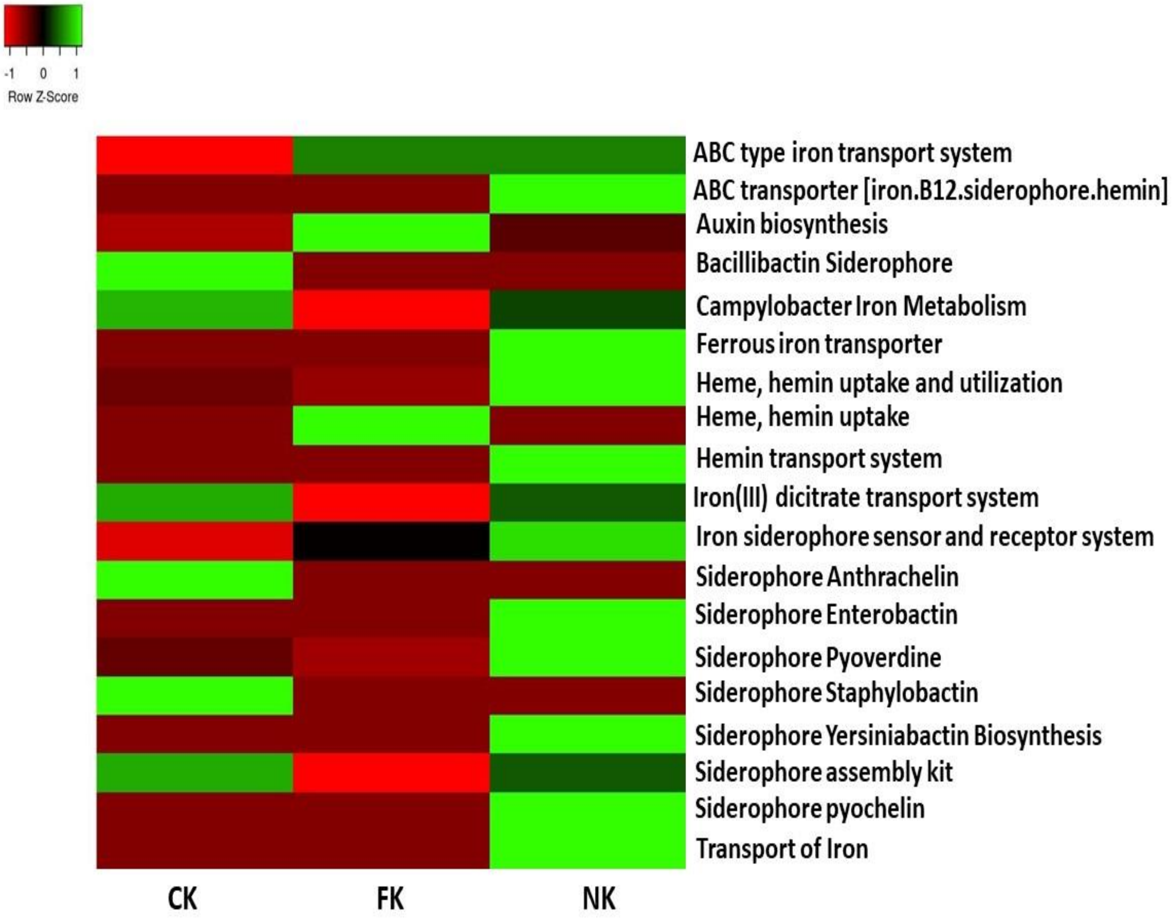


Figure 5.5C: Heat map showing the relative abundance of pathways involved in secondary and iron metabolisms in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

5.4 Discussion

Maize being one of the major food crops in South Africa can be cultivated using different agricultural practices depending on the interest of the farmers. Due to these rising concerns on the health and environmental implications of inorganic fertilizers, the global demand for organic fertilizers application in sustainable agriculture is increasing. However, going by the fact that samples from organic fertilizer (FK) sites had the highest relative abundance of endophytic bacteria as observed in this study, it was suspected that maize cultivated with organic fertilizer (FK) would harbor a high diversity of microorganisms compared with other treatments.

Previous studies on the metagenomic analysis of endophytes from maize have employed 16S rRNA and pyrosequencing (Correa-Galeote et al., 2016, Mashiane et al., 2018). However, 16S rRNA gene sequencing has been reported to be biased due to unequal gene amplification throughout the species, whereas shotgun metagenomic does not only yield read for16S rRNA but sufficient reads to capture and detect even rare species in a sample (Shah et al., 2011, Puri et al., 2018). This enabled us to discard the maize plant DNA sequence from the sequenced data while focusing only on the maize endophytic community. Although a recent study showed that the use of locked nucleic acid (LNA) PCR can help avoid or reduce the plant DNA amplification (Ikenaga et al., 2016, Puri et al., 2019), analysis of the sequenced data using subsystems showed that approximately 85% of the endophytic microbes were bacterial endophytic (Supplementary Figure S1). Using only these microbes, we studied the structure and diversity of endophytic bacteria using different fertilizations.

Firmicutes, Bacteroidetes, Actinobacteria, *Proteobacteria*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Tenericutes*, *Planctomycetes*, *Cyanobacteria,* and *Chlorobi* were predominant at the phylum level and more abundant at sites with organic fertilizer (FK). However, *Firmicutes* and *Bacteroidetes* dominated sites without fertilizers (CK) as compared to other sites, while *Proteobacteria* dominated inorganic fertilizers sites (NK). These major phyla identified in this study are similar to the phyla reported for maize plant by Correa-Galeote et al. (2018), Hardoim et al. (2015), Liu et al. (2017b), Puri et al. (2018) and Puri et al. (2019). To investigate this claim further, we examined the distribution of bacterial endophytes at the class level, of which *Bacilli* predominated FK and CK than NK sites. Also, *Flavobacteria*, *Sphingobacteria*, *Cytophagia*, *Clostridium* and *Actinobacteria* were more abundant in FK than other sites though they showed similar profiles (Figure 5.1). PCA graph showed that each site has a distinguishing bacteria phyla and it accounts for a combined 79.75% variance between all the fertilization sites (Figure 5.2). The position occupied by each endophytic bacteria reflects the makeup of sequences linked with each phylum; the vector arrows showed the phyla most strongly influenced by the distribution. Using this clue, it is possible to determine which bacterial phyla are more dominant in each sampling site as compared to others (Figure 5.2). As observed in this study bacterial phyla were more dominant in the FK site as compared to other sites (Figure 5.2).

Shannon and Evenness index calculated at all bacterial levels significantly differ ($P < 0.05$) within the family and genus levels, but phylum, class and order levels showed no significant difference ($P > 0.05$). Endophytic bacteria in maize cultivated with organic fertilizer were more diverse and evenly distributed than endophytic bacteria in maize plant cultivated with inorganic and without fertilizer (Table 5.1). The PCoA plot showed that the bacterial endophyte community structure of FK was different from that of CK and NK (Figure 5.4). This Euclidean dissimilarity matrix based plot showed a wide difference between the bacterial endophytes in the root of maize plants across the sampling sites.

However, it is interesting to know that organic fertilizers have gained prominence in the last two decades due to their immense benefits to plant growth, health and environment because little or no chemical input is involved (Reganold and Wachter, 2016, Van Bruggen and Finckh, 2016). Furthermore, farming practices using inorganic fertilizer boost soil health, enhance plant yields and growth, alongside enhancing plant resistance to abiotic and biotic stresses (Kniss et al., 2016, Xia et al., 2019). Some of the notable advantages of organic fertilizers over inorganic fertilizers are that it boosts water retention ability of the soil, soil texture and enhances resistance to soil erosion which might be responsible for the abundance and diversity of more microbial groups attributed to organic farming operation (Sharma and Chetani, 2017, Lin et al., 2019). Organic fertilizers make nitrogen available to the plants in usable form which help in plant growth enhancement and at the same time not destroying beneficial microbes in the soil (Figure 5.5A).

Bacteria endophytes have been reported to play a key role in modulation of plant development and growth through fixing of nitrogen, phosphorus solubilization, production of siderophore, phytohormones production, reduction of ethylene concentration, antibiotics production, secretion of antifungal metabolites, induced and systemic resistance (Hardoim et al., 2015, Vejan et al., 2016, Correa-Galeote et al., 2018, Fadiji and Babalola, 2020a). Some notable genera identified in this study have previously been reported to be diazotrophic bacteria (*Bacillus, Sphingobium,* and *Burkholderia*)(Correa-Galeote et al., 2018), others solubilize phosphorus (*Sphingobium*, *Burkholderia*, *Staphylococcus, Bacillus* and *Chitinophaga*) (Midekssa et al., 2015, Acevedo et al., 2014, Chung et al., 2012). Also, some are involved in siderophore production (*Staphylococcus, Sphingobium*, *Chitinophaga* and *Burkholderia*) (Esmaeel et al., 2016, Yu et al., 2017, Sauvêtre and Schröder, 2015) or indole acetic acid producers (*Sphingobium, Burkholderia, Chitinophaga, Staphylococcus, Bacillus*) (Sauvêtre and Schröder, 2015, Correa-Galeote et al., 2018). Others can exhibit ACC deaminase activity (*Sphingobium, Staphylococcus, Burkholderia*, and *Bacillus*) (Srivastava et al., 2013, Ahmad et al., 2008), biocontrol activity (*Sphingobium*, *Burkholderia*, *Bacillus*, *Geobacillus* and *Chitinophaga*) (Bulgari et al., 2011, Sivasakthi et al., 2014, Ormskirk et al., 2019), and phytoremediation (*Desulfobacterium*) (Li et al., 2012a).

Furthermore, it is important to state that, to the best of our understanding, some notable bacteria genera identified in this study such as *Neptunibacter*, *Leadbetterella*, *Desulfitobacterium*, *Microscilla*, *Prevotella* and *Shewanella* have not been previously identified as bacterial endophytes inhabiting maize. These new bacteria genera could possess some biotechnological potentials especially in the development of novel microbial inoculants. Also, the detection of *Staphylococcus* and *Bdellovibrio* is unusual in that some of their species are notable as human pathogens. These two genera have been identified in apple, roots of blackberry, grapevine shoots, and fresh orange fruits (Griffin and Carson, 2018, Phukon et al., 2013, Correa-Galeote et al., 2018). Moreover, recent studies reveal that some pathogenic bacteria inhabit the tissues of the plant (Szilagyi-Zecchin et al., 2014, Sandhya et al., 2017).

Furthermore, we using SEED level 3 database we were able to predict the major nutrient cycling pathways of endophytic bacteria in maize plants by farming practice. Even though, bacterial endophytes diversity varied with farming practice, the major plant beneficial metabolic pathways examined in this study do not differ significantly (P>0.05) except for pathways involved in carbohydrates (Figure 5.5A-C). This agrees with an earlier report in which bacteria structure was found to strongly rely on their functions as compared to their taxonomic relatedness (Burke et al., 2011). Also, Hardoim et al. (2015) earlier reported that the functions of bacterial endophytes do not depend on taxonomy groupings but environmental factors and the plant host. Nitrogen fixation and phosphorus uptake were observed to be highly represented in FK samples. Nitrogen, phosphorous metabolisms, secondary and iron mechanisms are some of the important mechanisms employed by endophytes in inhabiting and protecting the host plant (Fadiji and Babalola, 2020a, Hong et al., 2019). Similarly, auxin and siderophore-related traits notable in plant growth-promotion were greatly represented in samples from FK and NK sites respectively (Figure 5.5C). Auxin and siderophore-related traits were earlier detected in secondary and iron metabolic pathways of endophytic bacteria detected in ginseng plants (Hong et al., 2019). Our results further suggest that the interactions between bacteria endophytes and maize plant using different fertilizers are helpful for root growth.

5.5 Conclusion

In conclusion, using the shotgun approach, this present study revealed that the use of organic fertilizer for agricultural practices had a strong influence on the abundance, and diversity of bacteria endophyte communities as compared to inorganic fertilizers at phylum level alongside their nutrient cycling pathways. At genus level, it was further observed that maize planted with organic fertilizer housed more distinct endophytic bacteria. This showed a distinct taxonomical difference in the abundance of endophytic bacteria in the root of maize cultivated with organic fertilizer as compared to other fertilizer sites. Virtually, all major endophytic bacteria genera, identified in this study have the potential not only to enhance agricultural sustainability, and promote growth of the maize plant, but its application in agricultural practice can also bring about a reduction in chemical inputs which have been reported not to be ecofriendly. We hereby suggest co-culturing of the uncultivable bacteria species with other bacteria, as recreating the environment in the laboratory, and combining these approaches with micro-cultivation technology will increase throughput and access to rare species. Future studies should investigate the mechanisms used by endophytic bacteria and specific function attributed to them in plant growth promotion. Also, detailed studies on the effects of other organic-farming practices such as crop rotation, cover cropping, and crop integration could help in establishing the best practices which could enhance the abundance and diversity of plant-associated microbiomes which could subsequently promote plant growth, yield and health. Studies in this regards will boost the application of beneficial endophytes for sustainable agricultural practices. Understanding how to efficiently apply endophytic bacteria in agricultural practices will be key to their adoption for agricultural sustainability. This study advocates for acceptance of organic fertilizer for sustainable agriculture by agriculturists and policymakers.

CHAPTER SIX

6.0 ORGANIC FARMING ENHANCES THE DIVERSITY AND COMMUNITY STRUCTURE OF ENDOPHYTIC ARCHAEA AND FUNGI IN MAIZE PLANT: A SHOTGUN APPROACH

Abstract

This study compares, for the first time, the diversity and community structure of the endophytic archaea and fungi inhabiting the roots of maize plant cultivated using two different fertilizer regimes and the control field via shotgun metagenomics. This was used to verify our hypothesis that organic farming positively influences the diversity, beneficial effects and abundance of plant endophytic communities as compared to conventional farming. Total DNA extraction was carried out using the roots of maize plants cultivated with organic fertilizer (FK), inorganic fertilizer (NK) and without any fertilizer (CK) at different farming sites in an experimental field and sequenced using shotgun metagenomics. In all, 3 archaea phyla namely, *Crenarchaeota*, *Euryarchaeota*, *Thaumarchaeota* and 2 fungi phyla namely, *Ascomycota* and *Basidiomycota* were identified in the samples with higher abundance in samples from organic fertilizer site. Our diversity results revealed that the abundance of endophytic archaea and fungi species across the farming sites are in the order NK < CK < FK. The results indicate that organic farming enhances the abundance of endophytic archaea and fungi which might be a boost to sustainable agricultural practices. Furthermore, some new genera of endophytic archaea and fungi were identified from the maize roots. Future studies into how these novel endophytes can be cultured are important in a bid to explore their functions in sustainable agriculture as well as identify their biotechnological importance.

Keywords: Endophytes, Fertilizer application, Plant-microbe interactions, Metagenomes, Sustainable agriculture

This chapter has been published in *Journal of Soil Science and Plant Nutrition* 2020; 20:2587–2599. https://doi.org/10.1007/s42729-020-00324-9

6.1 Introduction

Chemical and biological fertilizers are often used for the improvement of soil fertility, still the dependence on inorganic fertilizers for agricultural practice has led to many health problems and severe imbalance in the ecosystem (Sharma and Chetani, 2017, Savci, 2012, Chandini et al., 2019). Thus, in a bid to reduce or possibly eliminate the side effects of inorganic fertilizers, a new agricultural practice called sustainable or organic agriculture had been adopted (Chowdhury, 2004). Organic agriculture was defined by the U.S. Department of Agriculture as an ecological management method that promotes biological cycles, biodiversity and biological activities of the soil through the utilization of off-farm inputs and management strategies that maintain, restore and enhance ecological synergy (Xia et al., 2019). Organic agriculture has recently gained importance globally and is currently being adopted by most farmers, policymakers and other collaborators in agriculture because of the limited application of chemicals attributed to it (Kniss et al., 2016). It has also been reported to enhance the growth and yield of most food crops such as tomato, maize, and pepper in developed countries of the world (Xia et al., 2019).

Maize (*Zea mays* L.) is one of the most important cereal crops largely cultivated in Africa under diverse environmental conditions (Alori et al., 2019). In most developing countries, maize is often consumed raw and serves as a major staple diet for above 200 million people. Most people in these countries adopt maize as their major breakfast cereal (Du-Plessis, 2003, Alori et al., 2019). Maize is a household food crop that can be grown in all soil types (Liu et al., 2017b, Marag et al., 2018), most times with different degrees of yield. Plants shelter a lot of beneficial and to some extent species-specific microbiota (Berg and Smalla, 2009, Wagner et al., 2016). These microbial communities play an important role in improvement of plant growth, adaptation, health yield and diversification (Bulgarelli et al., 2013). Out of all plant associated microbes such as epiphytes and, rhizobiomes among others (Compant et al., 2019), endophytes have gained much attention recently as a result of their beneficial roles.

Endophytes are classes of microbes (such as fungi, archaea, bacteria and actinomycetes) which inhabit the tissues of healthy plants without causing any harm or disease (Omomowo and Babalola, 2019), though a recent study showed that some could be pathogenic (Brader et al., 2017b). They are reservoirs of bioactive metabolites and phytochemicals which have been reported to promote the growth and yield of the plant, alongside boosting the plant's fitness against biotic and abiotic stresses (Furtado et al., 2019, Hardoim et al., 2015, Fadiji and Babalola, 2020a). Endophytic fungi can be beneficial to their host and can also be pathogenic at some specific stages of their growth when exposed to certain environmental conditions (Jia et al., 2016). Likewise, fungal endophytes can boost plant resistance to insect and pathogen attacks (Cui et al., 2017), alongside enhancing plant defense against abiotic stresses such as high salinity and drought (Azad and Kaminskyj, 2016). Endophytic fungi have been reported to improve sustainable agriculture through the secretion of beneficial metabolites which enhance their interactions with the host (Xia et al., 2019).

However, in the last few decades, the interest of researchers in the diversity, composition and metabolic potentials of archaea in different environments has increased (Julian et al., 2018). Archaea represent an essential component of plant microbial communities where its impact is under-investigated (Moissl-Eichinger et al., 2017). They are most times found in the endosphere and rhizosphere, but not often in the phyllosphere, this can be attributed to diverse abiotic factors dominating these environments (Chelius and Triplett, 2001, Julian et al., 2018). Although many factors influencing the functionality of archaea under anaerobic conditions have been studied (Sher et al., 2011), their diversity and composition in endophytic environments have been under-investigated (Chelius and Triplett, 2001, Müller et al., 2015). Going by their presence in tissues of most healthy plants (Odelade and Babalola, 2019), we conclude that archaea have positive interactions with plants, which are not well understood.

Interestingly, the advent of next-generation sequencing methods and omics tools have enhanced metagenomics study in plants, in which all the plant microbiome can be studied without difficulties (Fadiji and Babalola, 2020c). Molecular methods such as internal transcribed spacer (ITS) have been used to study the composition and diversity of endophytic fungi in crops especially maize (Renuka and Ramanujam, 2016, Russo et al., 2016, Potshangbam et al., 2017, Xia et al., 2015), while few studies exist on endophytic archaea using 16S rRNA (Müller et al., 2015, Chelius and Triplett, 2001). However, the shotgun metagenomics has proven to be more advantageous over other metagenomics methods in that it allows the study of the entire microbial community in an environment. Limited studies exist on the metagenomic analysis of endophytic microbes in the plant using shotgun (Hong et al., 2019) and the studies have focused only on bacterial species due to its dominance in the metagenome, thereby being silent and sometimes leaving out fungi and archaea endophytes. The above mentioned limitations hereby enhanced our interest in investigating the community structure of the endophytes. Being conscious of the fact that each beneficial microbial community has its notable role in plant growth promotion and should not be neglected, this study, therefore, focused on investigating the community structure of endophytic archaea and fungi in maize plants. To the best of our understanding, no study exists on the influence of different farming practices on the diversity and community structure of endophytes in maize using shotgun metagenomics. Hence, this study presents the first effort to assess the influence of farming practices on the diversity and community structure of endophytic archaea and fungi in maize plants using shotgun metagenomics. The study hypothesized that organic farming boosts the abundance and diversity of endophytic archaea and fungi in maize plants as compared to other farming practices involving chemical fertilizers.

6.2 Material and Methods

6.2.1 Seed collection

Maize seed (WE 3127) planted in this study was obtained from North-West University School Farm, Molelwane, Mafikeng, North West Province, South Africa.

6.2.2 Experimental design and climatic conditions of the site

The study was carried out separately in the organic and inorganic fields of the North-West University School farm, Molelwane, Mafikeng, North West Province (S25^{o}47'25.24056", E25^{o}37'8.17464"), South Africa. A high presence of shrubs and trees is often used to describe this province. The average temperatures witnessed in Northwest province vary from 17 to 31^{o}C and 3 to 21^{o}C in the summer and winter, respectively. This farm has been in existence for over 15 years. The pH and other chemical properties of the soil in the farm were checked. The soil of the farm had physicochemical properties (22% Sand, 66% Silt, 12% clay, pH 6; 0.48% organic C, 101.5 ppm P, 0.15% total N, 0.962ppm K) (Supplementary Table S1). The annual rainfall experienced in this province is evaluated at 360 mm per annum. These experimental fields have been devoted to crop rotation involving sorghum-maize-soybean for an extended period. Soybean was the last planted crop for the year 2018. Two fertilizer regimes were employed in this study namely organic fertilizer (FK) and inorganic fertilizer (NK), while the third site had no fertilization (CK). Cow dung and NPK has been in constant use for more than 15years in the organic and inorganic sites respectively complying with standard procedure (USDA, 2014), while no fertilizer has ever been applied to the third site. Dosage of inorganic fertilizer which had been in use is 150 N, 75 P_2O_5 and 75 K_2O all in kg ha^{-1} for the inorganic site. Urea was used as N fertilizer, Potassium sulfate as K fertilizer, and calcium superphosphate as P fertilizer. The organic fertilizer site has been experiencing the application of cow dung with a dosage of 10,625 kg ha^{-1} for over 15 years. The dimension for each planting site for this study was taken as 10 m x 4 m and the maize seeds were planted. The experiment was carried in the summer of 2019. The entire sites were continuously irrigated to avoid drought stress. Weeding of the farming sites was carried out manually.

6.2.3 Root sampling strategy

As a result of the triangular shape possessed by the experimental field, each farming site was divided into 3 regions for the root sampling. Ten (10) fresh plants were randomly selected from each region of the sites and their roots pooled to represent a biological replicate, making up 30 plants per planting site. The maize plants were harvested after 65 days of planting, during the post-tasseling stage. Generally, 90 maize plants were analyzed, and the 3 regions from each site represent 3 replicates for each farming sites. The plants were then transported immediately to the laboratory for further processing.

6.2.4 Root surface washes

Soil particles that accompanied the roots from the field were first of all removed, while surface washing of the fresh roots was carried out using the method described by Liu et al. (2017b). The roots were soaked in 70% ethanol for 3 min, then washed with 2.5% NaOCl solution for 5 min, rinsed again with 70% ethanol for 30 s and finally washed with sterilized distilled H_2O. To ascertain whether the surface washing process was properly done and epiphytes removed some pieces of the washed roots were plated on yeast extract-mannitol medium (YEM). Petri plates were checked for microbial growth after the 72 h incubation at 30 °C (Marag et al., 2018, Correa-Galeote et al., 2018). Maize roots from Petri dishes where no contamination was observed were used for DNA extraction (Correa-Galeote et al., 2018, Chelius and Triplett, 2001).

6.2.5 Extraction of DNA and shotgun sequencing

The maize roots were cut into small pieces using a sterilized scalpel and macerated immediately using TissueLyser (Qiagen, USA). Completed metagenome DNA was instantly extracted from the root tissues via the Qiagen DNeasy Plant Mini Kit (USA). Shotgun metagenomic sequencing was carried out at MR DNA molecular laboratory, Texas, USA. DNA library was prepared using the Nextera DNA Flex kit (Illumina) following standard guidelines. Qubit® dsDNA HS Assay Kit was used to ascertain the DNA concentration. The library was prepared using fifty (50) ng of the DNA concentration. The concentration of the final libraries was evaluated using Qubit® dsDNA HS Assay Kit while the Agilent 2100 Bioanalyzer was used to ascertain the size of the library. The library size ranges from 683 to 877 bp with an average of 731 bp while the pooling of libraries was done using 0.6nM ratios, and the paired-end sequencing was done with 300 cycles via the NovaSeq 6000 system (Illumina).

6.2.6 Data and statistical analyses

After uploading the sequences obtained for each metagenome on metagenomics rapid annotation online server (MG-RAST) (Hong et al., 2019), quality control (QC) assessment which includes removal of adapter, filtering of ambiguous bases and low reads sequences from the raw data was carried out via the Trimmomatic v 0.33 program (Bolger et al., 2014). After QC analysis, sequence annotation was carried out using a BLAST-like alignment tool called BLAT (Kent, 2002), against M5NR database (Wilke et al., 2012), which allows a non-redundant integration of several databases. Taxonomic profiling of endophytic archaea and fungi were performed employing the SEED database with defined parameters such as maximum e-value of 1 x 10^{-5} cut-off and minimum 60% sequence similarity to a subsystem. Sequences that declined annotation were removed. However, since our concentration is on endophytic fungi and archaea, we similarly discarded sequences for bacteria and viruses. Data normalization was carried out on MG-RAST. The obtained endophytic fungi and archaea tables were assembled according to each taxon and unclassified sequence reads were kept for statistical analysis. Relative abundance in percentages, for each taxon, was calculated after an independent analysis of the 9 sequences using MG-RAST**.** The average values of the relative abundance of the 3 replicates for each sampling sites (CK, FK and NK) were used for statistical analysis. These standard sequences have been deposited at the National Center for Biotechnology Information's Sequence Read Archive (NCBI SRA) dataset with the accession number PRJNA607664.

The Shinyheatmap was used for plotting the relative abundance graph of endophytic fungi and archaea communities at class level after data normalization (Khomtchouk et al., 2017). Paleontological Statistics software package (PAST) version 3.20 (Carrell and Frank, 2015) was used to assess the Shannon diversity and Pielou evenness index for each of the sampling sites and the indices between the sites was examined via Kruskal–Wallis test. The beta diversity was described using the principal coordinate analysis (PCoA) based Bray-Curtis dissimilarity matrix, while the one-way analysis of similarities (ANOSIM) via PAST software was used in assessing the differences in the community structure across the sites (Carrell and Frank, 2015). The principal component analysis (PCA) using the Bray-Curtis dissimilarity matrix was employed to reveal the dispersal of the identified endophytic fungi and archaea class across the farming sites. The PCoA and PCA were designed and plotted using CANOCO version 5.0

6.3 RESULTS

6.3.1 Shotgun sequencing datasets.

The average number of uploaded sequences were 1613298509 for CK, 1493819190 for FK and 2651625071 for NK sequences. After quality control assessment was executed in MG-RAST, the quantity of retained mean sequences was 334259767 for CK, 415505341 for FK and 817699487 for NK respectively (Supplementary Table S2).

6.3.2 Phylum level distribution from maize samples across the sites.

Metagenomic analysis using the Subsystem database revealed a total of 3 archaea phyla namely *Crenarchaeota*, *Euryarchaeota* and *Thaumarchaeota* in all the samples across the sites with more abundance in the FK site (Figure 6.1). The difference observed for *Crenarchaeota* and *Euryarchaeota* was not significant across the sites ($P>0.05$), while *Thaumarchaeota* differs significantly ($P<0.05$) across the sites (Supplementary Table S5). Besides, 2 fungi phyla namely *Ascomycota* and *Basidiomycota* were identified across the sites. The difference that exists between was not significant ($P>0.05$) across the sites (Figure 6.1, Supplementary Table S6). Their abundance across the sites followed the order NK < CK <FK respectively.

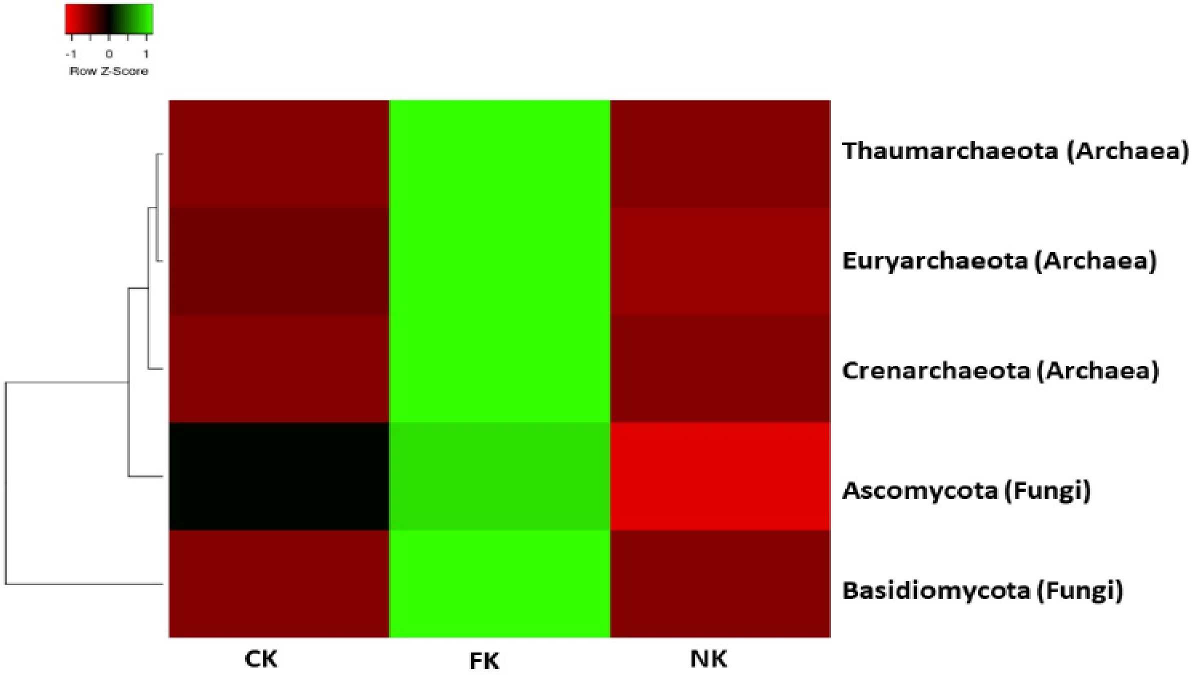


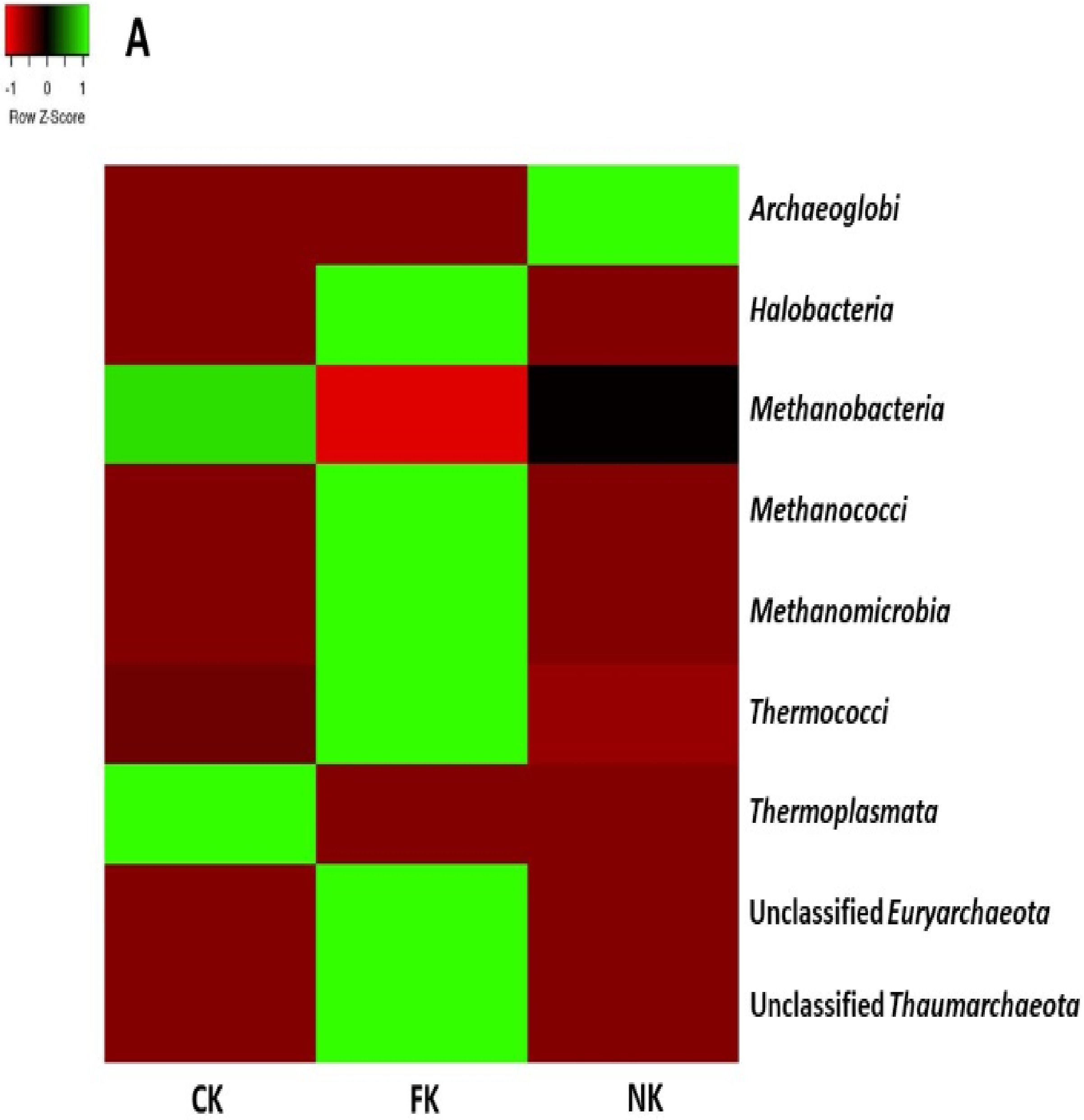
Figure 6.1: Phyla distribution of the identified endophytic archaea and fungi from samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic archaea and fungi

6.3.3 Community composition and abundance of endophytic archaea from maize samples across the fertilizer sites.

The three (3) major phyla identified in this study, were most abundant in the FK site with *Crenarchaeota* (71.4%), *Euryarchaeota* (68.7%) and *Thaumarchaeota* (81%). However, the difference observed for *Crenarchaeota*, and *Euryarchaeota* across the sites was not significant (P>0.05), while *Thaumarchaeota* differed significantly (P<0.05) across the sites.

At the class level, unclassified *Thaumarchaeota* dominated the FK site followed by *Halobacteria*, *Methanomicrobia, Thermococci*, unclassified *Euryarchaeota*, *Methanomicrobia* and *Methanococci* (Figure 6.2A). *Methanobacteria* and *Thermoplasmata* dominated the CK site, while only *Archaeoglobi* predominated the NK site. The difference that exist between them do was not significant (P>0.05) across the sites, except for unclassified *Thaumarchaeota* which differed significantly (P<0.05) across the sites (Supplementary Table S5).

Furthermore, at the genus level, *Candidatus Nitrososphaera, Halomicrobium, Methanococcoides, Pyrococcus, Cenarchaeum, Nitrosopumilus, Methanosarcina, Methanosaeta, Aeropyrum*, *Desulforococcus*, *Thermofilum*, *Halalkalicoccus*, *Methanospirillum*, *Candidatus Micrarchaeum* and *Haloferax* were more abundant at FK sites. *Methanosphaerula, Natrialba*, *Staphylothermus*, *Methanoculleus*, *Thermococcus* and *Thermoplasma* dominated the CK site, while *Methanococcus* was dominant at the NK site (Figure 6.2B). The PCA was used to show the distribution of the identified archaea genus between the fertilizer sites with FK sites having the highest distribution (Figure 6.3).


-1 0 1
Row Z-Score
A
Archaeoglobi
Halobacteria
Methanobacteria
Methanococci
Methanomicrobia
Thermococci
Thermoplasmata
Unclassified Euryarchaeota
Unclassified Thaumarchaeota
CK
FK
NK

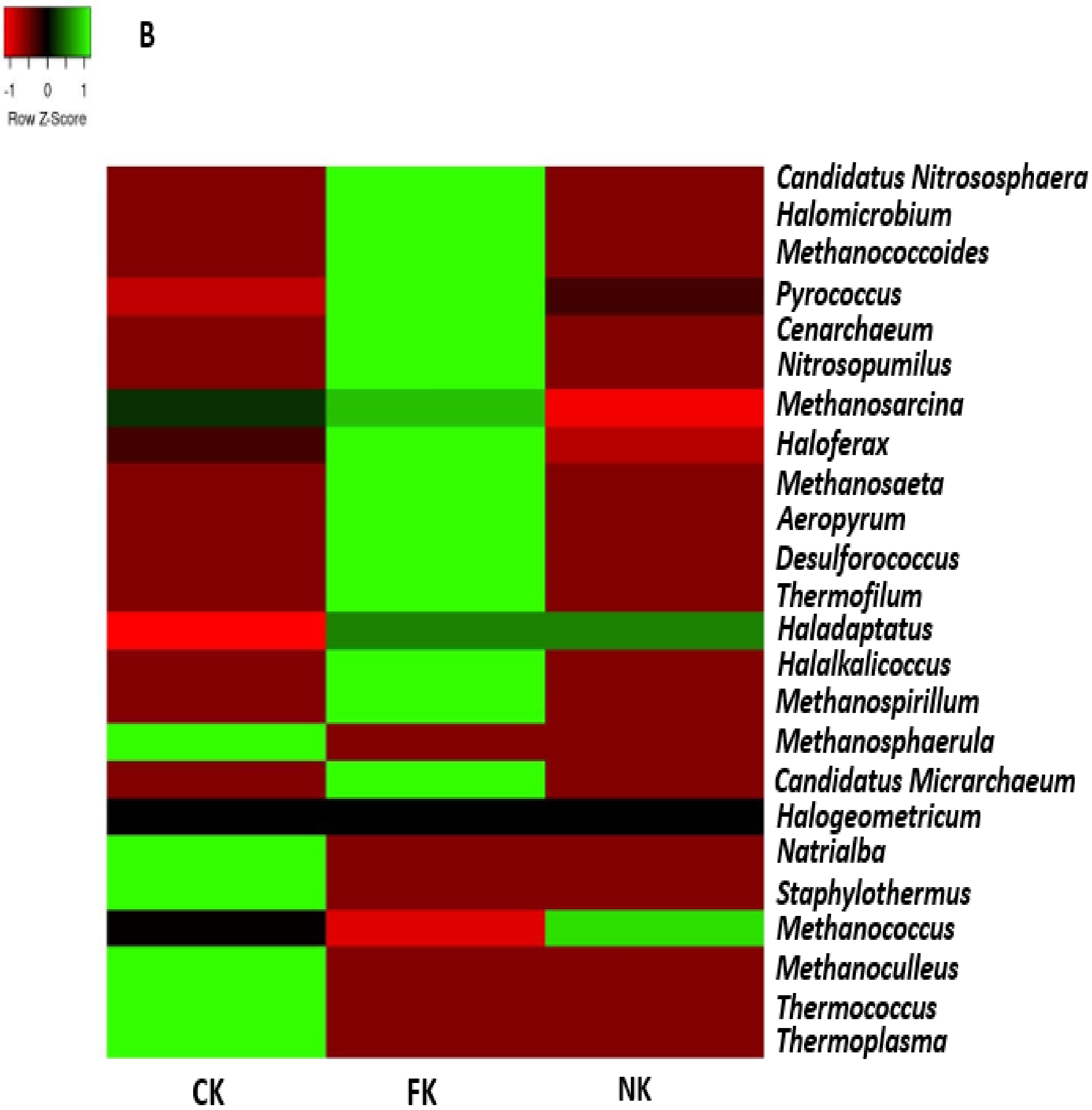


Figure 6.2: Heatmap of endophytic archaea communities (A) Class (B) Genus. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic archaea taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

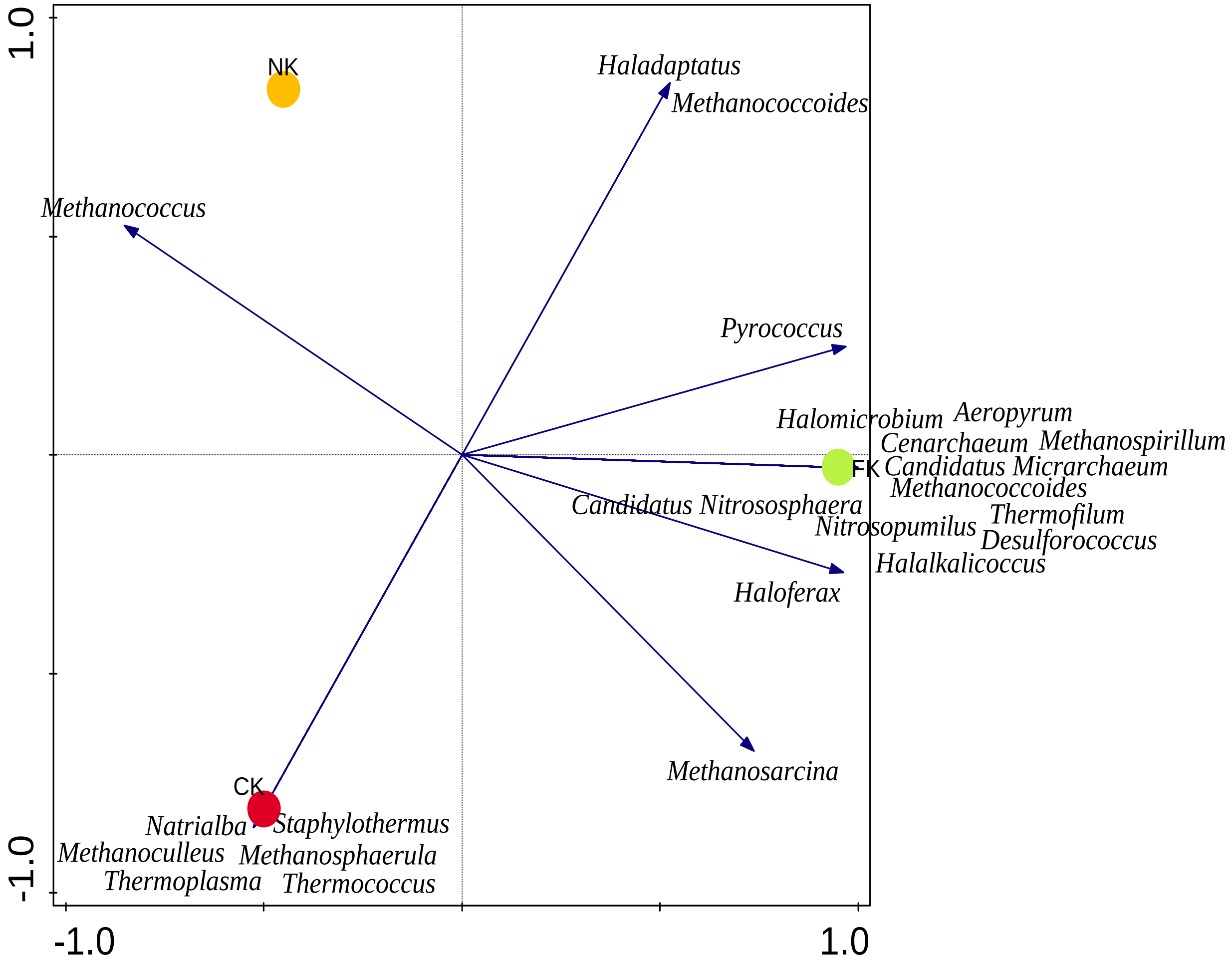


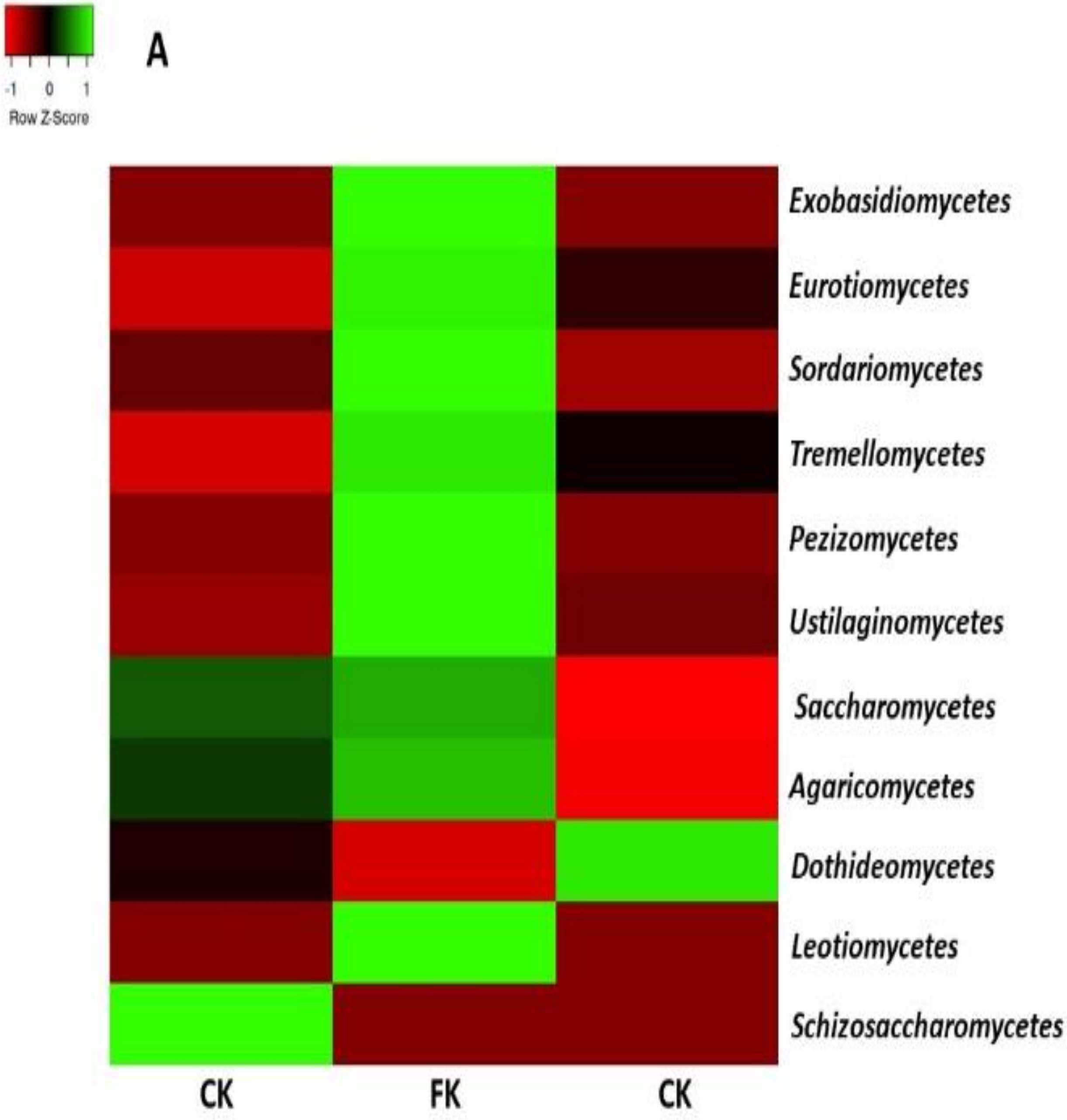
Figure 6.3: PCA graph of endophytic archaea metagenomes. The vector arrow represents the influence of endophyte archaea metagenomes. Axis 1 (95.1%) and Axis 2 (4.9%) explained the variations. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

6.3.4 Community composition and abundance of endophytic fungi

Two major fungi phyla were identified in this study, namely *Ascomycota* (51%) and *Basidiomycota* (84.7%) and predominant in samples FK site though the difference between them was not significant (P > 0.05) across the sites (Supplementary Table S6).

At the class level, *Exobasidiomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Tremellomycetes*, *Pezizomycetes*, *Ustilaginomycetes*, *Saccharomycetes*, and *Agaricomycetes* predominated the FK site (Figure 6.4A). *Leotiomycetes* and *Schizosaccharomycetes* dominated the CK site while only *Dothideomycetes* dominated the inorganic fertilizer site (NK), the difference between them was not significant ($P > 0.05$) across the sites (Supplementary Table S6).

In addition, at the genus level, *Malassezia*, *Filobasidiella*, *Ustilago*, *Tilletia*, *Metarhizium*, *Sordaria*, *Coprinopsis*, *Sclerotinia*, and *Schizophyllum* dominated FK sites. *Ajellomyces*, *Filobasidiella*, *Gibberella*, *Aspergillus*, *Schizosaccharomyces*, and *Talaromyces* were dominant in CK site, while *Leptosphaeria* was found dominant in the NK site (Figure 6.4B). However, *Podospora* dominated both CK and FK sites while *Phaeosphaeria* dominated both CK and NK sites.The PCA was used to show the distribution of the identified fungi between the fertilizer sites with the highest distribution observed at the organic fertilizer (FK) site (Figure 6.5).


-1
0
1
Row Z-Score
A
Exobasidiomycetes
Eurotiomycetes
Sordariomycetes
Tremellomycetes
Pezizomycetes
Ustilaginomycetes
Saccharomycetes
Agaricomycetes
Dothideomycetes
Leotiomycetes
Schizosaccharomycetes
CK
FK
CK

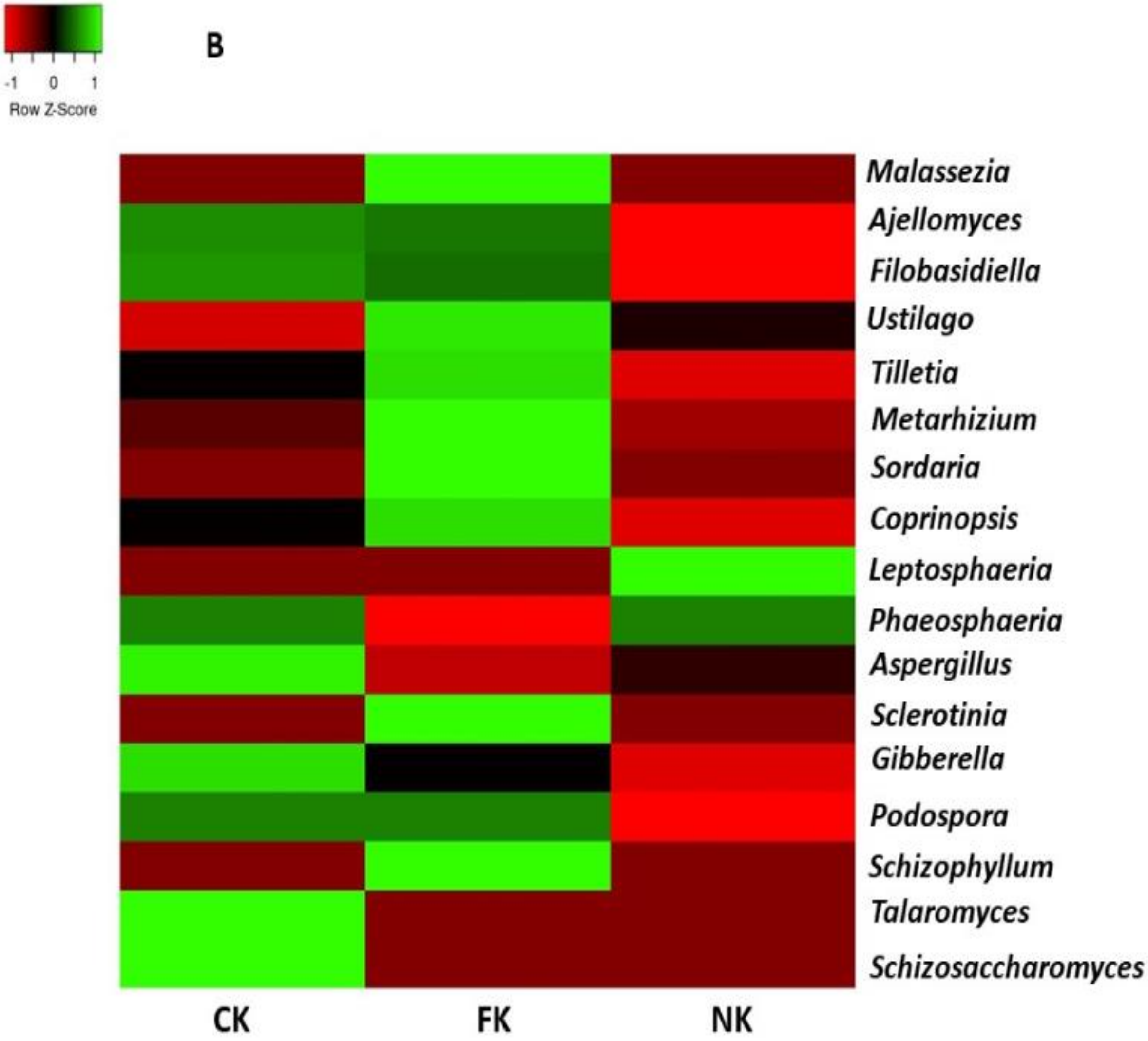


Figure 6.4: Heatmap of endophytic fungi communities (A) Class (B) Genus. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic fungi taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

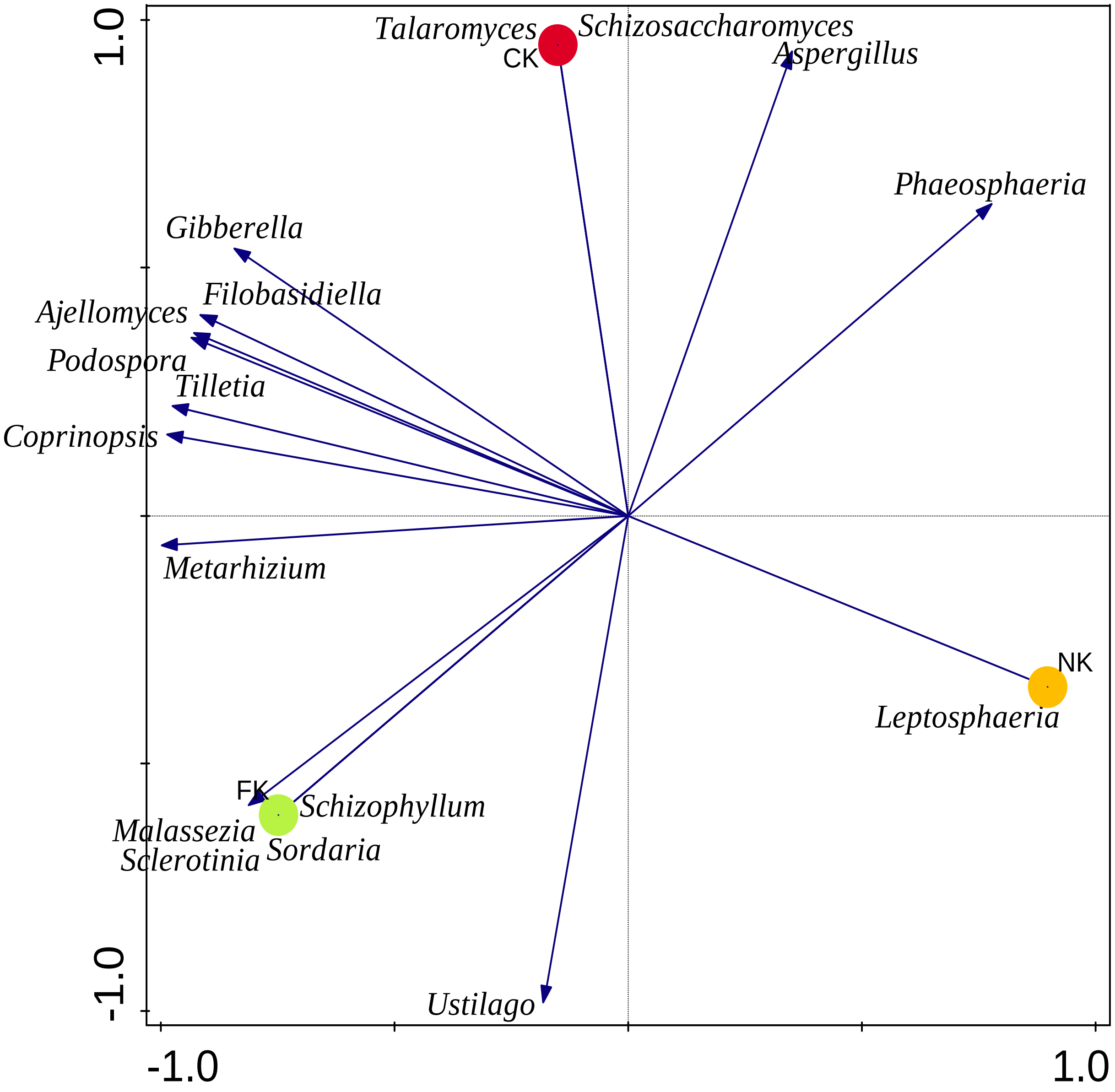
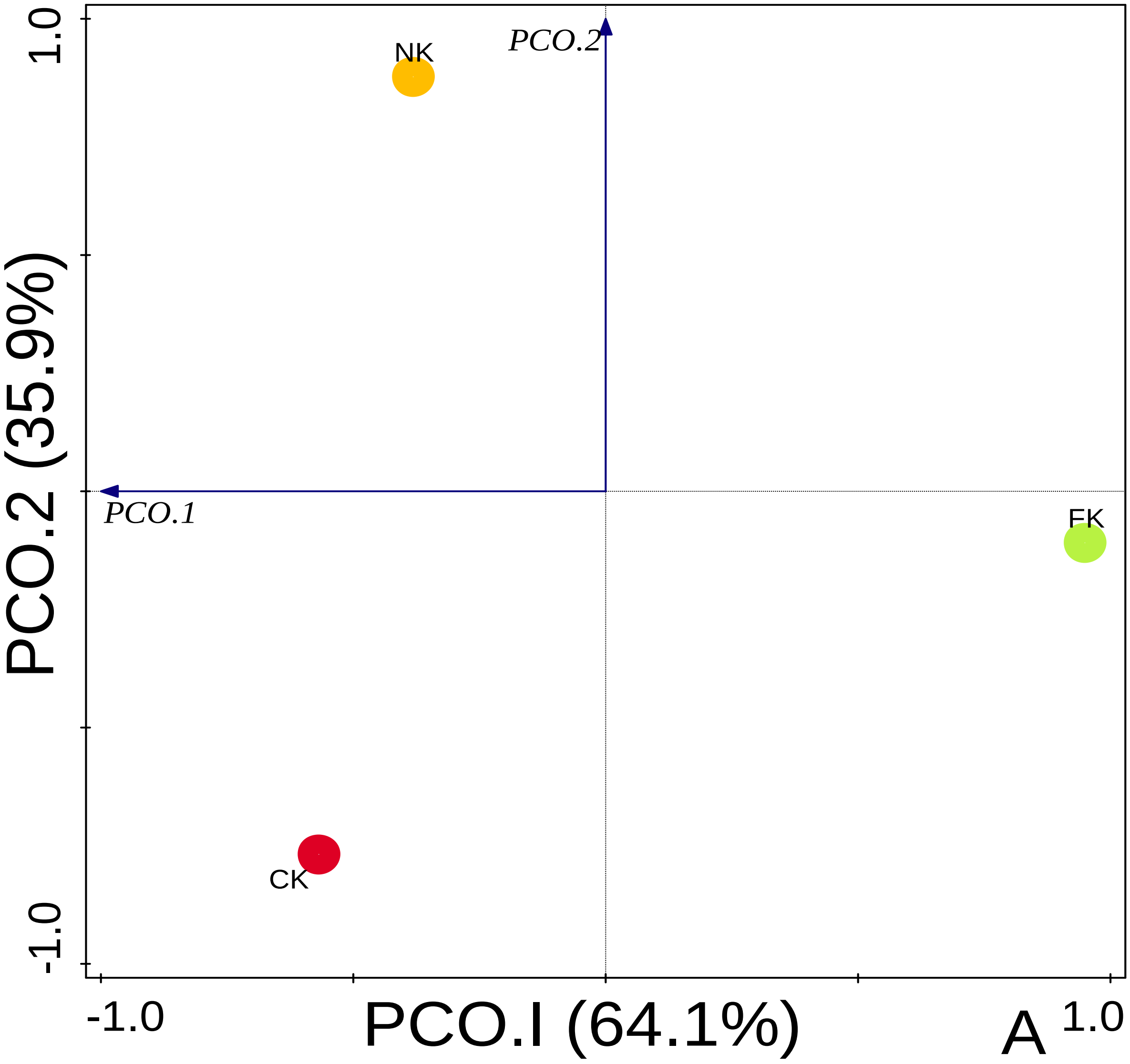


Figure 6.5: PCA graph of endophytic fungi metagenomes. The vector arrow represents the influence of endophytic fungi metagenomes. Axis 1 (72.4%) and Axis 2 (27.6%) explained the variations. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

6.3.5 Alpha and beta diversity in endophytic archaea and fungi communities across the farming sites.

The diversity assessment using the Shannon index and evenness index calculated for the archaeal endophytes do not differ significantly ($P > 0.05$) within the phylum, while significant difference ($P < 0.05$) was observed at the genus level (Table 6.1) across the sites. The PCoA using the test was employed in analyzing the endophytic archaea composition across the sites (Figure 6.6A). Similarly, diversity assessment using the Shannon index and evenness index calculated for the endophytic fungi do not differ significantly ($P > 0.05$) within the phylum, while significant difference ($P < 0.05$) was observed at the genus level (Table 6.1) across the sites. The Bray-Curtis dissimilarity based PCoA was similarly employed in analyzing the endophytic fungi composition across the sites (Figure 6.6). Furthermore, the PCoA plot for the endophytic archaea and fungi revealed that FK samples differ significantly from CK and NK (Figure 6.6A and 6.6B respectively). ANOSIM revealed a significant difference in the diversity of endophytic archaea and fungi from samples across the sites ($P=0.01$ and $R=0.67$).


1.0
PCO.2
NK
PCO.2 (35.9%)
PCO.1
FK
CK
-1.0
-1.0
PCO.I (64.1%)
A
1.0

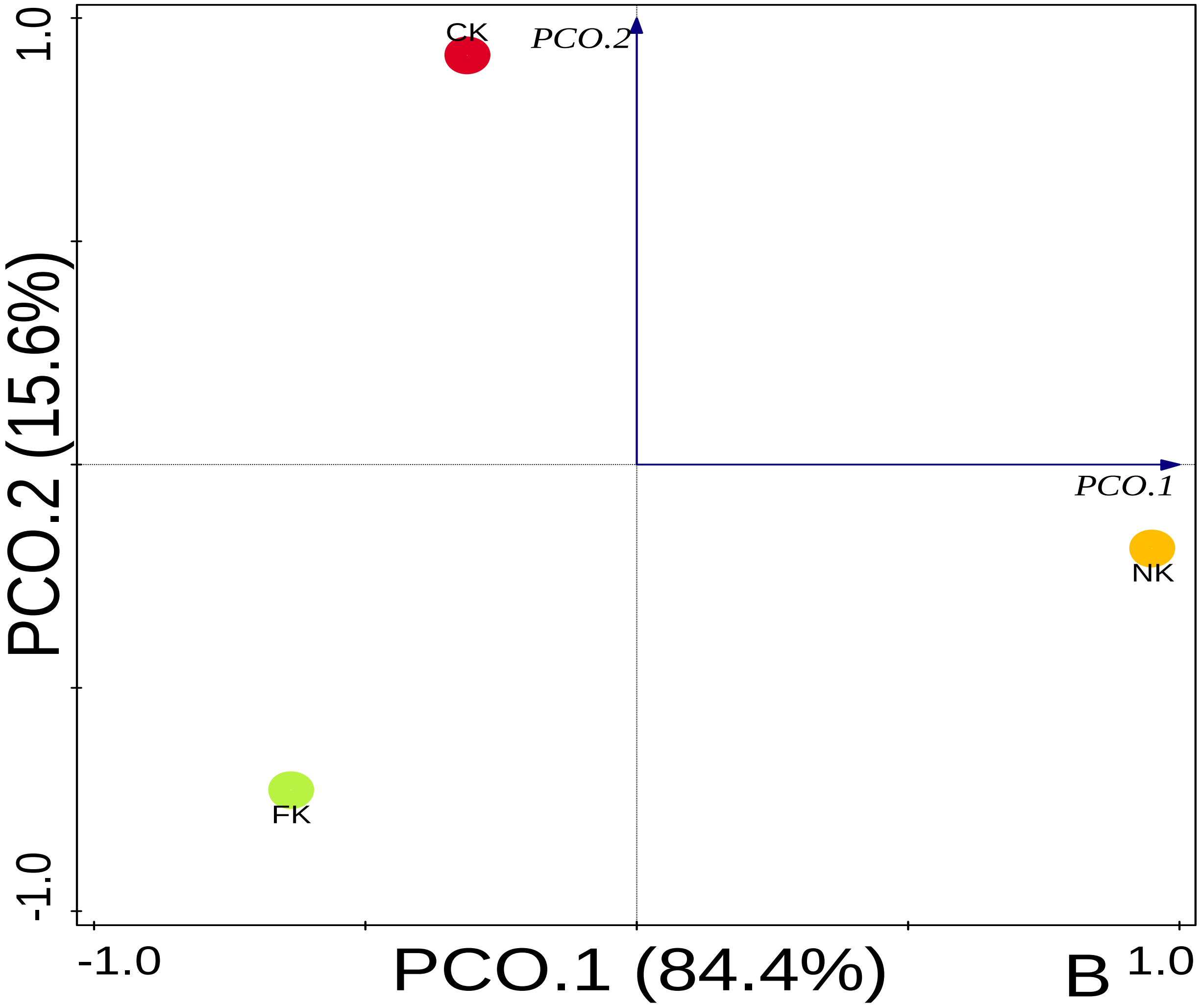


Figure 6.6: Principal coordinate analysis (PCoA) plot of (A) endophytic archaea and (B) endophytic fungi community compositions across the maize sites based on Bray-Curtis dissimilarities. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

Table 6.1: Diversity and evenness assessment of endophytic archaea and fungi across the sampling sites

Level	Indices	CK	FK	NK	P-value
		Endophytic Archaea			
Phylum	Shannon_H	0.50±0.02	0.80±0.16	0.26±.0.14	0.16
	Evenness_e^H/S	0.55±0.03	0.74±0.09	0.65±0.13	
Genus	Shannon_H	2.01±0.38	2.44±0.23	1.70±0.23	0.001
	Evenness_e^H/S	0.83±0.24	0.63±0.15	0.91±0.19	
		Endophytic Fungi			
Phylum	Shannon_H	0.56±0.02	0.67±0.32	0.49±0.01	0.16
	Evenness_e^H/S	0.88±0.04	0.98±0.03	0.78±0.15	
Genus	Shannon_H	1.62±0.09	1.69±0.33	0.45±0.21	0.003
	Evenness_e^H/S	0.36±0.06	0.90±0.16	0.10±0.09	

Mean ± standard deviation (n = 3). p-values obtained via the Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

6.4 Discussion

In the last two decades, organic farming has gain prominence all over the world because of low chemical input it required alongside their immense benefits to the environment, crop growth and health (Van Bruggen and Finckh, 2016, Reganold and Wachter, 2016, Abdelrahman et al., 2020). Consequently, these farming practices have a direct impact on the diversity, functions and abundance of soil microbial communities, and hence can be linked to crop yield and growth improvement, enhancement of crop to abiotic and biotic stress alongside improved soil health

(Kniss et al., 2016, Gamboa et al., 2020, Flores-Rentería et al., 2020). In this study, we assessed the impact of different farming practices on the diversity of endophytic archaea and fungi communities inhabiting the root of maize cultivated with different fertilizer regimes using shotgun metagenomics. The sequenced metagenome data obtained were analyzed using MG-RAST. The data were analyzed by identifying sequences that are for endophytic microbes while sequences of plant origin were abandoned.

Our results showed that the phyla *Crenarchaeota*, *Euryarchaeota Thaumarchaeota* were the major endophytic archaea phyla in maize plants across the sites and these phyla were most dominant in the FK site as compared to other sites. The identified phyla of endophytic archaea in this study agree with an earlier study on maize, alpine bog and coffee (Chelius and Triplett, 2001, Julian et al., 2018, Oliveira et al., 2013). Other studies revealed that *Thaumarchaeota* is a major component of soil microbiomes (Probst et al., 2013). *Euryarchaeota* groups such as *Thermoplasmata, Methanomicrobia*, and *Halobacteria* identified in this study have earlier been reported as endophytic archaea groups known to inhabit the tissues of the plant such as coffee and olive (Müller et al., 2015). At the genus level, *Haloferax*, *Candidatus Nitrososphaera*, *Methanobacterium* and *Thermoplasma* were identified to be dominant at the FK site. This also echoes previous findings on rice (Chelius and Triplett, 2001, Sun et al., 2008, Suman et al., 2016, Oliveira et al., 2013). However, to the best of our understanding, the following archaea genera; *Halomicrobium, Methanococcoides, Pyrococcus, Cenarchaeum, Nitrosopumilus, Methanosarcina*, *Methanosphaerula, Natrialba*, *Staphylothermus* and *Methanococcus* have not been reported as endophytes in maize plant before. This is an indication that numerous endophytic archaea have great prospects for plant growth, yield and health improvement under natural environments (Julian et al., 2018). Low abundance and diversity of endophytic archaea observed in this study agree with an earlier study by Finkel et al. (2011) in which a low abundance of endophytic archaea was reported in the tissues of plants. It is also important to state that endophytic archaea could help to protect the plant against pathogenic microorganisms and in maintaining plant health, as a result of their ammonia-oxidizing ability, in which they regulate the pH and local ammonia production especially in a natural environment (Müller et al., 2015). However, due to the fact that all archaea phyla were more abundant in the FK site, we thereby use endophytic archaea genus to plot our PCA graph, which revealed that each fertilizer site has different endophytic archaea and accounts for a combined 95.1% variation across the site (Figure 6.3). The abundance of endophytic archaea in the FK site can be linked to an earlier study in which endophytic archaea are reported to thrive well in natural environment (Suman et al., 2016). The position retained by each endophytic archaea class indicates the sequences linked to its makeup, while the vector arrows revealed the genus that is most affected by the distribution.

Furthermore, this study identified two major phyla of endophytic fungi namely *Ascomycota* and *Basidiomycota* across the sites with high abundance in samples from the FK sites compared to CK and NK sites. These major phyla have earlier been reported as dominant endophytic fungi phyla in most crops such as maize and rice (Suman et al., 2016, Sun et al., 2019b, Sun et al., 2019c). Our results further revealed that the organic fertilizer employed in this study positively influences the abundance and diversity of endophytic fungal diversity. Our results agree with an earlier study on soybeans, maize and grapevines in which diversity and abundance of endophytic fungi were higher in crops cultivated on organic site than in the inorganic site (Radić et al., 2014, da Costa Stuart et al., 2018, Kazemi et al., 2019).

Some of the endophytic fungi genera identified in this study such as *Malassezia, Filobasidiella, Ustilago, Tilletia, Metarhizium, Sordaria, Coprinopsis, Sclerotinia, Gibberella, Phaeosphaeria, Podospora Ajellomyces, Aspergillus, Schizosaccharomyces, Talaromyces*, *Phaeosphaeria* and *Leptosphaeria* have earlier been identified as beneficial endophytic fungi in similar studies carried out on sunflower, cotton and maize (Waqas et al., 2015, Potshangbam et al., 2017, Yuan et al., 2017). Similarly, *Schizosaccharomyces* have been previously identified as an endophytic fungus and implicated for industrial importance (Xia et al., 2019, Benito et al., 2016).

It is also important to state that the detection of *Malassezia* is strange, as they are notable human skin pathogens (Gupta et al., 2004). However, recent studies have revealed that some pathogenic organisms inhabit the tissues of the plant (Szilagyi-Zecchin et al., 2014, Sandhya et al., 2017). Beneficial endophytic fungi are known to enhance plant yield and growth through various mechanisms such as phytohormone production, an increment of nutrient uptakes such as iron, phosphate and nitrogen and activation of plant growth-promoting genes (Martínez-Medina et al., 2011, Xia et al., 2019, Fadiji and Babalola, 2020a, Durán et al., 2018). Due to the dominance of all the fungi phyla in FK sites, we used the endophytic fungi genus to plot our PCA graph, which revealed that each fertilizer site has different endophytic fungi and accounts for a combined 72.4% variance across the sites (Figure 6.5). The abundance of endophytic fungi in the FK site agrees with an earlier study in which endophytic fungi are reported to thrive well through organic farming (Xia et al., 2019, Yang et al., 2019). The position retained by each endophytic fungi class indicates the sequences linked to its makeup, while the vector arrows revealed the genus most affected by the distribution.

Shannon and Evenness index calculated at class level for endophytic archaea and fungi class showed no significant difference ($P > 0.05$) across the sampling sites. As expected, our results revealed a higher diversity of endophytic archaea and fungi at the genus level than the phylum level. This can be as a result of high richness and abundance of both endophytes at the genus level as compared to the phylum level. However, endophytic archaea and fungi were more diverse and evenly distributed in maize cultivated in FK site as compared with NK and CK sites (Table 6.1). This result, therefore, confirmed our hypothesis and earlier findings that organic farming has a positive influence on diversity and abundance of plant microbiomes such as endophytic archaea and fungi as observed in this study. The PCoA plot showed that the endophytic archaea and fungi community structures in organic fertilizer (FK) site were different from those of CK and NK sites (Figure 6.6A and 6.6B respectively). This result further revealed that endophytic archaea and fungi are more abundant in maize planted in FK site than other sites.

Previous studies have revealed that the abundance and diversity of soil and plant microbiomes is greatly influenced by different farming practices, one reason attributed to this is that difference in water use can influence the interaction of the soil environment across the farming types (Lori et al., 2017, Hartmann et al., 2015). For instance, the growth and survival of microorganisms in soil can be directly influenced by the water content in the soil, which significantly affect the access of microorganisms to essential nutrient which are critical for their survival (García-Orenes et al., 2013, Lienhard et al., 2013, Abdelrahman et al., 2020).

Fertilizer application has been reported as one of the major factors that affects soil microbial diversity (Leskovar and Othman, 2018, Abdelrahman et al., 2020). For instance, the quality and quantity of organic fertilizers have been reported to perform major roles in the diversity of soil microbes (Hartmann et al., 2015, Leskovar and Othman, 2018). Also, the diversity of microbial communities in plants can be more obvious when there is large application of pesticides, soil tillage, and also when crop rotation is not adequately practiced (Xia et al., 2019, Gamboa et al., 2020). Since most soil microbes often end up becoming endophytes (Wang et al., 2016), these farming practices might affect the endophytic microbial community as revealed in this study.

6.5 Conclusion

Taken as a whole, the present shotgun study revealed that maize cultivation using organic fertilizer (FK) positively influenced the diversity and abundance of endophytic archaea and fungi communities as compared to inorganic fertilizer (NK) and no fertilizer (CK) cultivations. The results will bring about a reduction in our dependence on chemical fertilizers. This study advocates for organic farming practice in the cultivation of maize plant. Furthermore, some novel endophytic fungi and archaea associated with maize roots were also identified in this study, future studies into how these novel endophytes can be cultured and experimented through pots and field trials are important. This will help in exploring their functions in sustainable agriculture as well as identifying their biotechnological importance. Although the functions of endophytic archaea in the plant are not fully understood, this study advocate for an in-depth understanding of their functions and mode of actions. On the contrary, though the roles of endophytic fungi in plant health and growth improvement have been reported, further studies on the mechanisms involved in carrying out these beneficial functions are advocated, this is important to enhance their application in sustainable agriculture. This study also suspects a harmonious interaction between endophytic microbial communities inside the tissue of the plant. We call for further studies into the mechanisms behind their synergistic association in the tissues of the plant and how these interactions can sustain agroecosystems.

CHAPTER SEVEN

7.0 SHOTGUN METAGENOMICS REVEALS THE FUNCTIONAL DIVERSITY OF ROOT-ASSOCIATED ENDOPHYTIC MICROBIOMES IN MAIZE PLANT

Abstract

In this study, we used shotgun metagenomics to analyze the whole DNA from maize root planted with different fertilization and without fertilization in a bid to profile the impact of fertilizer applications on the functional diversity of endophytic microbiomes. Complete DNA extraction from roots of maize plant grown on different farming sites such as organic (FK), inorganic (NK) and no fertilizer (CK) sites was carried out, and sequenced using a shotgun metagenomic approach. The raw sequenced data obtained were analyzed using an online database called MG-RAST. Through MG-RAST analysis, endophytic microbiome sequences were identified while sequences of maize origin were discarded. The prediction of the functions of the endophytic microbiomes was done using the SEED subsystem. Our results revealed that no significant difference ($P > 0.05$) exist in the relative abundance of the 28 functional groups identified within the endophytic microbiomes across the sites. Also, some functional groups and metabolic pathways associated with plant growth promotion such as carbohydrate, secondary metabolism, nitrogen metabolism, iron acquisition and metabolism alongside phosphorus metabolism were observed in the endophytes across the sites. Alpha diversity study revealed no significant difference exist among the functional groups of the endophytes across the sites, while beta diversity study indicated that there was a significant difference ($P=0.01$) among the functional groups of the endophytes across the fertilizer sites. Going by the high abundance of functional groups observed in this study, especially in FK samples, it is evident that different farming practices influenced the functions of endophytic microbiomes. We recommend that further studies should explore the functional genes in endophytic microbiomes with the aim of assessing their usefulness in promoting sustainable agriculture.

Keywords: Endophytes, Metagenomes, SEED subsystems, *Zea mays*

This chapter has been published in *Current Plant Biology* 2021; 25:100195. https://doi.org/10.1016/j.cpb.2021.100195

7.1 Introduction

Maize is the staple food for the largest number of people in the world, particularly in South, West, East and Northern part of Africa (Alori et al., 2019). However, in South Africa, about 8 million tons of maize grain are produced annually on almost 3.1 million ha of land. Half of this production is white maize, which is majorly consumed by humans (Du-Plessis, 2003). The continuous increase in maize consumption demands increased yields and improved management practices (Sessitsch et al., 2012). Maize roots have been observed to passively secret natural compounds such as sugars, nucleosides, amino acid, mucilage, and organic acids, which help to entice microbes from the bulk soil to its rhizospheric environment and subsequently endosphere for plant growth promotion (Edwards et al., 2015). Notable among the organisms that are attracted to the endosphere is the endophytes.

Endophytic bacteria and fungi have been reported to be beneficial to plant growth enhancement via several mechanisms among which are fixing of nitrogen, production of ammonia, siderophore and phytohormones (Gomes et al., 2018, Khan et al., 2014, Yadav, 2018, Fadiji and Babalola, 2020a). Endophytes are organisms that inhabit the tissues of plants without causing harm to the host (Sessitsch et al., 2019, Fadiji and Babalola, 2020c). Studies have shown that endophytes perform notable roles in plant growth and health improvement (Li et al., 2016, Gupta et al., 2016, Kumar et al., 2017, Tumangger et al., 2018, Omomowo and Babalola, 2019). For about a decade, most researchers have concentrated on endophytic microbes from medicinal plants because these organisms are believed to possess a huge capacity to secrete many important secondary metabolites including antibiotics, antituberculosis, antimalarial, antiviral, antifungal insecticidal, anticancer, antifungal, volatile organic compounds, antiviral, immunosuppressant and plant growth improvement (Golinska et al., 2015, Ateba et al., 2018, Khunjamayum et al., 2017, Rajamanikyam et al., 2017, Fadiji and Babalola, 2020a). However, recent metagenomics studies have focused on the diversity and community structure of endophytes in maize, rice and other plants (Sessitsch et al., 2012, Correa-Galeote et al., 2016, Correa-Galeote et al., 2018, Hong et al., 2019) while limited studies exist on the functions of these endophytes (Sessitsch et al., 2012, Hong et al., 2019). Considering all these functions and benefits, endophytic microbiomes are still underexplored, because they have the prospect of replacing our dependence on chemical fertilizers through their potentials as a biofertilizer and enhancement of better agricultural practices.

In order to have an in-depth understanding of other contribution and function of these microbes, it is important to unravel their adaptations and beneficial characteristics. However, assessing the function of endophytes is faced with many challenges especially in culturing the microbes, because most of these microbes inhabit the tissue of their host and most times they are not responsive to genetic or biochemical analyses (Sessitsch et al., 2012). Endophytic cells inside plant tissues firmly stick to the host cells, and are often difficult to separate and isolate from plant cells alongside at the risk of being contaminated with epiphytes. However, the advent of next-generation sequencing technology has now simplified the process, in which, endophytic genomes can be extracted from the total metagenome dataset of a plant without the fear of being contaminated by plant genome (Hong et al., 2019).

Understanding the functions, host-microbe interactions, adaptations and purported beneficial traits, strongly depend on culturable endophytic microbes from maize (Menéndez et al., 2016, Sandhya et al., 2017) and other plants (Compant et al., 2010). Cellular capacities and functions of uncultured microbial communities can be unraveled using metagenomic techniques (Dinsdale et al., 2008). Interestingly, cluster analysis and evaluation of plant microbial metabolism and interactions have been previously attributed to the uncultivable microorganisms (Hong et al., 2019). However, studies in this regard concentrated on rhizospheric microbes, while studies on endophytes are restricted to tomato (Tian et al., 2015), rice (Sengupta et al., 2017), peony (Yang et al., 2017), grape (Campisano et al., 2014), aloe (Akinsanya et al., 2015b), maize (Correa-Galeote et al., 2018), *Panax* (Hong et al., 2019) among others, with limited information about the functional roles of the identified endophytic microbes. At present, to the best of our understanding, no report exists on the functional diversity of endophytic microbiomes from maize plant cultivated with different fertilizers using shotgun metagenomics. Shotgun metagenomics is preferred recently over other techniques because it enables the functional profiling of microbial communities inhabiting an environment (De Tender, 2017). In shotgun metagenomic sequencing, all DNA samples pulled out from a particular environment will be analyzed, instead of focusing on a distinct genomic locus. This novel globally recognized technique is dependent on 2 major steps. Firstly, the splitting of the DNA molecules into some tiny gene fragments, followed by independent sequencing. The second step involves the reassembling of gene fragments (Bouchez et al., 2016, Fadiji and Babalola, 2020c)**.** This study hypothesized that the functional diversity of endophytic microbiomes will increase in maize plant in cultivated with organic fertilizer sites as compared to those cultivated in inorganic site.

In this study, we investigate, for the first time, the functional diversity of endophytic microbial communities in the root of maize plant using shotgun metagenomic approach**.**

7.2 Materials and Methods

7.2.1 Seed collection

The WEMA (WE 3127) maize seed used in this experiment was collected from North-West University School Farm Molewane, Mafikeng, North West Province, South Africa.

7.2.2 Experimental design and site description.

The long-existing (15 years) organic and inorganic experimental fields located in North-West University School farm Molelwane, Mafikeng, North West Province (S25°47'25.24056", E25°37'8.17464"), South Africa, was used for the study. North West Province of South African borders Botswana. This province is characterized by shrubs and trees. The mean temperatures experienced in the province ranges from 3- 21°C in winter and 17- 31°C in summer. The rainfall of the province is estimated at 360 mm per annum, having severe rains experienced between October and April. This major plant cultivated in this experiment site had been the rotation of sorghum, maize, and soybean for a long time, with soybean planted in 2018. In this study, the experimental field was divided in to three different sites. The soil samples were analyzed for pH and other soil chemical parameters. The soil samples from the experimental sites had similar chemical and physical properties (22% Sand, 66% Silt, 12% clay, pH 6; 0.48% organic C, 0.15% total N, 101.5 ppm P, 0.962ppm K) (Supplementary Table S1).

Two fertilization regimes were used in this study, the organic fertilizer site (FK) and inorganic fertilizer site (NK) which has been in operation for over 15 years following standard methods as described by the U S Department of Agriculture (Xia et al., 2019), while the third site is the no fertilizer site (CK) (Table 7.1). The planting was carried out during October- December 2019.

Irrigation was provided across the sites in required volumes to prevent drought stress. The weeding process was handled manually.

Table 7.1: General information on the selected farming sites

Information	**Organic fertilizer site (FK)**	**Inorganic fertilizer site (NK)**	**No fertilizer site (CK)**
Years of existence	over 15 years	over 15 years	over 15 years
Type of fertilizer continually used	Cattle manure	NPK Urea is used as fertilizer N, Potassium sulfate taken as fertilizer K, and calcium superphosphate as P fertilizer	No fertilizer application
Constant fertilizer dosage over the years	10,625 kg ha^{-1}	150 N, 75 P_2O_5 and 75 K_2O all in kg ha^{-1}	Nil
Maize cultivar planted	WE 3127	WE 3127	WE 3127
Dimension adopted for the study on each site	10 m x 4 m	10 m x 4 m	10 m x 4 m

7.2.3 Root sampling

Each farming site was divided into three different regions representing three (3) replicates for root sampling purpose. Each replicate sample for sequencing came from the roots of 10 randomly selected fresh plants in each region of the sites which were pooled (Figure 7.1). The plants were collected at fruiting stage of the plant growth (Xia et al., 2015). In total, 90 plant samples were evaluated; the three regions represent three replicates for each sampling site. The plant samples were kept with ice and transported to the laboratory the same day, where they were processed immediately.

Figure 7.1: Representative samples of maize roots used in the study.

7.2.4 Surface washing of maize roots

Surface washing was carried out on the fresh maize roots using the method described by Liu et al. (2017b) and Correa-Galeote et al. (2018). To sure that the process of sterilization was perfectly carried out and epiphytes removed, small parts of the sterilized roots were cut and plated on yeast extract-mannitol medium using a Petri dish (Vincent, 1970). After 72 h incubation at 30°C the Petri dishes were checked bacterial growth. Maize roots from Petri dishes without contamination were chosen for DNA extraction (Correa-Galeote et al., 2018, Marag et al., 2018).

7.2.5 Extraction of DNA and shotgun sequencing

The roots were cut into 1 cm using a sterile scalpel and instantly macerated using a Qiagen TissueLyser. Total metagenome DNA was immediately extracted from the root tissue samples using the Qiagen DNeasy Plant Mini Kit. Shotgun metagenomic sequencing was done at the Molecular Research LP, Texas, USA. The preparation of library was carried out with Nextera DNA Flex kit (Illumina) following standard procedure. The actual DNA concentration in all the samples was evaluated making use of the Life Technologies Qubit® dsDNA HS Assay Kit. The library preparation was carried out using 50 ng of the DNA. The samples passed through fragmentation and adapter sequences were added. These adapters were then used for limited-cycle PCR with specific indices being added to the samples. After the library has been prepared, the final concentration was measured using the Qubit® dsDNA HS Assay Kit, and the Agilent 2100 Bioanalyzer was to ascertain the size of the library. The library size varies from 683 to 877 bp with an average of 731 bp pooling of libraries were done using 0.6nM ratios, and the paired-end sequencing was done with 300 cycles via the Illumina NovaSeq 6000 system.

7.2.6 Data analysis

The obtained sequences of each metagenome were transferred to an online server called MG-RAST (Hong et al., 2019). Inside this online server, quality control of the raw data was carried out. This include, removal of the adapter and low reads sequences from the sequenced data using the Trimmomatic v 0.33 program (Bolger et al., 2014) for the quality trimming of the sequenced data. The quality control process also includes the removal of artificial sequences, filtering of ambiguous bases, specification of minimum read size, and length filtering. After quality control analysis, annotation of the processed sequences was carried out using BLAT (Kent, 2002), against M5NR database (Wilke et al., 2012), which allows nonredundant integration of several databases. Also, profiling of the functional categories of endophytic microbiomes was performed using the SEED Subsystem level 1, 2 and 3 databases with specified parameters such as a 10^{-5} e-value cut-off and minimum 60% sequence similarity to a subsystem. No further analysis was carried on the sequences that could not be annotated. However, since our concentration is on endophytic microbiomes, which accounts for a large percentage of the whole sequences, we, therefore, discard maize plant sequences. To suppress the influence of experimental error/noise, data normalization option was selected on the MG-RAST. The functional table obtained was aggregated to functional level and uncategorized sequence reads were kept for statistical analysis. Furthermore, the relative abundance of the functional categories was calculated in percentages, after the independent analysis of the 9 sequences using MG-RAST. For the statistical analysis, the average figure of the obtained relative abundance of the 3 replicates for each sampling sites (CK, FK and NK) were used. These standard sequences can be found on NCBI SRA dataset with the accession number PRJNA607664.

7.2.7 Statistical analyses

Shinyheatmap via z-score was used for the plotting of heatmaps using the relative abundance of the functional groups. The abundance and distribution of the major functional group at subsystem level 2 were visualized using a column bar graph via Microsoft Excel software. Shannon and Pielou indices for diversity assessment were employed for samples across the fertilizer sites and Kruskal–Wallis test was used to compare these indices. The analyses were performed via PAST version 3.20 (Chauhan et al., 2019). The Euclidean based principal coordinate analysis (PCoA) and ANOSIM through 999 permutations were used for the β diversity study and for the assessment of functional differences in the samples across the fertilizer sites respectively (Carrell and Frank, 2015). A Euclidean based PCA was used to assess the distribution of the different functional categories from samples across the fertilizer sites. CANOCO version 5.0 was used for the plotting of both PCoA and PCA graphs.

7.3 Results

7.3.1 Metagenome sequencing, quality control and protein annotation

A total of 56087796311 sequenced reads were recorded for the three (3) sampling sites, with individual sequence reads of samples as CK (4839895527), FK (2977205570) and NK (48270695214) respectively. After quality control analyses were carried out in MG-RAST, the sequenced reads for CK was 334259767 having a mean G+C content of 44%, FK had 415505341 having a mean G+C content of 44% and while NK had 817699487 with a mean G+C content of 49% (Supplementary Table S2). Among the sequences that passed the quality control check, sequences that mapped for identified proteins in the samples were 325439 (CK), 371329 (FK) and 643141 (NK), respectively (Supplementary Table S2).

7.3.2 Functional analysis of endophytic microbiomes associated with maize plant

The results obtained at SEED subsystem level 1 showed 28 key functional categories attributed to the endophytic microbiomes from all the sites. The functional categories such as carbohydrates (C), cell division and cell cycle (CDCC), cell wall and capsule (CWC), clustering-based subsystems (CBS), cofactors, vitamins, prosthetic groups and pigments (CVPGP), DNA metabolism (DNAM), dormancy and sporulation (DS), fatty acids, lipids, and isoprenoids (FLI), iron acquisition and metabolism (IAM), membrane transport (MT), metabolism of aromatic compounds (MAC), and miscellaneous (Mis), motility and chemotaxis (MC), nitrogen metabolism (NM), phages, prophages, transposable elements, and plasmids (PPTP), photosynthesis (P), potassium metabolism (PoM), regulation and cell signaling (RCS), secondary metabolism (SecM), stress response (SR), and virulence, disease and defense (VDD) dominated samples from the organic fertilizer site (FK) (Figure 7.2). However, functions associated with nucleosides and nucleotides (NN), protein metabolism (ProM), RNA metabolism (RNAM), and respiration (R) predominated samples from the no fertilizer site (CK). While, amino acids and derivatives (AAD), iron acquisition and metabolism (IAM), motility and chemotaxis (MC), phosphorus metabolism (PM), and sulfur metabolism (SulM) were abundant in samples from inorganic fertilizer site (NK). The differences observed in all the functions identified did not vary significantly (P>0.05) across the sites (Supplementary Table S7). PCA was used to assess how the distribution of the identified functional categories between the CK, FK and NK sites (Figure 7.3), and this revealed that eighteen (18) major functional categories dominated samples from the inorganic fertilizer sites (FK), six (6) functional categories dominated the NK site while four (4) dominated samples from the CK site.

The functions unknown predominated the SEED Subsystem level 2 hierarchy for annotation of the gene across all the samples. The relative abundances for unknown protein in the samples were the most dominant with 17.149% (CK), 21.65% (FK) and 25.55% (NK) samples (Figure 7.4).

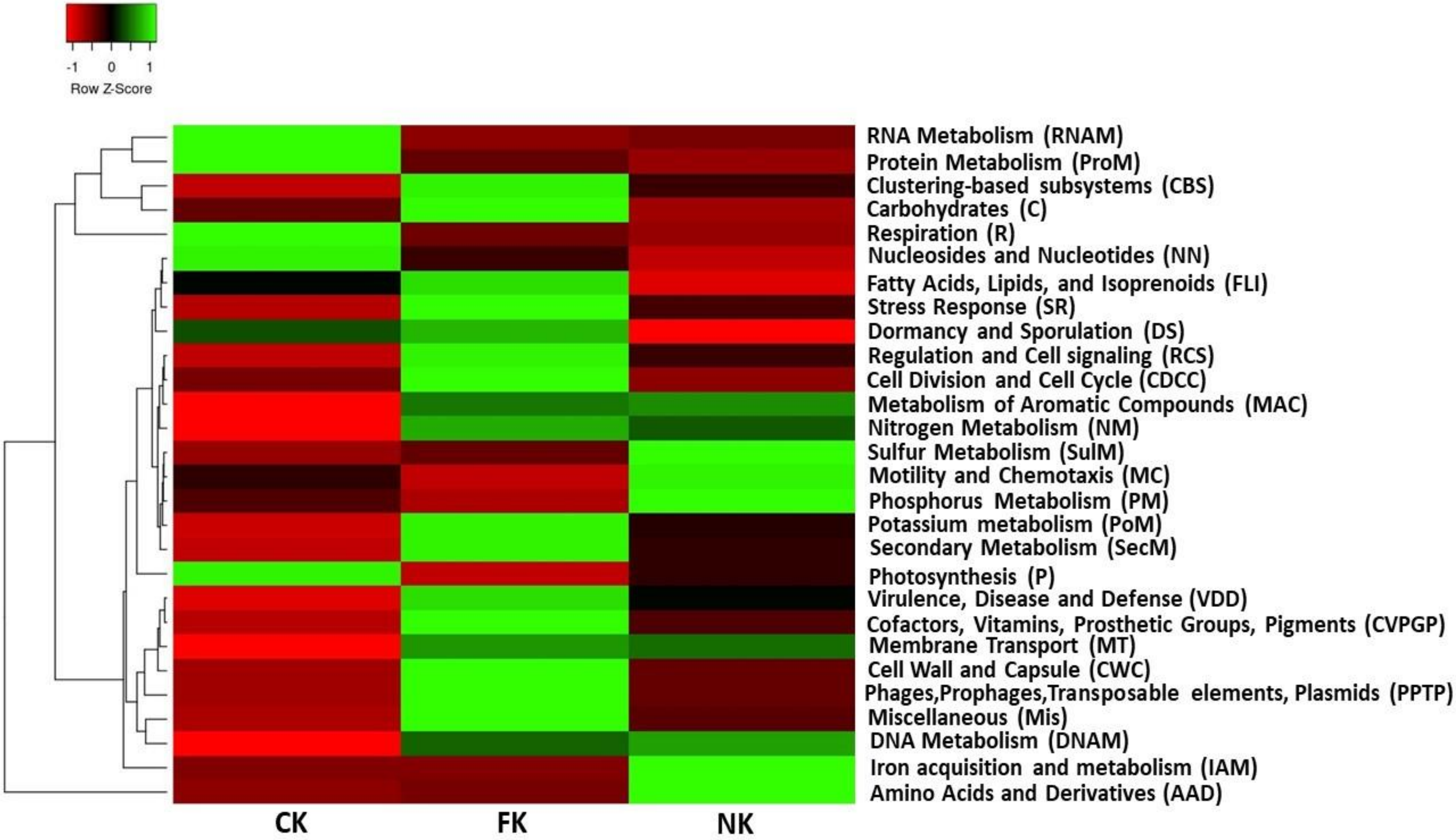


Figure 7.2: Sequences similar to key metabolisms in samples from the maize plant in different sites. Relative abundance is indicated with the different colors as represented with the scale bar with z-score. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

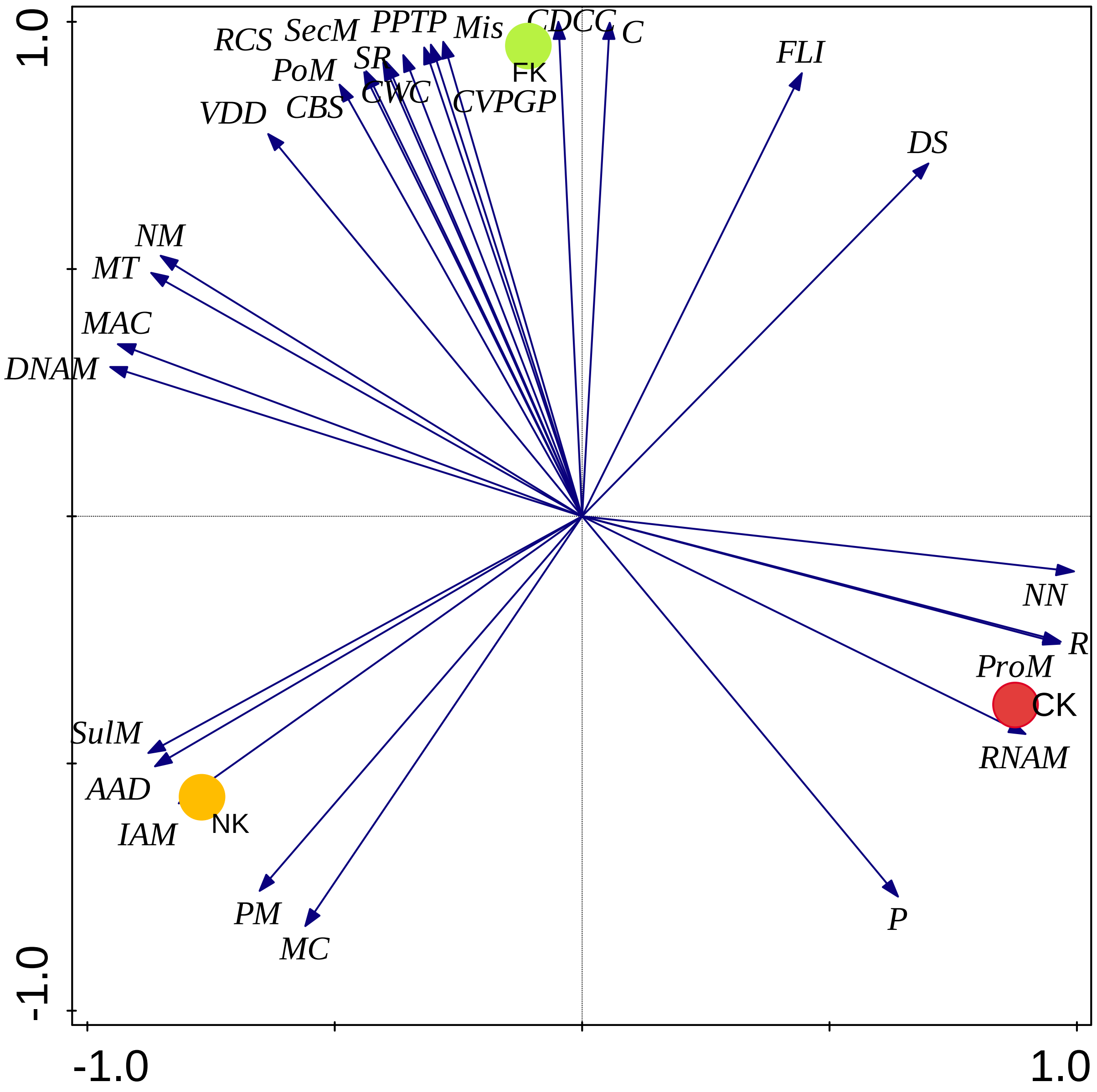


Figure 7.3: PCA graph showing the functional analysis of endophytic microbiomes. The vector represents the impact of each metabolic process. Axis 1 (60.3%) and Axis 2(39.7%) explained the variations based on Euclidean dissimilarities**.** NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

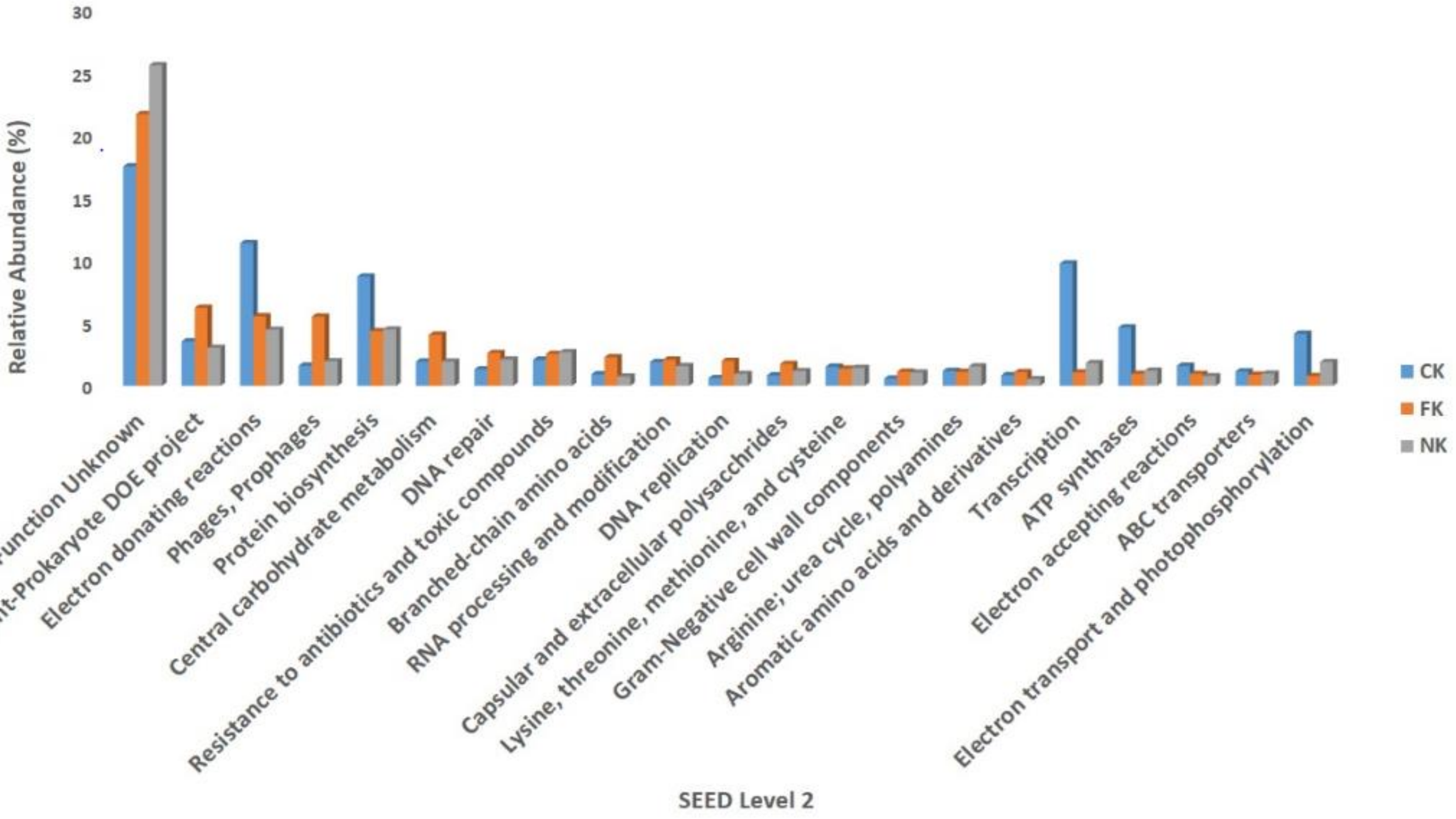


Figure 7.4: Functional groups obtained at level 2 of the SEED subsystems. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

7.3.3 Alpha and Beta diversity assessment of the functional groups across the sampling sites

The diversity of the functional groups at level 1 of the SEED subsystem was evaluated using the evenness index and Shannon index, and they were observed not to differ significantly ($P > 0.05$) (Table 7.2). Using the Kruskal–Wallis test, the extent of the differences in diversity between all samples from each site were assessed and no significant difference was observed ($P=0.77$). The PCoA plot revealed a clear difference in the abundance of the 28 functional categories identified at SEED Subsystems level 1 in FK as compared to CK and NK (Figure 7.5). Similarity test using one-way ANOSIM revealed a significant difference in the 28 functional categories from samples across the sites ($P=0.01$ and $R=0.67$).

Table 7.2: Evenness and diversity examination of the functional categories endophytic microbiomes at level 1 of the SEED subsystem from each site

Indices	**CK**	**FK**	**NK**	**P-value**
Shannon_H	2.73±0.17	2.94±0.16	2.82±0.16	0.77
Evenness_e^H/S	0.55±0.08	0.68±0.09	0.60±0.09	

Mean ± standard error (n= 3). p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

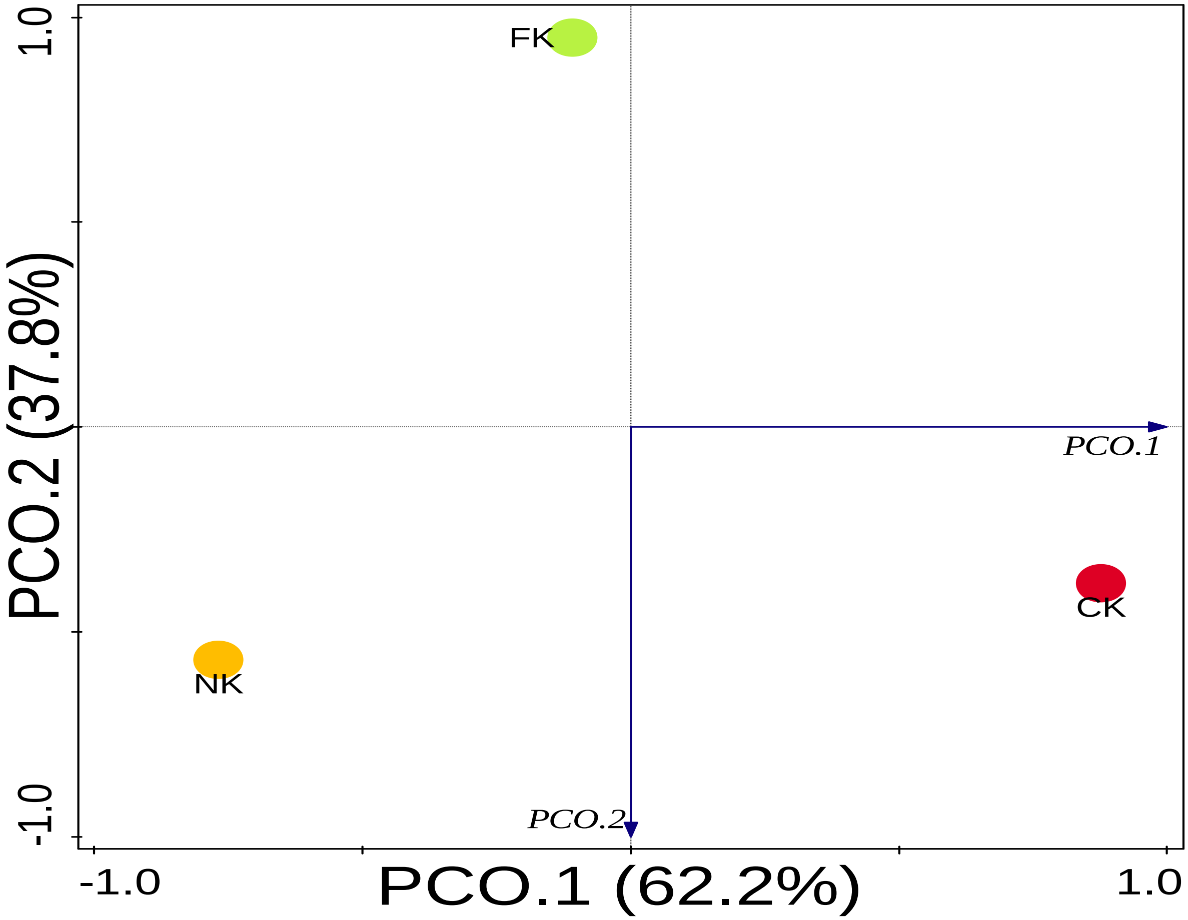


Figure 7.5: PCoA graph for the functional groups identified at the SEED subsystem level for all the endophytic microbiomes from each site based on Euclidean dissimilarities. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

7.4 Discussion

In this study, we assessed the effect different farming practice on the functional diversity of endophytic microbiomes in root of maize plant. To actualize this the whole DNA from maize root planted with different fertilization and without fertilization were extracted and sequenced using shotgun metagenomics. The raw sequenced data obtained were analyzed using an online database called MG-RAST. Using MG-RAST analysis, endophytic microbiome sequences were identified while sequences attributed to maize plant were discarded. The major endophytic microbiome phyla identified in this study are basically of bacterial, fungi and archaea origin. The SEED subsystem analysis was then used to predict the functions of identified endophytic microbiomes using different fertilizer applications.

The SEED is a categorization system that assembles functional gene groups into a hierarchy with 5 levels of subsystems. Level 1 which is the highest level of the subsystems includes different metabolisms such as catabolism and anabolism, while the lower levels show specific pathways or genes involved in the metabolisms (Overbeek et al., 2014). In this study, though the abundance of microbiomes varies across the different fertilizer sites, the functional difference identified at subsystems level 1 across the sites do not differ significantly ($P > 0.05$) (Table S3). Eighteen (18) major functional categories dominated samples from the organic fertilizer sites (FK), six (6) functional categories dominated the inorganic (NK) site while four (4) dominated samples from the on fertilizer (CK) site. This agrees with the claim that bacteria are grouped based on functional relatedness rather than taxonomic relatedness (Burke et al., 2011), the study suggests that the key level at which to address the assembly and structure of bacterial communities may not be “species” but rather the more functional level of genes. Other studies also revealed the functional roles of endophytes do not rely on their taxonomic classification but depend strongly on the environmental factors and host types (Hardoim et al., 2015, Hong et al., 2019).

Our results from the alpha diversity analysis showed the functions exhibited by the metagenomes in all the sites approached the theoretical limit of 2.81, indicating that virtually all the subsystems are present in the samples from all the sites (Dinsdale et al., 2008). In addition, low evenness value was observed (approximately 0.61, Table 7.2), indicating that there are few dominant metabolic processes (Such as protein metabolism, clustering-based subsystems and respiration) in each site. Through shotgun metagenomics, we were able to show the different predominant metabolisms and distinct characteristics of the identified functional categories in the endophytic metagenomes. Our results further revealed that all the functional groups attributed to the endophytes from all the sampling sites did not differ significantly (P >0.05) (Table 7.1). PCoA plot showed distinct separations (R=0.67) between all the fertilizer sites (Figure 7.5). This was further checked with ANOSIM which revealed that there was a significant difference between the functional groups of the endophytes across the fertilizer sites (P-values=0.01).

Furthermore, PCA was used to test the hypothesis that different farming practices have a major impact on the metabolic pathways of endophytes (Figure 7.3). The variance between the different sites obtained in this study is an indication that major functions are predicted by endophytic metagenomes. The position occupied by each metagenome in the PCA graph revealed the makeup of sequences linked to each subsystem, with the vectors showing that metabolism has considerable influence on the distribution. Going by this results, it is easier to predict which metabolism is important to the endophytes identified in each fertilizer site. For instance, amino acids and derivatives (AAD), iron acquisition and metabolism (IAM), motility and chemotaxis (MC), phosphorus metabolism (PM), and sulfur metabolism (SulM) were abundant and specific in endophytes from inorganic fertilizers sites (NK) as compared with endophytes found within FK and NK sites.

Our results also revealed that each fertilizer site has some predominant categories of functional gene attributed to them. Dominant sequences associated with fatty acid, lipids and isoprenoids metabolism, carbohydrate metabolism, stress response, phage, prophage, nitrogen metabolism and secondary metabolism were observed in samples from the organic fertilizers sites (FK) (Supplementary Table S7, Figure 7.2). This is expected because organic fertilizers sites (FK) are known to have higher organic matter and the plant cultivated in such site is expected to have a higher level of carbon (Sharma and Chetani, 2017). Endophytes will successfully thrive well in such an environment because they depend solely on the plant as their energy source. Therefore, higher fatty acid, lipids and isoprenoids metabolism, and carbohydrate from organic fertilizer samples as obtained in this study are in agreement with the earlier reports by Sharma and Chetani (2017) and Lin et al. (2019) where high level of carbon are reported as part of the major characteristics of organic farming sites.

This was further buttressed at lower levels with the abundance of sequences linked with major metabolic pathways involved in the carbon cycle, such as glycolysis, gluconeogenesis and TCA cycle, being dominant in FK samples (Supplementary Figure S2A). Higher stress response, phage, and prophage observed in the organic fertilizer sites were much as expected, these attributes can help in plant protection against many environmental factors. This agrees with earlier studies in which agricultural practices with organic fertilizer increases soil microbial activities and enhances plant resistance to disease and pest attack (Chang et al., 2010, Zhang et al., 2012). Similarly, sequences related to nitrogen metabolism were abundant in endophytes from FK site. This is expected because studies have shown that organic fertilizers produce nitrogen in usable form, which helps in plant growth promotion without causing root burn in plants or destroying beneficial microorganisms inhabiting the soil (Sharma and Chetani, 2017). At a lower level, sequences associated with metabolic processes involved in the nitrogen cycle like allantoin utilization, ammonia assimilation, nitrogen fixation, denitrification, nitrate and nitrite ammonification, alongside nitrilase (Supplementary Figure S2B) were dominant in FK samples. This agrees with an earlier study in which high nitrate and ammonium were observed in tomato cultivated with organic fertilizer (Grunert et al., 2019). In addition, sequences associated with secondary metabolism were dominant in FK samples. At a lower level, auxin associated trait such as auxin biosynthesis (Supplementary Figure S2E) was identified, which have been reported in plant growth promotion (Hong et al., 2019).

Although sequences linked with phosphorus metabolism, iron acquisition and metabolism, sulfur metabolism and motility and chemotaxis were dominant in inorganic fertilizer sites (NK), they do not differ significantly ($P< 0.05$) across the sites. High application of inorganic fertilizers can be linked to high phosphorus and sulfur metabolism observed in the NK site, though inorganic fertilizers have been reported to have some side effects on microorganisms in the environment (Sharma and Chetani, 2017, Savci, 2012). This was further confirmed at lower levels with notable metabolic processes such as thioredoxin disulfide reductase, alkanesulfonate assimilation, sulfatide metabolism, inorganic sulfur assimilation, and galactosylceramide (Supplementary Figure S2C). Equally, some key metabolic pathways involved in phosphate metabolism observed at the lower level are alkylphosphonate utilization, phosphorus uptake and phosphate binding DING proteins (Supplementary Figure S2D).

Moreover, sequences associated with iron acquisition and metabolism were dominant in NK samples. At the lower level, this was further confirmed with notable metabolic pathways (such as bacillibactin siderophore, iron siderophore sensor and receptor system, siderophore pyochelin, siderophore achromobactin, siderophore enterobactin, siderophore assembly kit, siderophore pyoverdine, siderophore yersiniabactin biosynthesis, siderophore [Alcaligin-like], and siderophore staphylobactin (Supplementary Figure S2E). Iron is important in the secretion of siderophore which are believed to abundant in inorganic fertilizer sites (Shenker and Chen, 2005), as observed in this study. This agrees with a study by Rajkumar et al. (2009) where high iron for the roots of plants increased the production of siderophores by phytosiderophore-iron complex. Siderophore linked traits have been reported in plant growth promotion, this suggests that the association between endophytes and maize plants can enhance the growth of the root (Hong et al., 2019, Marag et al., 2018). Also, siderophore biosynthesis has been reported in inducing systemic resistance of the plant to pathogens (Dube et al., 2019).

Sequences associated with motility and chemotaxis were also observed in NK samples. Motility and chemotaxis can aid the performance of endophytes; in that, it will enhance movement, networking and regulation of nutrient acquisition within the host (Enagbonma et al., 2019, Hardoim et al., 2015). Equally, clustering-based subsystems were observed to be predominant in all the samples, especially samples from FK sites. Clustering-based subsystems have been reported to harbor several functional genes whose functions are unknown (Castañeda and Barbosa, 2017). In this study, they were the second most abundant functional category in all the samples (Figure 7.2, Supplementary Table S7). This high distribution of clustering-based subsystems coupled with unknown function at level 2 of the subsystem (Figure 7.4) showed that many notable endophytic genes are present in endophytes whose functions are not yet explored.

7.5 Conclusion

We carried out the first functional diversity study of endophytic microbiomes in maize plant using shotgun metagenomics. Our study has shown that the functional diversity of endophytic microbiome in maize plant is influenced by different farming practices. To a greater extent, major functional categories were most abundant in endophytic microbiome from organic fertilizer sites (FK). Alpha diversity study revealed no significant differences exist among functional groups of the endophytes across the sites, while beta diversity study indicated that there was a significant difference among the functional groups of the endophytes across the fertilizer sites. Our study presents a high abundance of functional groups whose functions are unknown, indicating the prospect of identifying peculiar genes from the endophytic microbiomes. Therefore, we advocate for further studies that will explore the functional genes in endophytic microbiomes. Also, this study indicates that traits associated with plant growth promotion were highly represented in endophytes from plants cultivated with organic fertilizer. Our findings suggest a basis for the improvement of maize cultivation by exploring the beneficial properties of endophytes, this study advocate for the use of organic fertilizers in maize cultivation and in promoting sustainable agriculture.

CHAPTER EIGHT

8.0 UNVEILING THE PUTATIVE FUNCTIONAL GENES PRESENT IN ROOT-ASSOCIATED ENDOPHYTIC MICROBIOME FROM MAIZE PLANT USING THE SHOTGUN APPROACH

Abstract

To ensure food security for the ever-increasing world population, it is important to explore alternatives for enhancing plant productivity. This study is aimed at identifying the putative plant growth-promoting (PGP) and endophytic gene clusters in root-associated endophytic microbes from maize root and also to verify if their abundance is affected by different farming practices. To achieve this, we characterize endophytic microbiome genes involved in PGP and endophytic lifestyle inside maize root using the shotgun metagenomic approach. Our results revealed the presence of genes involved in PGP activities such as nitrogen fixation, HCN biosynthesis, siderophore, 4-hydroxybenzoate, ACC deaminase, phenazine, phosphate solubilization, butanediol, methanol utilization, acetoin, nitrogen metabolism, and IAA biosynthesis. We also identify genes involved in stress resistance such as glutathione, catalase and peroxidase. Our results further revealed the presence of putative genes involved in endophytic behaviors such as aerotaxis, regulator proteins, motility mechanisms, flagellum biosynthesis, nitrogen regulation, regulation of carbon storage, formation of biofilm, reduction of nitric oxide, regulation of beta-lactamase resistance, type III secretion, type IV conjugal DNA, type I pilus assembly, phosphotransferase system (PTS), and ATP-binding cassette (ABC). Our study suggests a high possibility in the utilization of endophytic microbial community for plant growth promotion, biocontrol activities, and stress mitigation. Further studies in ascertaining this claim through culturing of the beneficial isolates as well as pot and field experiments are necessary.

Keywords: Endophytic genes, Metagenome, PGP genes, Shotgun, *Zea mays*

This chapter has been published in *Journal of Applied Genetics* 2021; 62:339–351. https://doi.org/10.1007/s13353-021-00611-w

8.1 Introduction

Maize (*Zea mays* L.) is a major staple diet of the native peasants of most African countries. Maize being a household food crop can be grown in almost all soil types (Liu et al., 2017b) most times with a different degree of yield. More than fifty species are cultivated depending on the region; the species vary in taste, texture, size and shape. Plants form beneficial association with diverse microorganisms that provide them with specific benefits (Khare et al., 2018, Toju et al., 2018). Most common among these beneficial microorganisms are the endophytes. Endophytes are microorganisms that colonize the interior tissues of plants asymptomatically (Omomowo and Babalola, 2019, Fadiji and Babalola, 2020c), but recent studies have shown that some of them can lead to disease development in their host plant (Van Overbeek et al., 2014, Brader et al., 2017b). Similar studies have also revealed that plant microbiome could have considerable side effects on the health of humans when plants are consumed raw (Blaser et al., 2013, Van Overbeek et al., 2014, Liu et al., 2017b).

Some endophytes enhance plant growth, help in disease suppression, and boost stress tolerance (Syranidou et al., 2017, Sun et al., 2019a, Zolti et al., 2020). Endophytes secrete a notable plant hormone, indole-3-acetic acid (IAA), which promotes plant growth and development (Mefteh et al., 2019, Fadiji and Babalola, 2020a). Endophytes also aid phosphate solubilization and nitrogen uptake, which are considered as the most important elements for the growth and development of plants (Slama et al., 2019). Similarly, they produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which is notable for ethylene production (Alenezi et al., 2017). Furthermore, endophytes possess high ACC deaminase activity which helps in plant growth promotion (Mefteh et al., 2019). The plant also resists growth inhibition by a number of ethylene-inducing stresses (Lumactud and Fulthorpe, 2018). Production of HCN by endophytes has been found to be beneficial to their host and aid an indirect increase in nutrient availability (Rijavec and

Lapanje, 2016). Equally, the production of siderophore by endophytes indirectly enhances plant growth promotion (PGP) and it is considered important for plants to thrive in polluted environments (Aloo et al., 2019).

A lot of current and past studies on plant endophytic microbiomes have generated a large quantity of sequenced data and numerous information on the abundance and diversity of the different taxonomic groups of endophytic microbiomes present in maize plant using next-generation techniques (Liu et al., 2017b, Correa-Galeote et al., 2018, Fadiji and Babalola, 2020c). However, limited studies exist on the functional importance of endophytic microbiomes for plant growth, yield and health (Sessitsch et al., 2012, Mashiane et al., 2018, Carrión et al., 2019). Furthermore, the genes responsible for most of these important functions, in most cases, are still unknown (Carrión et al., 2019).

Plant roots are often regarded as the point in which most interactivity between microorganism take place (Sessitsch et al., 2012). In this study, to unravel the plant growth-promoting and endophytic genes in the root-associated endophytic microbiomes in maize plant, we carried out shotgun metagenomic sequencing on the DNA extract from the root of maize plants cultivated with organic fertilizer, inorganic fertilizer and those without fertilizer. Shotgun metagenomics is now being embraced over similar metagenomic techniques because it enables a comprehensive functional study of entire microbial communities present in an environment (De Tender, 2017). Limited studies exist on the functional genes of endophytic microbes in the plant using shotgun metagenomics (Sessitsch et al., 2012, Hong et al., 2019). However, to the best of our knowledge no report exists on the effects of different farming practices on plant growth-promoting (PGP) and endophytic genes in endophytic microbiomes from maize plants using shotgun metagenomics. Hence, this study presents the first attempt to unveil the putative PGP and endophytic gene clusters present in root-associated endophytic microbes of maize plants alongside assessing the effect of different farming practices on their expression using shotgun metagenomics. The study hypothesized that functional genes would be more represented in endophytes from maize plants cultivated with organic fertilizer than inorganic fertilizers.

8.2 MATERIALS AND METHODS

8.2.1 Seed collection

The maize seed (WE 3127) used in this experiment was collected from North-West University School Farm Molewane, Mafikeng, North West Province, South Africa.

8.2.2 Experimental design and site description

The field located in North-West University School farm Molewane, Mafikeng, NorthWest Province (S25°47'25.24056", E25°37'8.17464"), South Africa, used for the study has been in existence for over 15 years. The chemical and physical properties of soil sample from this experimental field were assessed as 22% Sand, 66% Silt, 12% clay, pH 6; 0.48% organic C, 0.15% total N, 101.5 ppm P, and 0.962ppm K (Supplementary Table S1). The NorthWest province is characterized by shrubs and trees. The mean temperatures experienced in the province ranges from 3°C to 21°C in winter and 17°C to 31°C in summer. The rainfall in the province is estimated at 360 mm per annum, having heavy rains between October and April. The major plants cultivated in this experiment site had been the rotation of sorghum**,** maize and soybean for a long time, with soybean planted in 2018. In this study, three different sites in the experimental field were used.

The maize seeds were planted at different maize sites, each site with 10 m x 4 m in dimension. The planting was carried out during October-December 2019. Two fertilization sites namely Organic fertilization (FK), inorganic fertilization (NK) and no fertilization (CK) were used in this study. These sites have been in existence for over 15 years. Urea (N), potassium sulfate (K) and calcium superphosphate (P) has been in use as the inorganic fertilizer and applied to the site at 150, 75, and 75 kg/ha for N, P, K respectively. The organic fertilizer (Cattle manure) has been in a consistent application at 10,625 kg ha^{-1} approximately for the organic site for more than 15years complying with standard procedures (USDA, 2014), while no fertilizer has ever been applied to the third site. Irrigation was provided across the sites in required volumes to prevent drought stress. The weeding process was handled manually.

8.2.3 Root sampling

Each site was divided into three regions for sampling purposes. Each replicate sample for sequencing came from the pooled roots of 10 randomly selected from fresh plants from each region of a treatment site. The plants were collected during the fruiting stage (Xia et al., 2015). A total of 90 plant samples (representing 30 plants per site) were evaluated; the three regions represent three replicates for each sampling site. The plant samples were kept with ice and transported to the laboratory the same day where they were processed immediately.

8.2.4 Root surface washing

Surface washing was carried out on the maize roots using the method described by Liu et al. (2017b) after soil particles have been removed. 70% ethanol was used to submerge the roots for 3 min, 2.5% solution having sodium hypochlorite was used to rinse for 5 min; again the roots were washed for 30 s with 70% ethanol and lastly washed with sterile distilled water. To ensure that the process of sterilization was correctly carried out, sterilized roots were plated on yeast extract-mannitol medium using a Petri dish (Vincent, 1970). After 72 h of incubation at 30^{o}C, the plates were checked for bacterial growth. Maize roots from Petri dishes without contamination were chosen for DNA extraction (Correa-Galeote et al., 2018).

8.2.5 Extraction of DNA and shotgun sequencing

The roots were cut into 1 cm using a sterile scalpel and instantly macerated using a Qiagen TissueLyser. Total metagenome DNA was extracted from the root tissue samples using the Qiagen DNeasy Plant Mini Kit (Qiagen, USA). Shotgun metagenomic sequencing was done at the Molecular Research LP, Texas, USA. The preparation of the library was carried out with Nextera DNA Flex kit (Illumina) following standard procedure. The DNA concentration in the samples was evaluated using Life Technologies' Qubit® dsDNA HS Assay Kit. The library preparation was carried out using 50 ng of the DNA. After the library has been prepared, the final concentration was measured using the Qubit® dsDNA HS Assay Kit, and the Agilent 2100 Bioanalyzer was used to ascertain the size of the library. The library size varies from 683 to 877 bp with an average of 731 bp, pooling of libraries were done using 0.6nM ratios, and paired-end sequencing was done with 300 cycles using the Illumina NovaSeq 6000 system.

8.2.6 Metagenome assembly and gene annotation

The obtained sequences of each metagenome were transferred to an online server called MG-RAST(Hong et al., 2019). Inside this online server, quality control analysis was carried out using the Trimmomatic v 0.33 program (Bolger et al., 2014). Other quality control processes also include the removal of chimeras, filtering of ambiguous bases, specification of minimum read size, and length filtering. After quality control analyses, annotation of the processed sequences was carried out using BLAT (Kent, 2002), against M5NR database (Wilke et al., 2012), which allows nonredundant integration of several databases. Classification of the endophytic microbiomes and protein-coding genes was carried out using SEED Subsystem. The identified putative functional genes were manually selected from the SEED functional level databases with specified parameters such as a 10^{-5} e-value cut-off and a minimum 60% sequence similarity to a subsystem. No further analysis was carried on the sequences that could not be annotated. However, since the main focus of this work is on endophytic microbiomes which include bacteria, fungi, and archaea, which account for about 99% of the whole sequences we, therefore, discard the plant and viral sequences after mapping against a reference genome database (Jayakodi et al., 2018, Hong et al., 2019). To suppress the influence of experimental error/noise, data normalization option was selected on the MG-RAST. Furthermore, the relative abundance of the functional genes was calculated in percentages, after taking the average of the independent analysis of the 3 sequences for the sampling sites FK, CK and NK respectively using MG-RAST. These sequences can be found on NCBI SRA dataset with the accession number PRJNA607664.

8.2.7 Statistical analyses

Shinyheatmap via z-score was used to visualize the abundance and distribution of the major endophytic microbiomes at the phylum level and the plotting of the relative abundance of the identified functional genes. The abundance of each functional gene across the sites was plotted using Circos software (www.circos.ca/software). Simpson, Shannon and Pielou indices for diversity assessment were employed for samples across the sites, and Kruskal–Wallis test was used to compare these indices. The analyses were performed with PAST version 3.20 (Chauhan et al., 2019). The Bray-Curtis based principal coordinate analysis (PCoA) and ANOSIM were used for the β diversity study and to assess the differences in the functional genes present in the samples across the sites (Carrell and Frank, 2015). The PCoA and PCA plots based on Bray-Curtis dissimilarity matrix were performed using CANOCO version 5.0.

8.3 Results

8.3.1 Metagenome sequencing, quality control, and protein annotation

After quality control analyses were carried out in MG-RAST, the sequenced output for CK was 334259767 bp, FK had 415505341 bp, and NK had 817699487 bp, while the mean G+C content of 44%, 44%, and 49% for CK, FK, and NK respectively (Supplementary Table S2). Among the sequences that passed the quality control check, sequences that mapped predicted proteins with known functions in the samples were 643141, 371329, and 325439 sequence reads from metagenomes originating from plants grown under inorganic fertilized (NK), organic fertilized (FK) and no fertilizer (CK) sites respectively. The species richness was obtained by rarefaction analysis through MG-RAST (Figure 8.1).

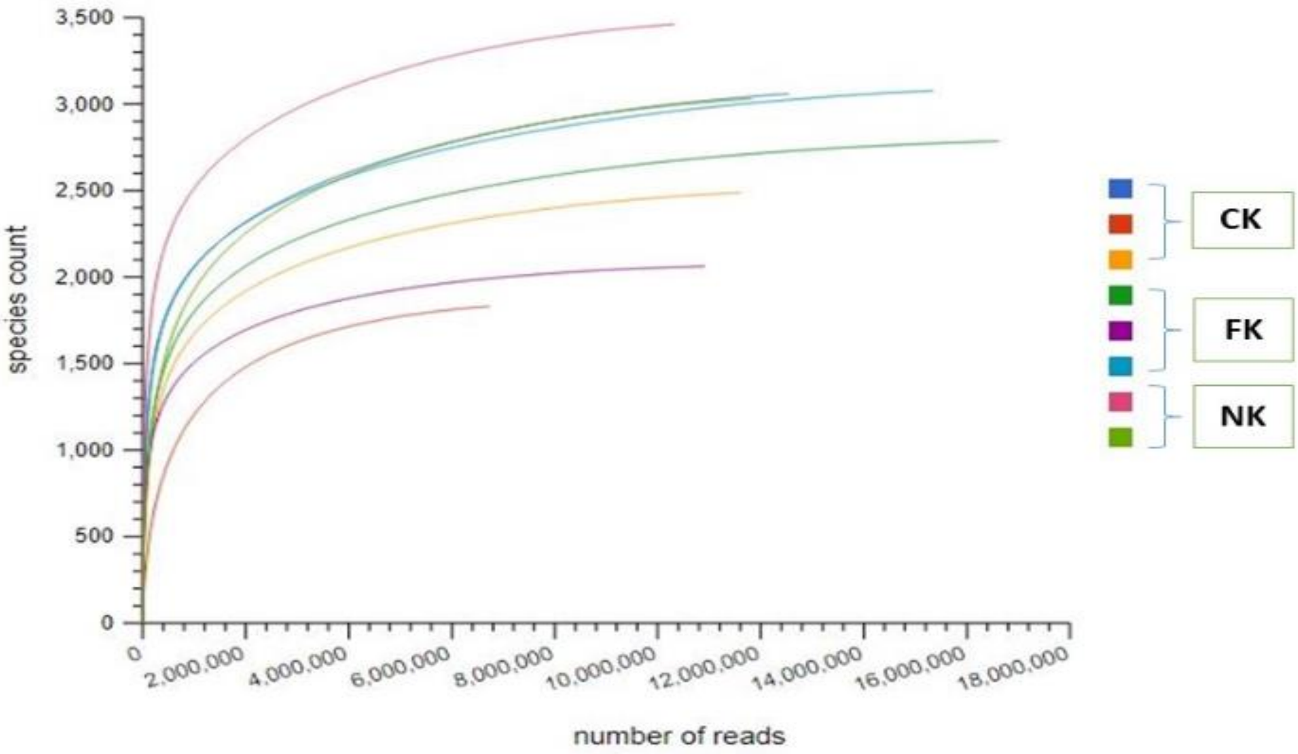


Figure 8.1: Rarefaction curves used to ascertain the species richness of sequences across the sites. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

8.3.2 Distribution of endophytic microbiomes in the maize plant

Although twenty-nine (28) bacterial phyla were identified from the samples, 23 phyla were dominant in the sites, some of which include *Firmicutes*, *Proteobacteria* and *Bacteroidetes*.

Three (3) archaea phyla were also identified, namely *Crenarchaeota*, *Euryarchaeota*, and *Thaumarchaeota*. In contrast, two (2) fungal phyla, namely *Ascomycota* and *Basidiomycota* were identified. The most dominant phyla of endophytic microbiomes identified are presented in Figure 8.2. No significant difference (p= 0.292, Kruskal-Wallis) was observed among the identified dominant endophytic microbiomes across the sampling sites.

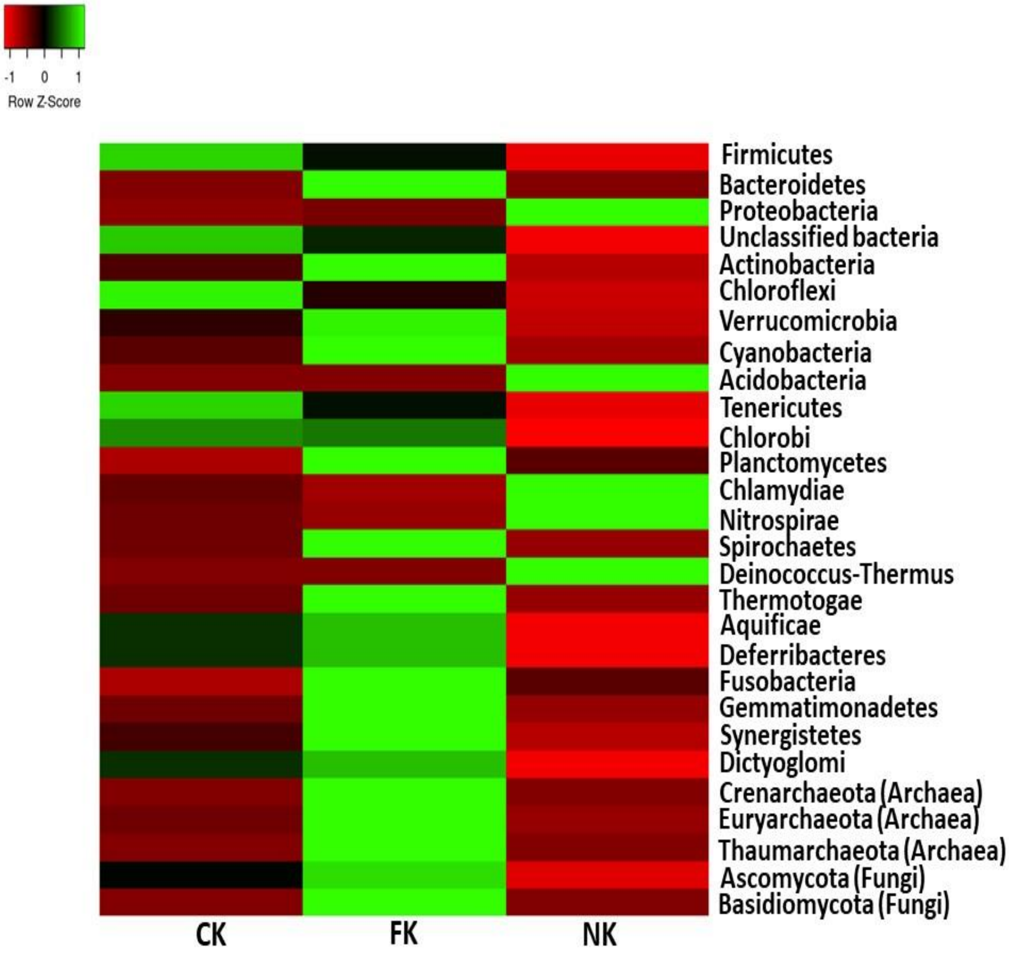


Figure 8.2: Heatmap showing the distribution of endophytic microbiomes in maize samples across the sites. The scale bar represents colour saturation gradient based on the relative abundances with z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

8.3.3 Functional genes in maize endophytic microbiomes

A total of 58 functional genes were detected in maize endophytic microbiomes across the fertilizer sites. The identified functional genes were classified into plant growth-promoting and endophytic genes.

8.3.3.1 Plant growth-promoting genes in maize endophytic microbiomes

Twenty-two (22) out of the putative genes were identified as plant growth-promoting (PGP) genes, notable among them include genes involved in nitrogen fixation (*nifH*), siderophore (*pchB*, and *entF*), HCN biosynthesis (*hcnB*), ACC deaminase (*acdS*), phenazine (*phzF*), phosphate solubilization (appA), butanediol (*butB*), methanol utilization (*xoxF*), acetoin (*budC*), nitrogen metabolism (*glnA*, *glnB*, *gltB*, *gltD* and *nirB*), and IAA biosynthesis (*ipdC* and *iaaM*). We also identify genes involved in stress tolerance such as glutathione, catalase, and peroxidase (*btuE*, *gst*, *katE*, and *sod1*) (Figure 8.3; Supplementary Table S8). No significant differences ($p>0.05$) were observed in the abundance of these functional genes across the sampling sites. Principal component analysis (PCA) was used to show the distribution of PGP genes present in endophytic microbes across the farming sites with FK sites having the highest distribution (Figure 8.4).

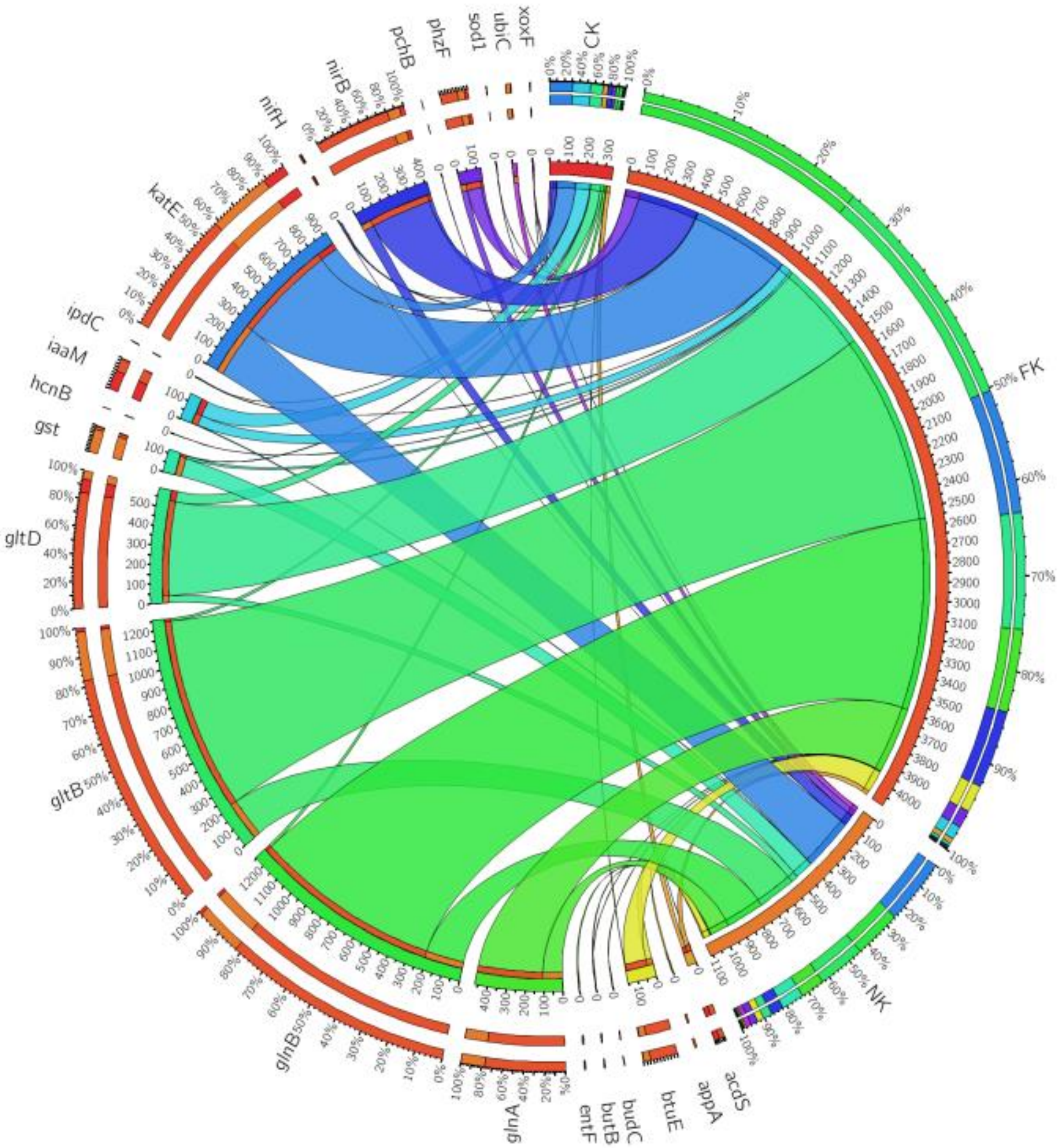


Figure 8.3: Relative abundance of genes involved in plant growth promotion observed across the sampling sites plotted using Circos software. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

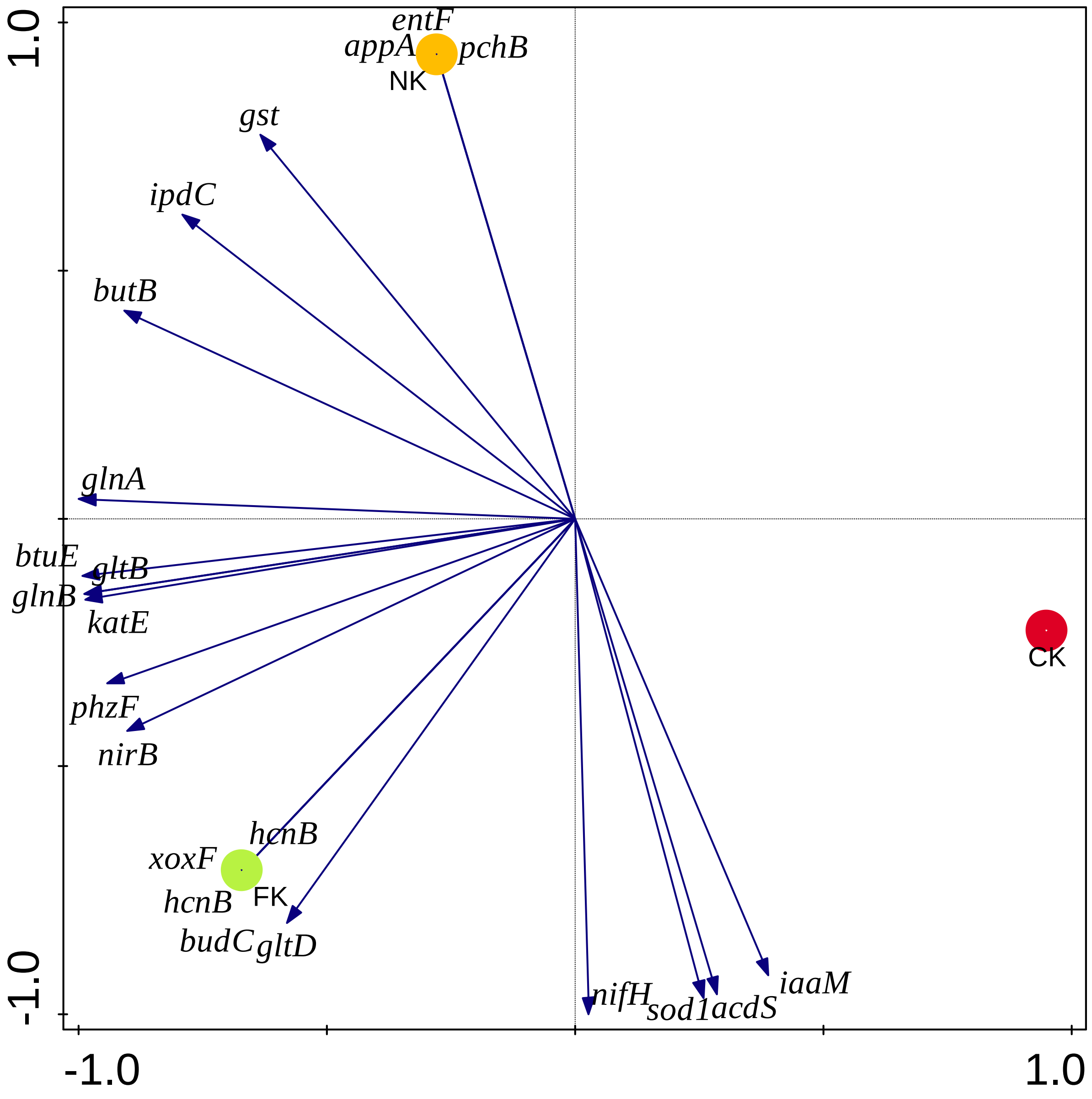


Figure 8.4: PCA graph of plant growth-promoting genes. The vector arrow represents the influence of plant growth-promoting genes. Axis 1 (71%) and Axis 2 (29%) explained the variations. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

8.3.3.2 Endophytic genes in maize endophytic microbiomes

Furthermore, 36 genes out of the functional genes were associated with endophytic behaviors. Notable among these genes are those involved in chemotaxis and motility such as aerotaxis (*aer*), regulator of proteins (*cheC*, *cheD*, *cheV* and *cheZ*) and motility (*flhA*, *flhB*, *flhF*, and *fliL*). Others include, transcriptional regulators such as nitrogen regulation (*nifA* and *nadR*), regulation of carbon storage (*sdiA*), formation of biofilm (*crp*), reduction of nitric oxide (*norR*) and regulation of beta-lactamase resistance (*ampR*). Also, we identified genes involved in secretion systems such as type III secretion (*yscJ*), type IV conjugal DNA (*virB2*), and type I pilus assembly (*fimA*). Furthermore, genes involved in transport system were identified to include twitching movement, phosphotransferase system (PTS), an ATP-binding cassette (ABC), multidrug transporter, tricarboxylic transporter (*tctA*) and methyl-dicarboxylate (*dctA*) (Figure 8.5; Supplementary Table S9). No significant differences ($p>0.05$) were observed in the abundance of these endophytic genes across the sampling sites. Principal component analysis (PCA) was used to show the distribution of endophytic genes present in the endophytic microbes across the farming sites with FK sites having the highest distribution (Figure 8.6).

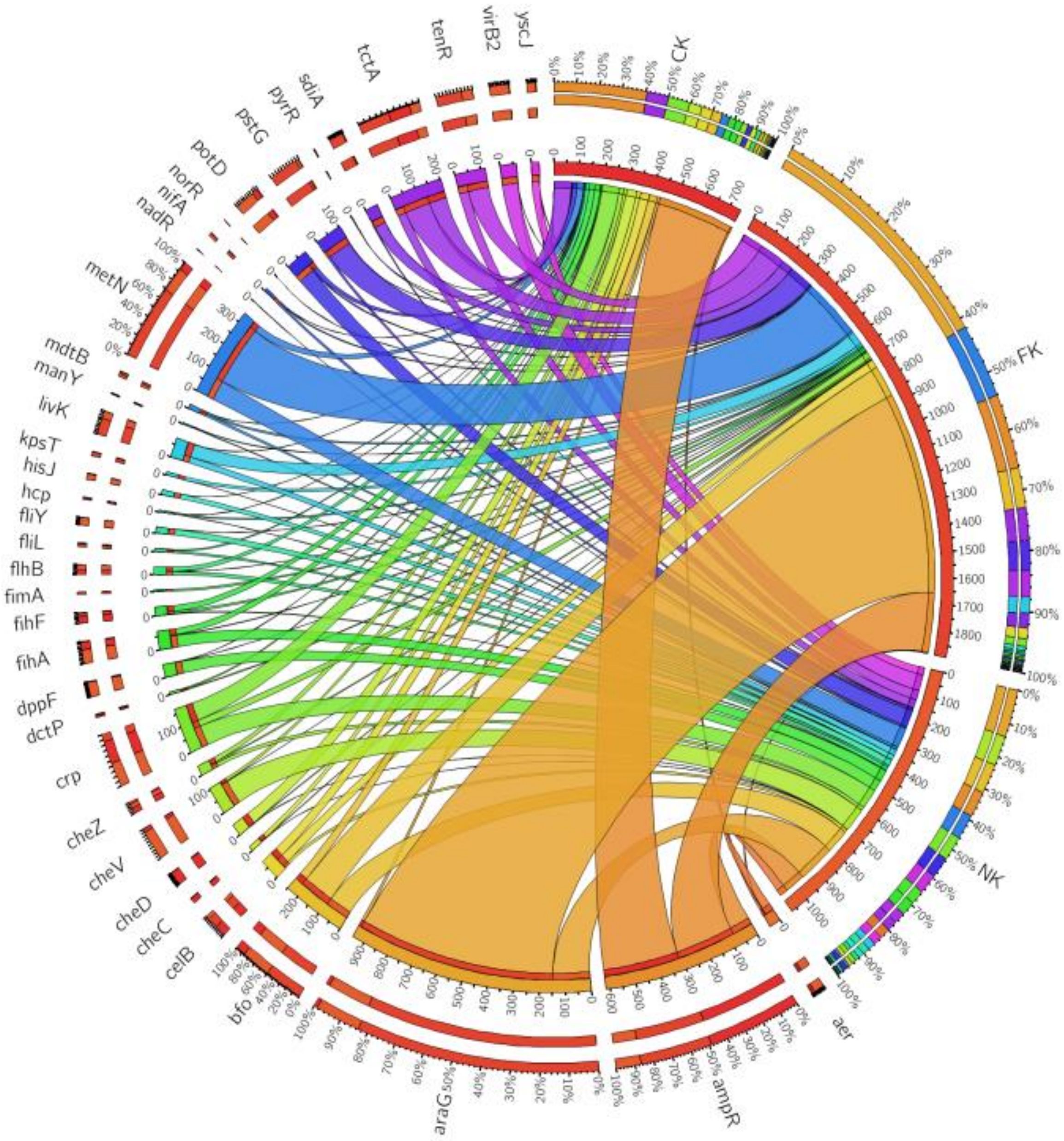


Figure 8.5: Relative abundance of genes involved in endophytic behaviors observed across the sampling sites plotted using Circos software. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

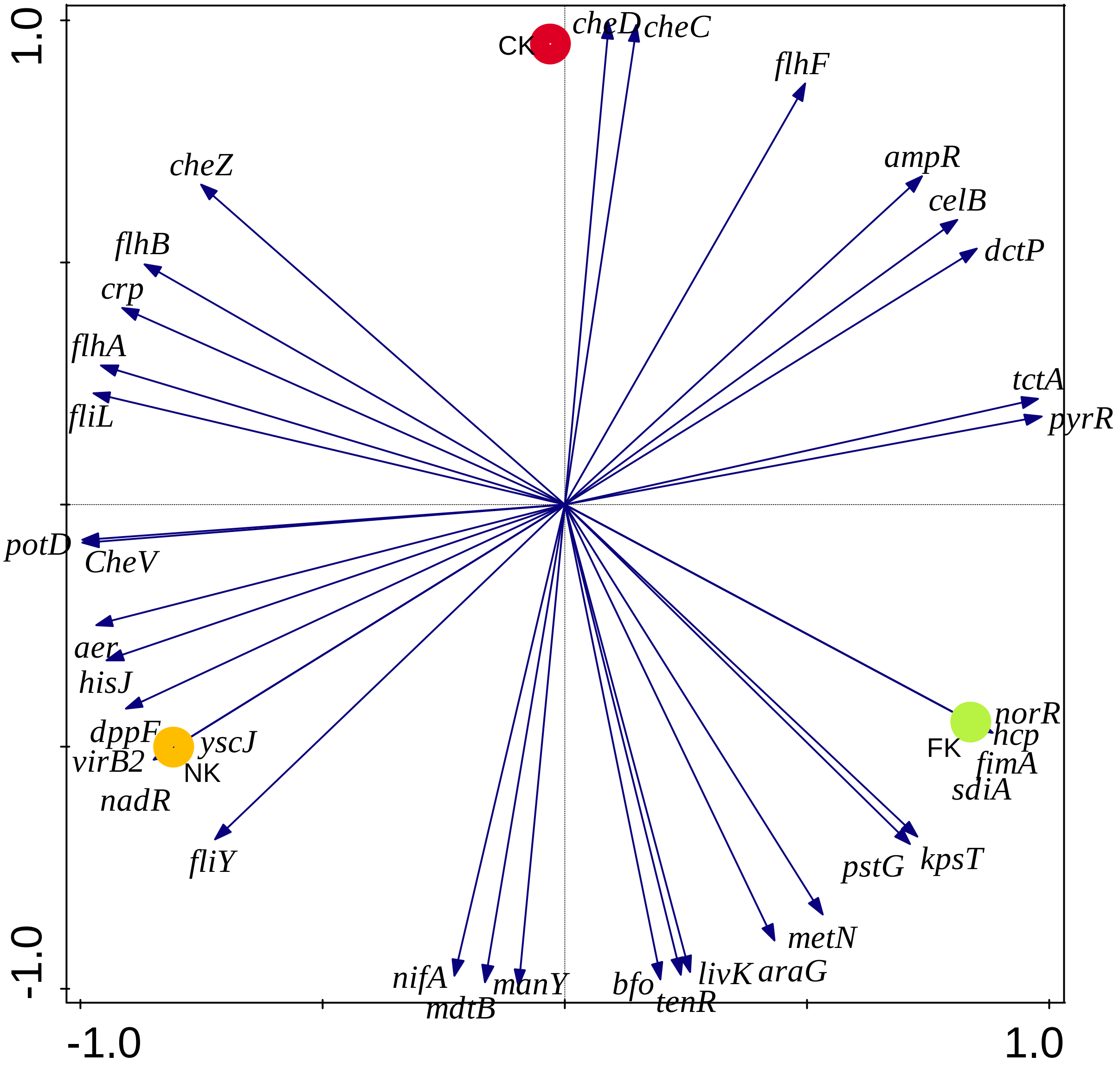
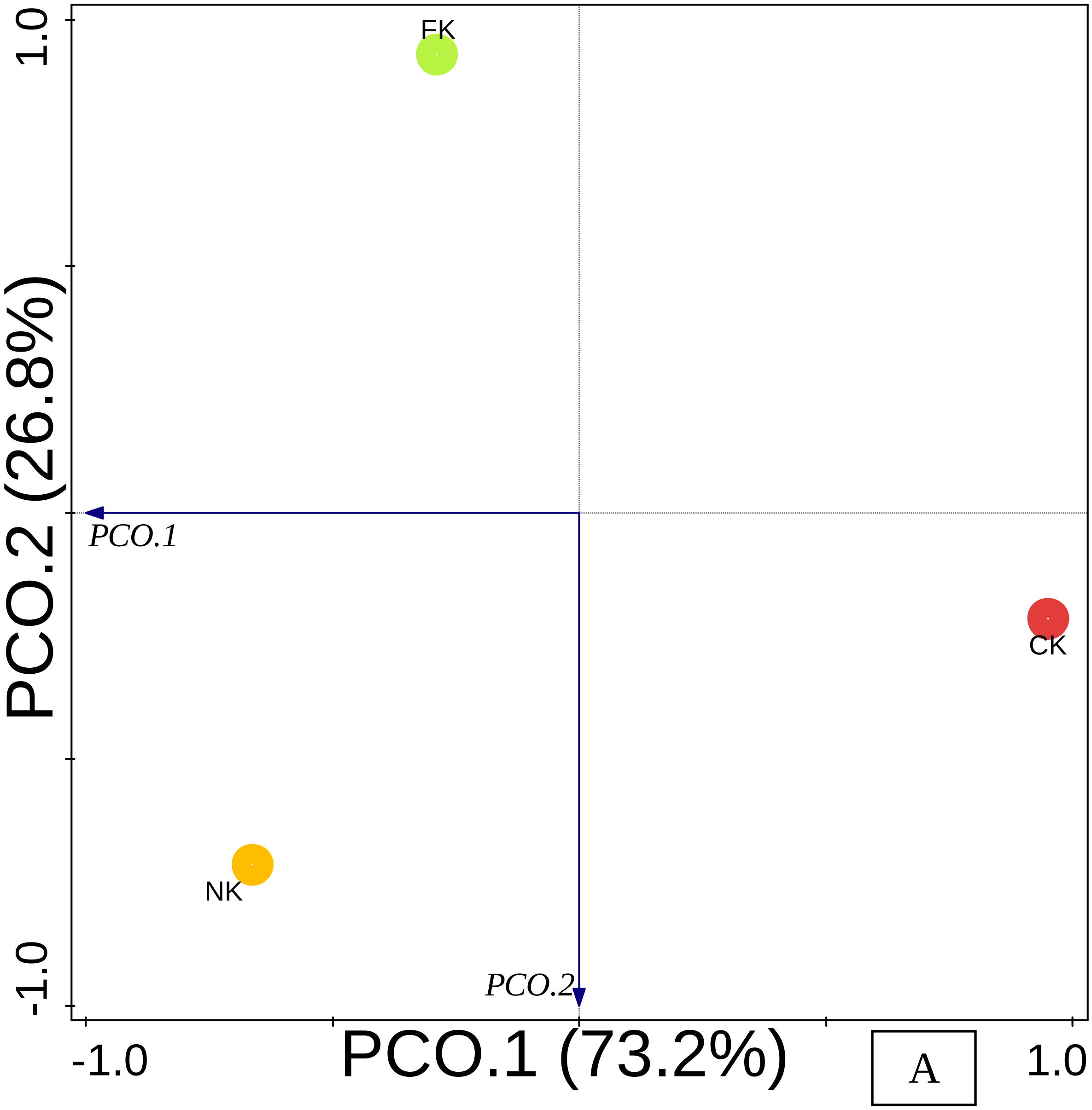


Figure 8.6: PCA graph of plant gowth-promoting genes. The vector arrow represents the influence of plant gowth-promoting genes. Axis 1 (51.4%) and Axis 2 (48.6%) explained the variations. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

8.3.4 Alpha (α) and beta (β) diversity estimation of functional genes in endophytic microbiomes across the sampling sites

Shannon, Simpson, and evenness indices were used to estimate the alpha diversity of the functional genes across the sampling sites. The alpha diversity results showed that there were no significant differences (p-value = 0.162 and 0.09, Kruskal–Wallis) among the identified PGP and endophytic genes across the sampling sites (Table 8.1). However, beta diversity using analysis of similarity (ANOSIM) revealed a significant difference (P-value = 0.01; R = 0.67) among the functional genes across the sites. PCoA further revealed a clear separation across the sites (Figure 8.7A and 8.7B).


FK
CK
NK
PCO.1
PCO.2
PCO.2 (26.8%)
PCO.1 (73.2%)
1.0
-1.0
-1.0
1.0
A

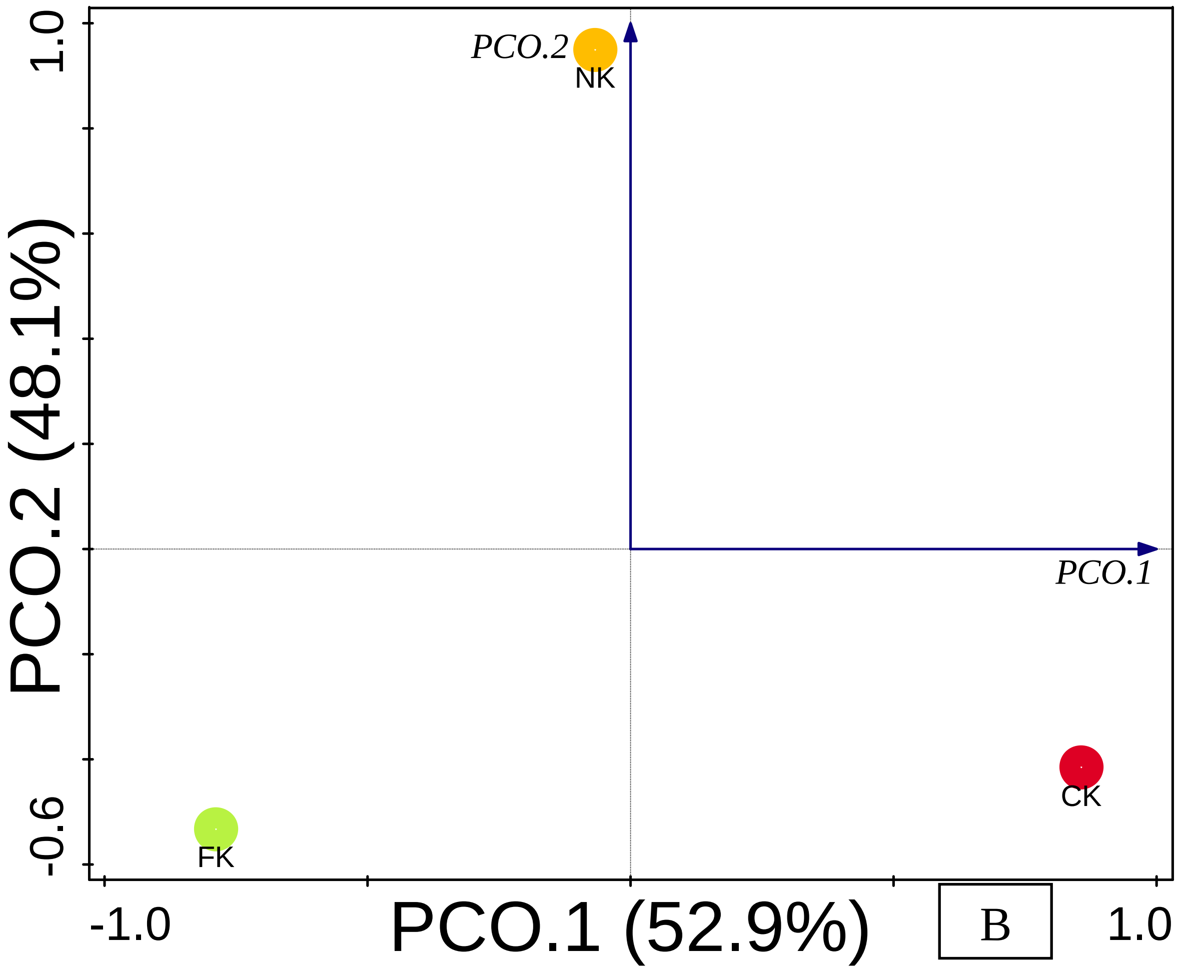


Figure 8.7: Principal coordinate analysis (PCoA) plot of (A) plant growth-promoting genes (B) endophytic genes composition across the maize sites based on Bray–Curtis dissimilarities. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

Table 8.1: Diversity and evenness assessment of putative functional genes in endophytic microbiome with different treatments in the sampling sites

Diversity Indices	**CK**	**FK**	**NK**	**p-value**
		Plant growth-promoting genes		
Simpson_1-D	0.82±0.01	0.77±0.01	0.94±0.01	0.162
Shannon_H	2.36±0.09	2.01±0.05	2.96±0.06	
Evenness_e^H/S	0.37±0.40	0.23±0.03	0.61±0.04	
		Endophytic genes		
Simpson_1-D	0.81±0.02	0.83±0.01	0.84±0.01	0.09
Shannon_H	1.94±0.09	1.95±0.03	2.04±0.05	
Evenness_e^H/S	0.50±0.10	0.37±0.04	0.43±0.04	

Mean ± standard error (n= 3). p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

8.4 Discussion

In this study, the shotgun metagenomic analysis was carried out on maize root cultivated with different fertilization levels and without fertilization. Using MG-RAST, the sequenced data were analyzed by recognizing sequences that are for the endophytic microbiome while discarding the whole genome sequences for maize plants. In the bacteria sequence, we identified dominant bacteria phyla such as *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Tenericutes*, *Planctomycetes*, *Cyanobacteria*, and *Chlorobi*. The identified fungi are *Ascomycota* and *Basidiomycota*, while we found *Crenarchaeota*, *Euryarchaeota*, and *Thaumarchaeota* as the dominant endophytic archaea. Most of the identified endophytic bacteria, fungi and archaea have been reported as notable plant growth-promoting microbes and they possess important genes linked with plant growth and health promotion

(Müller et al., 2015, Correa-Galeote et al., 2018, Hong et al., 2019, Xia et al., 2019). These PGP genes are involved in nitrogen metabolism, mitigation of environmental stress, phosphate solubilization, methanol utilization, and nutrient accessibility (Figure 8.3). Previous studies have revealed that endophytes promote the growth of plant via direct and indirect mechanisms. Some direct mechanisms employed include the production of phytohormones, phosphate solubilization, siderophore production and 1-Aminocyclopropane-1-carboxylate (ACC) utilization (Babalola, 2010, Singh and Dubey, 2018, Fadiji and Babalola, 2020a). The indirect mechanisms include the induction of plant resistance, secretion of secondary metabolites, hyper-parasitism and biocontrol activities (Olanrewaju et al., 2017, Latz et al., 2018, Fadiji and Babalola, 2020a).

In this study, from the metagenomes obtained for endophytic microbes, we observed gene *ipdC* and *iaaM* which encodes indole-3-pyruvate decarboxylase and tryptophan 2-monooxygenase respectively, which participates in the biosynthesis of an important hormone called IAA from tryptophan (Sugawara et al., 2015) represented only in FK and CK sites. Our result is consistent with Da Costa et al. (2013) in which IAA traits significantly improved the growth of rice cultivated in a moderate nutrient environment. Indole acetic acid (IAA) is a plant hormone that triggers plant cell division, differentiation and extension. It stimulates seed and tuber germination; increases the rate at which root and xylem develop, enhances lateral initiation, controls the rate of vegetative growth and formation of adventitious root (Singh and Dubey, 2018, Carrión et al., 2019). It also helps in the formation of pigments and biosynthesis of metabolites, controlling responses to gravity, light and fluorescence, photosynthesis, and resistance to extreme conditions (Hassan, 2017). Similarly, we identified *acdS* gene, which encodes ACC deaminase which was moderately represented in CK and FK sites and poorly represented in NK site. Our result suggests that at CK and FK sites, the interactions between endophytes and maize plants significantly enhanced growth promotion. This enzyme helps hydrolyze 1-aminocyclopropane-1-carboxylic acid (ACC) and reduce ethylene production in plants (Singh and Dubey, 2018, Hong et al., 2019). Ethylene is an important plant hormone that enhances seed germination, and it is the major regulator of bacterial colonization in the tissues of the plant (Iniguez et al., 2005, Babalola, 2010). However, its excessive accumulation can be detrimental to plant health and growth (Eid et al., 2019, Yurgel et al., 2019). Some studies also reported that ACC deaminase could likewise hydrolyze ACC into ammonia and α-ketobutyrate, which supply nitrogen for microbial growth (Xing et al., 2012). Endophytic bacteria genera such as *Bacillus*, *Arthrobacter*, and *Microbacterium* (belonging to *Firmicutes* and *Actinobacteria*) isolated from *Capsicum annum* L. exhibited ACC deaminase activity by supporting growth in plants exposed to drought stress (Sziderics et al., 2007).

Furthermore, we identified *appA* gene, which encodes for 4-phytase, an important enzyme involved in P mineralization in endophytes from FK and NK sites but with high abundance in the samples from NK site. This may occur due to the high phosphorus content present in the inorganic fertilizer. Our results, therefore, agree with an earlier report where inorganic fertilizer application improved phosphate solubilizing traits in microbes (Da Costa et al., 2013). Phosphorus is one of the major elements necessary for plant growth and other biological processes such as enhancing organ development in plants, glucose transport and stimulation of cell growth, but plants can not directly utilize phosphorus found in the soil without being solubilized to phosphate (Ahemad and Kibret, 2014, Singh and Dubey, 2018). Endophytes play a major role in phosphate solubilization and also enhance its availability for plant use through redox changes, chelation, mineralization of organic phosphorus and acidification (Van Der Heijden et al., 2008). Many endophytic microbes solubilize phosphate complexes and convert them into forms like ortho-phosphate, which is readily available for use (Otieno et al., 2015). Organic phosphorous mineralization coupled with the secretion of phytase was reported in *Streptomyces* sp. an endophyte (belonging to the phylum *Actinobacteria*) isolated from *Triticum aestivum* which significantly improved plant growth (Jog et al., 2014). We also found genes *budC, butB*, and *xoxF,* which codes for acetoin reductase, butanediol dehydrogenase, and methanol dehydrogenase, respectively. These genes were poorly represented in samples from NK and CK sites, but are more frequent in samples from the FK site. A recent study by Hong et al. (2019) revealed that 2,3-butanediol, and acetoin are novel volatile molecules that aid increased root length in *Panax ginseng*. It was also reported that 2,3-butanediol, and acetoin could enhance plant growth (Hardoim et al., 2015). Similarly, we found a poor representation of genes *pchB* and *entF*, which encode isochorismate pyruvate lyase and enterobactin synthetase component F, respectively, in samples from NK sites only. These genes are associated with iron and siderophore production and have been reported to be beneficial in plant growth promotion (Tsurumaru et al., 2015, Singh and Dubey, 2018). For iron to be utilized by plants, it must undergo solubilization. A study by Rajkumar et al. (2009) revealed that the provision of iron for the roots of plants was increased by the production of siderophores by phytosiderophore-iron complex or bacterial siderophore-iron complex. *Streptomyces tendae* F4 (belonging to *Actinobacteria*) has been reported to be a great producer of siderophore which enhances the growth of cowpea (*Vigna unguiculata*) exposed to nickel stress (Dimkpa et al., 2009).

Nitrogen fixation is often performed by endophytes in a bid to improve the fitness of their host, especially in an environment deficient of nitrogen. This is one of the mechanisms endophyte use to reduce the effect of environmental stress on the host plant (Ruppel et al., 2013). In this study, we found gene *nifH*, which encodes nitrogenase involved in nitrogen fixation alongside *glnA*, *glnB*, *gltB*, *gltD* and *nirB* that are associated with nitrogen metabolism. These genes were well represented in all samples, though more expressed in FK samples. Our result is similar to an earlier study in which organic fertilizer significantly improved nitrogen metabolism in peanuts (Purbajanti et al., 2019). Numerous root-associated endophytes are involved in nitrogen fixation, such as *Acetobacter* spp, *Herbaspirillum* spp, and *Azoarcus* spp; all belonging to the phylum *Proteobacteria* (Eid et al., 2019). An endophytic bacterium, *Paenibacillus* P22 (belonging to the *Firmicutes*) was also reported to fix nitrogen as well as effect changes in host plant metabolism (Hardoim et al., 2015). Similarly, we found gene *hcnB,* which encodes hydrogen cyanide synthase in only FK samples. This gene enhances HCN biosynthesis and HCN has been reported to be beneficial in the growth promotion of the host plant (Rijavec and Lapanje, 2016).

Furthermore, we identified genes *ubiC* and *phzF,* which encodes chorismate lyase and phenazine biosynthesis respectively in endophytes across the sites. These genes encode phenazine and 4-hydroxybenzoate which help in antibiosis and biocontrol activities in the host (Enagbonma and Babalola, 2020). Similarly, plant growth and health are affected by environmental stresses, and endophytes can protect their host from both abiotic and biotic stresses (Hardoim et al., 2015, Azad and Kaminskyj, 2016, Omomowo and Babalola, 2019). We identified genes involved in the mitigation of stress such as *btuE, gst, katE,* and *sod1* which codes for glutathione peroxidase, glutathione-S-transferase, catalase and superoxide dismutase respectively. The genes *btuE, gst*, and *katE,* were represented in all samples and more frequent in the FK samples while *sod1* was poorly expressed only in CK and FK samples. In a bid to overcome stresses, plants produce antioxidant defense mechanisms which include non-enzymatic and enzymatic components, which help to avert the buildup of reactive oxygen species (ROS) (Miller et al., 2010a). The enzymatic components are catalase, superoxide dismutase, glutathione reductase, and ascorbate peroxidase, while non-enzymatic components include ascorbic acid, glutathione, and cysteine (Vardharajula et al., 2017). However, the frequent abundance of PGP genes in samples from organic fertilizer site (FK) confirmed our hypothesis. PCA plot showed that each site has a different plant growth-promoting (PGP) genes and is responsible for a combined 71% variance witnessed across the fertilization sites (Figure 8.4). The position occupied by each plant growth-promoting (PGP) gene reflects their functional makeup, while the vector arrows showed the PGP gene most influenced by the distribution. Employing this indicator, it is easier to detect which of the identified PGP genes is more dominant in the endophytes from each sampling site as compared to others.

Additionally, we identified genes that are linked to the endophytic behavior of microorganisms inside the plant hosts (endophytic genes). Some of these genes include those involved in motility, regulation of transcription, transport system, and secretion systems (Figure 8.5). We identified genes *aer*, *cheZ*, *cheC*, *cheD*, and *cheV*, which encodes aerotaxis and regulator proteins that were moderately expressed in all samples across the sites. Similarly, we found genes *flhA*, *flhB*, *flhF* and *fliL*, which encode motility mechanisms and flagellum biosynthesis that could help endophytes survive in their host. These genes are connected with chemotaxis, motility, and adhesion, and were represented in all samples. One major factor that drives the colonization of microbes is the capacity to detect and respond to environmental cues (Hartmann et al., 2009, Porter et al., 2011). The response regulator proteins and flagellum biosynthesis were moderately present in all samples across the sites. The presence of these genes might contribute to a successful endophytic lifestyle in plants.

Similarly, we identified putative genes involved in transcriptional regulation in all endophytic microbiomes across all sites. The genes are *nifA* involved in nitrogen regulation, *sdiA* involved in the regulation of carbon storage, *crp* involved in the formation of biofilm, *norR* involved in the reduction of nitric oxide, and *ampR* involved in the regulation of beta-lactamase resistance. Others include *tenR* involved in thiamine metabolism, *pyrR* involved in pyrimidine regulator, and *nadR* involved in NAD regulation, which were more frequently detected in samples from sites FK and NK. Transcriptional regulators are of great importance to prokaryotes because they aid cellular homeostasis, response to environmental stress, new niche colonization, and adaptation flexibility (Berg et al., 2005). Regulatory genes involved in stress response, carbon metabolism, vitamins, and nucleotides alongside carbon and nitrogen metabolism might be of great support to endophytic lifestyles in plants (Hardoim et al., 2015, Rodríguez-Blanco et al., 2015). However, it is important to state that plant pathogens and nodule-forming symbionts that survive inside the inner tissues of plants use an entirely different mechanism from that of endophytes, indicating that all groups of microbes inhabiting the tissues of plants have a set of regulatory genes peculiar to their behavioral feedback (Hardoim et al., 2015, Olanrewaju et al., 2019).

Furthermore, nutrient transport is an important role for organisms to survive and thrive inside plants (Mitter et al., 2013). In this study, we observed identify genes for phosphotransferase system (PTS) such as *cleB*, *pstG*, and *manY*, which encode cellobiose, glucose and mannose respectively from endophytes across the sites. We also found genes for ATP-binding cassette (ABC) such as capsular polysaccharide (*kspT*), spermidine/putrescine (*potD*), dipeptide (*dppF*), branched-chain amino acid (*livK*), cystine (*fliY*), methionine (*metN*), histidine (*hisJ*) and L-arabinose (*araG*), which were represented in all samples, but more abundant in endophytes from site FK. Others include multidrug transporter (*mdtB*), tricarboxylic transporter (*tctA*) and methyl-dicarboxylate (*dctp*). Genes involved in the uptake of peptides, organic ions, amino acids, carbohydrate and capsular polysaccharides were detected among endophytes across the sites. The results showed a complex nature of the nutrient transport systems among the identified endophytes, reflecting different strategies of nutrient acquisition, which might support life inside the plant. The identified genes are similar to the ones detected in *Burkholderia* spp (belonging to *Proteobacteria*) (Santoyo et al., 2016).

Interestingly, protein secretion plays a major role in plant-microbe interactions (Downie, 2010, Reinhold-Hurek and Hurek, 2011). In this study, we identify a putative gene involved in type III secretion systems (*yscJ*) in all our samples across the sites but more abundant in samples from the NK site. Though identified in endophyte, the gene has an earlier record of being involved in phytopathogens and nodule-forming symbionts than in endophytes (Hardoim et al., 2015). This secretion system is often used by phytopathogens for manipulating their host metabolism (Abramovitch et al., 2006). In like manner, we found a gene associated with type IV conjugal DNA-protein transfer secretion systems (*vrB2*) which were poorly represented in all our samples but more abundant in samples from the NK site. The type IV secretion system has earlier been reported to be involved in DNA conjugation and host colonization (Salomon et al., 2014). Our results also revealed the presence of genes *hcp* and *fimA* in endophyte across the sampling sites and only at FK site, respectively. This suggests that endophytes in samples from FK sites, are more abundant than other samples. They encode secreted proteins and pilus assembly protein respectively. The genes are used most times for adhesion through type I pilus assembly, and twitching movement. These systems are suspected to play an important role in most successes recorded in host colonization by endophytes (Meng et al., 2005, Böhm et al., 2007). However, the frequent expression of these endophytic genes in samples from organic fertilizer sites (FK) confirmed our hypothesis. PCA plot showed that each site has different putative endophytic genes and is responsible for a combined 51% variance witnessed across the fertilization sites (Figure 8.6). The position occupied by each putative endophytic gene reflects the makeup of their sequences and the vector arrows showed the putative endophytic gene most dominant and influenced by the distribution. Employing this clue, it is not difficult to detect which of the identified putative endophytic genes is more dominant in the endophytes from each sampling site as compared to others.

Shannon, Simpson and Evenness indices calculated for the differences observed among the functional genes across the sites were not significant ($P > 0.05$) (Table 8.1). The alpha diversity analysis carried out on the functional genes from endophytes across the sampling sites revealed that the genes do not differ significantly (Kruskal–Wallis, P-value = 0.162, 0.09). The PCoA graph showed a clear separation of the sampling sites (CK, FK and NK). The ANOSIM test further establish the difference in the samples across the sites with the separation strength (R) of 0.67 and that the identified putative functional genes differ significantly ($P=0.01$) across the sites.

8.5 Conclusion

In summary, to the best of our understanding, this is the first attempt to identify putative functional genes in endophytic microbiomes in root of maize plants using shotgun metagenomics. The study identified genes that are putatively involved in plant growth-promoting and endophytic behavior (endophytic genes) in endophytes across the sites. These genes were more abundant in endophytes from maize plants cultivated in the organic fertilizer site (FK), which indicates that organic farming practices could be instrumental in the achievement of sustainable agriculture. However, there is a need for an extensive study of the mechanism of actions and colonization of endophytes in the endosphere. This will enhance their application in agricultural management practices such as biocontrol, plant nutrition and bioremediation. The sequenced data also revealed the abundance of more PGP genes in endophytic bacteria than endophytic fungi and archaea, thus presenting them as a potential source of biofertilizers for sustainable agriculture.

CHAPTER NINE

9.0 CONCLUDING REMARKS AND RECOMMENDATIONS

9.1 Concluding remarks

In a bid to reduce our reliance on chemical fertilizers and ensure food security, the world is gradually embracing biofertilizers and organic farming practices which are believed to be ecofriendly, boost the functions of plant microbiomes, and require less chemical input. Endophytes stand as promising alternatives in reducing our dependency on chemical fertilizer and mitigating the effects on the environment. This study explored the metagenomic methods used in understanding the diversity and functions of plant-associated microbiota with special focus on endophytes. It explored the potentialities of these endophytes in improving plant growth. Furthermore, this study elucidates the mechanisms of action of endophytes used in plant growth promotion and also highlights recent advances in beneficial bioactive metabolites secreted by endophytes. However, considering all the important benefits attributed to endophytes, factors that will favour the abundance and better performance of these beneficial microbes need to be explored. Hence, this study for the first time provided evidence that organic farming has significant influence on the diversity and functions of endophytic microbiomes in the maize plant using the shotgun approach. A total of 28 endophytic bacteria, 2 endophytic fungi and 3 endophytic archaea phyla were identified in all the samples. This study showed that the use of organic fertilizer for agricultural practices had a strong influence on the diversity, abundance, and functions of the endophytic microbiome as compared to inorganic fertilizers at the phylum level. At the genus level, it was further observed that maize planted with organic fertilizer housed a vast majority of distinct and novel endophytic bacteria, fungi and archaea as compared to other fertilizers. Basically, all major endophytic fungi, archaea and bacteria genera identified in this study have the potential to enhance agricultural sustainability. They not only promote growth, yield and health of the maize plant, but can also bring about a reduction in our dependence on chemical fertilizers which have been reported not to be ecofriendly.

Furthermore, a total of 28 functional groups were identified within the endophytic microbiomes. Some functional groups and metabolisms associated with plant growth promotion such as carbohydrate, secondary metabolism, nitrogen metabolism, iron acquisition and metabolism alongside phosphorus metabolism, while others linked with endophytic behaviors/lifestyles were observed in the endophytes across the sites. In addittion, this study also identified genes that are putatively involved in plant growth-promoting (PGP) and endophytic behavior (endophytic genes) across the sites. These genes were more prevalent in endophytes from maize plant cultivated in the organic fertilizer site (FK), thus indicating that organic farming practices could be instrumental in the achievement of sustainable agriculture and is strongly recommended in maize cultivation.

9.2 Recommendations and future research focus

More funding and support should be provided by the South Africa government towards organic farming practice. Also, laws that will encourage the implementation of organic farming practice on a large scale should be embraced as this holds a promising future for plant growth and yield improvement as well as being ecofriendly. Future studies should investigate the mechanisms used by endophytes and specific functions attributed to them in plant growth promotion. In addition, detailed studies on the impacts of other organic farming methods such as crop rotation, cover cropping, and crop integration would assist in establishing the best method for the abundance and diversity of endophytic microbiomes, which could subsequently promote plant growth, yield and health. Studies in this regard will enhance the applications of beneficial endophytes for sustainable agricultural practices.

Furthermore, there is a need for the development of a comprehensive open-source metagenome database that will be specific for the taxonomic classification of endophytic microbial communities. We also advocate for the development of more easy to use databases that will be specific for fungi and archaea because most current open-source databases favours the taxonomical classification of bacteria or are coding dependent. This will help in discovering more novel fungi and archaea species, give more insights into their beneficial functions for agricultural productivity and encouraging high throughput (HTS) studies in this regard. Similarly, it is important to state that one of the factors limiting the functional prediction and annotation of endophytic fungi and archaea through shotgun sequencing is the limited or lack of availability of easy bioinformatics tools and technical know-how on how to carry out this analysis. This is because most current analysis platforms involve the use of supercomputer and machine codes which are difficult to use by researchers in most developing countries. This study, therefore, advocates for the development of user-friendly bioinformatics tools which will encourage easy functional prediction of the individual groups of microorganisms present in the microbial community especially fungi species.

Also, using the shotgun metagenomic approach, more than three hundred (300) functional genes can be identified in any metagenome data. Therefore, it is very important to state that before carrying out any study, researchers should have a prior knowledge of the functional genes and pathways they intend to explore. This is important because one of the major factors limiting HTS methods is the difficulty in discovering new functional genes and pathways from processed sequence data. To overcome this, culturing of endophytes is required, though most endophytic microbes are still nonculturable. Recent attempts towards the culturing of nonculturable microbes explored co-culturing with culturable species, recreating laboratory environments, and combining both techniques with micro-cultivation technology to improve access to novel species. Although HTS methods have great prospects, we must bear in mind that culturing and experimental trials are also important. This study further advocates for collaboration among researchers from different disciplines such as biological sciences, environmental sciences, computer science and environmental engineering, to overcome the current limitations of the sequencing techniques which are believed to be suboptimal and which must be made fit for the data.

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SUPPLEMENTARY DATA

Table S1: Physicochemical characteristics of the experimental field

Soil Properties	Value
Sand (%)	22
Silt (%)	66
Clay (%)	12
pH	6.0
OC (%)	0.48
N (%)	0.15
P (ppm)	101.5
K (ppm)	0.962

Table S2: Analysis of sequenced data and diversity evaluation of the shotgun metagenomes of the maize plant from across the fertilizers sites

Sampling sites	**CK**	**FK**	**NK**
	Uploading Information		
bp count	1613298509	1493819190	2651625071
Sequence count	10302312	14294172	17449497
Mean sequence length	158±72bp	154±72bp	154±70bp
Mean GC content (%)	44±9%	43±9%	47±11%
	Post QC Information		
bp count	334259767	415505341	817699487
Sequence count	1765870	2171450	2176034
Mean sequence length	165±72bp	192±72bp	185±69bp
Mean GC content (%)	44±8%	44±8%	49±10%
	Processed reads		
Identified protein features	325439	371329	643141
Identified rRNA features	9808	19073	47651
	Aligned reads		
Identified protein features	86032	105456	177507
Identified rRNA features	2208	2053	2957

Values presented in the table are the means of the three replicates from each farming site. bp= basepair, GC= guanine, cytosine, QC=Quality control. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

Table S3: Diversity evaluation of major endophytic bacteria phyla in the maize plant across the fertilizers sites.

Phylum	CK	FK	NK	P value
Acidobacteria	0.055±0.04	0.060±0.016	1.416±1.356	0.49
Actinobacteria	0.496±0.007	1.325±0.542	0.210±0.165	0.20
Aquificae	0.007±0.004	0.009±0.002	0.003±0.003	0.62
Bacteriodetes	5.491±3.894	27.52±7.143	4.972±3.829	0.06
Candidatus poribacteria	0.000±0.000	0.002±0.001	0.001±0.001	0.73
Chlamydiae	0.021±0.020	0.002±0.001	0.073±0.065	0.56
Chlorobi	0.028±0.018	0.027±0.010	0.009±0.008	0.39
Chloroflexi	0.134±0.065	0.065±0.004	0.025±0.019	0.38
Chrysiogenetes	0.001±0.001	0.000±0.000	0.001±0.001	0.69
Cyanobacteria	0.087±0.021	0.233±0.104	0.057±0.039	0.18
Deferribacteres	0.004±0.003	0.005±0.005	0.001±0.001	0.79
Deinococcus-Thermus	0.009±0.003	0.020±0.007	1.703±1.686	0.43
Dictyoglomi	0.003±0.002	0.004±0.002	0.001±0.000	0.27
Elusimicrobia	0.000±0.000	0.000±0.000	0.001±0.000	0.96
Fibrobacteres	0.000±0.000	0.001±0.001	0.000±0.000	0.41
Firmicutes	35.85±27.95	22.38±6.628	5.755±3.491	0.39
Fusobacteria	0.004±0.003	0.009±0.006	0.004±0.003	0.73
Gammatimonades	0.004±0.002	0.014±0.003	0.003±0.002	0.11
Lentispaerae	0.001±0.001	0.006±0.003	0.002±0.001	0.59
Nitrospirae	0.017±0.01	0.012±0.06	0.067±0.044	0.65
Plantomycetes	0.023±0.015	0.050±0.010	0.028±0.021	0.59
Proteobacteria	5.061±1.915	6.838±2.305	40.21±18.81	0.19
Spirochaetes	0.011±0.005	0.066±0.042	0.006±0.005	0.06
Synergistes	0.04±0.002	0.012±0.010	0.001±0.001	0.53
Ternericutes	0.047±0.043	0.031±0.015	0.011±0.005	0.56
Thermotogae	0.008±0.005	0.028±0.010	0.060±0.004	0.10
Verrumicrobia	0.113±0.082	0.160±0.05	0.074±0.058	0.49
Unclassified bacteria	1.074±0.507	0.876±0.44	0.430±0.334	0.74

Mean ± standard error (n= 3). p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

Table S4: Diversity estimation of major nutrient cycling pathways in endophytes and evenness among the sampling sites

	CK	FK	NK	P value
		Nitrogen metabolic pathways		
Shannon_H	1.28	1.19	0.74	0.41
Evenness_e^H/S	0.51	0.43	0.23	
		Phosphorus metabolic pathways		
Shannon_H	1.15	1.60	0.74	0.68
Evenness_e^H/S	0.52	0.82	0.23	
		Secondary and Iron metabolic pathways		
Shannon_H	2.12	1.53	0.65	0.06
Evenness_e^H/S	0.70	0.51	0.12	

p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

Table S5: Diversity evaluation of major endophytic archaea phyla and class in the maize plant across the fertilizers sites.

	CK	FK	NK	p-value
		Archaea Phylum		
Crenarchaeota	0.001±0.001	0.005±0.005	0.001±0.001	0.43
Euryachaeota	0.018±0.015	0.068±0.042	0.013±0.019	0.19
Thaumarchaeota	0.002±0.002	0.089±0.080	0.000±0.000	0.04
		Archaea Class		
Archaeoglobi	0.000±0.000	0.000±0.000	0.001±0.001	0.56
Halobacteria	0.003±0.003	0.037±0.049	0.003±0.004	0.19
Methanobacteria	0.002±0.002	0.000±0.000	0.001±0.002	0.36
Methanococci	0.001±0.001	0.002±0.002	0.001±0.002	0.81
Methanomicrobia	0.006±0.006	0.029±0.036	0.006±0.006	0.43
Thermococci	0.003±0.002	0.013±0.017	0.002±0.002	0.20
Thermoplasmata	0.004±0.003	0.001±0.001	0.000±0.000	0.47
Unclassified Euryarchaeota	0.001±0.001	0.004±0.005	0.001±0.002	0.30
Unclassified Thaumarchaeota	0.002±0.003	0.137±0.162	0.000±0.000	0.04

Mean ± standard deviation (n = 3). p-values obtained via the Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

Table S6: Diversity evaluation of major endophytic fungi phyla and class in the maize plant across the fertilizers sites.

	CK	FK	NK	P-value
		Fungi Phylum		
Ascomycota	0.123±0.172	0.186±0.221	0.056±0.051	0.73
Basidiomycota	0.041±0.003	0.935±1.225	0.038±0.035	0.19
		Fungi Class		
Exobasidiomycetes	0.014±0.02	0.913±1.544	0.007±0.008	0.18
Eurotiomycetes	0.008±0.011	0.111±0.156	0.045±0.050	0.34
Sordariomycetes	0.012±0.017	0.033±0.025	0.008±0.008	0.48
Tremellomycetes	0.001±0.002	0.032±0.029	0.015±0.015	0.48
Pezizomycetes	0.000±0.000	0.020±0.017	0.000±0.000	0.95
Ustilaginomycetes	0.003±0.002	0.020±0.017	0.005±0.006	0.59
Saccharomycetes	0.011±0.018	0.013±0.018	0.003±0.004	0.87
Agaricomycetes	0.009±0.015	0.012±0.018	0.002±0.002	0.58
Dothideomycetes	0.010±0.014	0.004±0.002	0.018±0.024	0.79
Leotiomycetes	0.001±0.001	0.001±0.001	0.001±0.001	0.58
Schizosaccharomycetes	0.001±0.001	0.000±0.000	0.000±0.000	0.37

Mean ± standard deviation (n = 3). p-values obtained via the Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

Table S7: Average percentage of sequences similar to notable metabolisms in endophytic microbiomes across the fertilizer sites.

Functions	CK	FK	NK	P-value
Amino Acid Derivatives	5.80±2.22	6.83±3.52	21.68±13.93	0.56
Carbohydrates	8.34±2.79	12.75±2.43	7.58±2.60	0.49
Cell Division and Cell Cycle	0.63±0.25	1.24±0.08	0.59±0.26	0.06
Cell wall and Capsule	2.80±1.10	4.40±0.70	3.10±1.41	0.67
Clustering-based Subsystems	8.80±2.78	10.15±1.17	9.19±2.56	0.96
Cofactor, Vitamins, Prosthetic Groups, Pigments	2.52±0.93	3.30±0.51	2.71±1.03	0.96
DNA Metabolism	2.43±0.49	5.47±0.50	6.02±0.90	0.06
Dormancy and Sporulation	1.42±0.89	1.81±0.55	0.16±0.06	0.18
Fatty Acids Lipids and Isoprenoids	1.55±0.57	1.91±0.36	1.18±0.51	0.43
Iron Acquisition and Metabolism	0.86±0.27	0.91±0.34	6.19±4.43	0.19
Membrane Transport	2.89±0.90	3.63±0.35	3.45±1.36	0.86
Metabolism of Aromatic Compounds	0.49±0.13	1.01±0.19	1.05±0.23	0.07
Miscellaneous	4.08±1.12	6.90±1.37	4.60±0.39	0.19
Motility and Chemotaxis	0.55±0.22	0.52±0.09	0.61±0.28	0.96
Nitrogen Metabolism	0.43±0.25	1.74±1.03	1.48±0.74	0.29
Nucleosides and Nucleotides	1.81±0.66	1.64±0.29	1.57±0.75	0.67
Phages and Prophages, Transposable elements	1.72±1.14	6.05±4.46	2.31±0.93	0.67
Phosphorus Metabolism	0.61±0.18	0.51±.016	0.98±0.29	0.33
Photosynthesis	4.21±2.45	0.81±0.48	1.92±1.34	0.25
Potassium Metabolism	0.26±0.14	0.62±0.20	0.39±0.20	0.56
Protein Metabolism	10.63±2.29	6.53±0.61	5.93±2.45	0.43
RNA Metabolism	11.71±4.68	3.21±0.12	3.45±1.04	0.06
Regulation and Cell Signaling	0.71±0.27	1.30±0.16	0.88±0.46	0.56
Respiration	20.92±8.21	8.79±3.51	7.40±3.95	0.39
Secondary metabolism	0.07±0.05	0.15±0.09	0.10±0.03	0.73
Stress Response	1.50±0.53	2.28±0.50	1.71±0.49	0.73
Sulfur Metabolism	0.28±0.13	0.34±0.04	0.75±0.16	0.11
Virulence, Disease and Defense	2.59±0.99	3.28±0.12	2.95±0.83	0.96

Mean ± standard error (n= 3), p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site.

Table S8: Putative Genes contributing to plant-growth promotion as observed in root-associated endophytic microbiome of maize plant

Plant growth-promoting traits	Putative plant-growth promoting genes	CK	FK	NK	P-value
Siderophore production	*pchB*	0.00±0.00	0.00±0.00	0.33±0.58	0.37
	entF	0.00±0.00	0.00±0.00	5.00±8.66	0.37
IAA production	*ipdC*	0.67±1.22	2.00±2.65	0.00±0.00	0.11
	Iaam	81.33±138.28	55.66+74.27	3.00±3.00	0.78
Nitrogen fixation	*nifH*	0.67±1.01	1.33±1.53	0.33±0.57	0.11
Acetoin synthesis	*budC*	0.00±0.00	2.67±4.62	0.00±0.00	0.32
Butanediol synthesis	*butB*	0.00±0.00	1.53±2.65	0.33±0.57	0.11
Methanol utilization	*xoxF*	0.33±0.57	1.00±1.73	0.33±0.57	0.95
Phosphate solubilization	*appA*	0.00±0.00	2.34±4.31	7.00±12.12	0.37
Nitrogen metabolism	*glnA*	0.33±0.57	358.61±621.23	107.00±175.04	0.62
	glnB	15.00±13.89	1031.33±185.00	211.33±345.46	0.07
	nirB	19.67±29.83	335.00±544.09	56.66±52.17	0.50
	gltB	15.00±13.89	1031.33±185.00	211.33±345.46	0.07
	gltD	56.00±94.39	1498±134.62	116±56.90	0.07
Chorismate pyruvate	*ubiC*	0.67±1.15	0.67±1.15	22.00±27.62	0.36
Hydrogen cyanide production	*hcnB*	0.00±0.00	0.33±0.57	0.00±0.00	0.37
Phenanzine biosynthesis	*phzF*	15.00±20.22	77.00±67.10	32.00±48.66	0.55
ACC deaminase activity	*acdS*	22.00±38.10	24.00±30.27	1.00±1.73	0.62
Stress-related activities	*btuE*	2.00±2.00	117.67±118.34	32.00±44.64	0.55
	Gst	3.00±2.65	13.33±17.21	99.33±148.46	0.50
	katE	102.67±148.46	535.67±444.35	282.00±428.26	0.39
	Sod1	0.67±1.15	0.67±1.15	0.00±0.00	0.56

Mean ± standard deviation (n = 3). p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

Table S9: Putative genes contributing to endophytic lifestyle as observed in root- associated endophytic microbiome of maize plant

Traits associated with endophytic lifestyle	Putative endophytic genes	CK	FK	NK	P-Value
Chemotaxis and motility	*Aer*	8.00±13.86	4.00±6.08	33.00±54.79	0.64
	cheZ	18.33±28.36	6.33±10.96	14.00±15.09	0.72
	cheC	16.00±17.69	1.33±2.31	1.00±1.00	0.47
	cheD	41.67±48.56	1.67±1.52	0.67±1.15	0.37
	CheV	11.33±10.40	1.00±1.73	93.67±158.78	0.21
	flhA	26.33±37.02	3.00±2.65	43.67±45.06	0.50
	flhB	15.33±19.73	0.33±0.57	14.00±12.29	0.17
	flhF	22.33±27.79	9.33±16.17	8.00±13.00	0.42
	fliL	4.00±4.58	0.00±0.00	8.00±10.58	0.22
Transcriptional Regulators	*NifA*	0.67±0.58	3.67±4.62	5.67±5.13	0.47
	sdiA	0.33±0.57	2.33±3.21	45.00±63.73	0.37
	Crp	70.33±59.69	23.67±31.01	76.66±94.00	0.59
	norR	0.00±0.00	0.67±1.00	0.00±0.00	0.11
	nadR	0.00±0.00	0.00±0.00	0.67±1.15	0.37
	ampR	302.00±266.61	232.67±203.00	81.33±91.88	0.43
	pyrR	1.33±1.53	1.67±2.89	0.00±0.00	0.35
	tenR	0.00±0.00	82.33±129.83	37.00±41.58	0.24
Transport system	*Kpst*	0.00±0.00	12.33±20.53	0.33±0.57	0.24
	potD	16.67±28.86	65.33±101.28	5.00±2.00	0.66
	dppF	1.33±1.15	1.00±1.00	45.33±58.33	0.54
	livK	1.00±1.00	51.00±77.35	23.33±39.55	0.22
	fliY	0.00±0.00	0.33±0.57	22.67±19.86	0.20
	metN	26.00±32.05	224.00±115.88	78.33±86.66	0.11
	hisJ	0.33±0.58	0.00±0.00	18.33±23.63	0.20
	AraG	12.00±8.66	738.00±1249.73	147.67±242.04	0.58
	celB	35.67±50.93	36.33±57.73	12.67±13.58	0.92

	pstG	3.33±2.89	93.00±161.08	8.66±14.15	0.96
	manY	0.00±0.00	1.67±2.89	2.67±3.79	0.28
	mdtB	0.67±0.57	6.67±11.55	9.00±9.00	0.79
	tctA	70.33±64.58	102.00±139.76	33.33±46.14	0.73
	Dctp	5.00±8.66	4.67±8.08	0.00±0.00	0.56
Secretory system	*yscJ*	0.33±0.58	0.33±0.58	29.00±45.13	0.20
	virB2	0.33±0.58	0.33±0.58	62.00±107.39	0.96
	Hcp	0.67±1.15	5.00±5.00	0.67±1.15	0.20
	fimA	0.00±0.00	7.67±13.27	0.00±0.00	0.37
	Bfo	31.67±38.83	124.67±170.46	93.33±144.68	0.73

Mean ± standard deviation (n = 3). p-values based on Kruskal–Wallis test. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

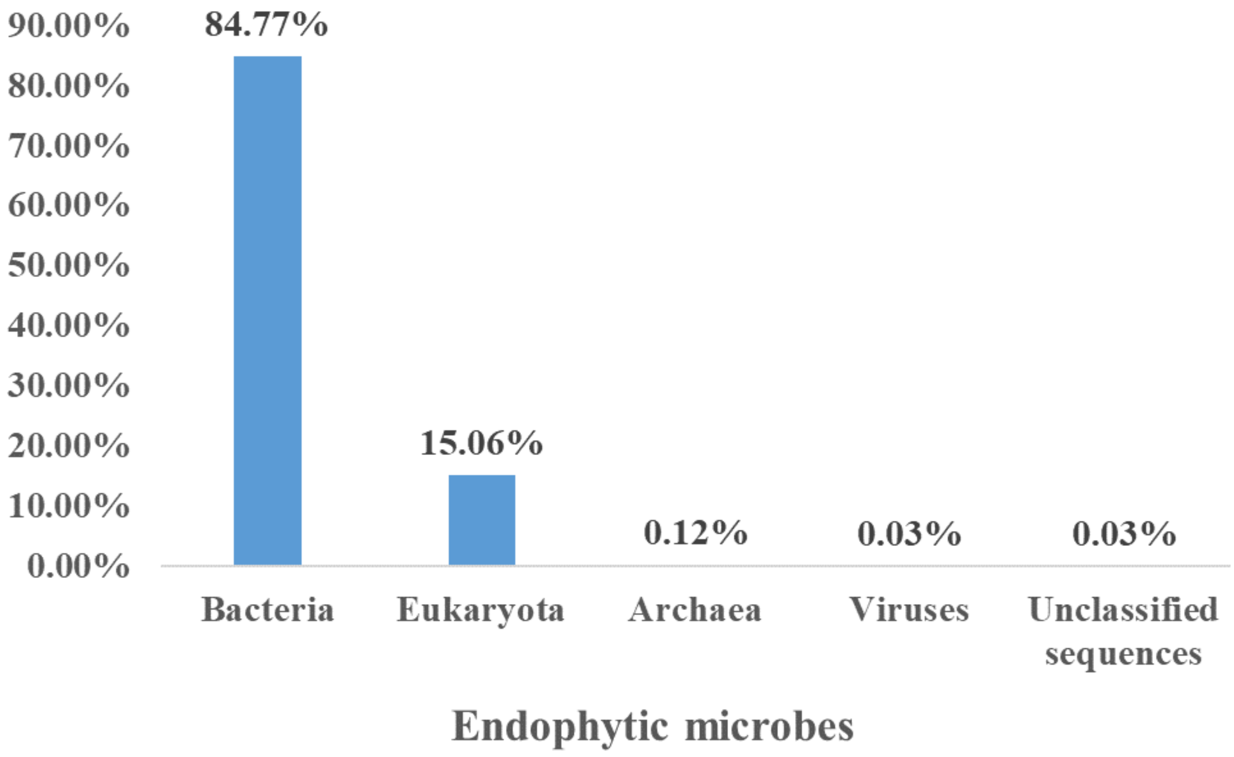

Figure S1: Distribution of endophytic microbes in the processed sequenced data.

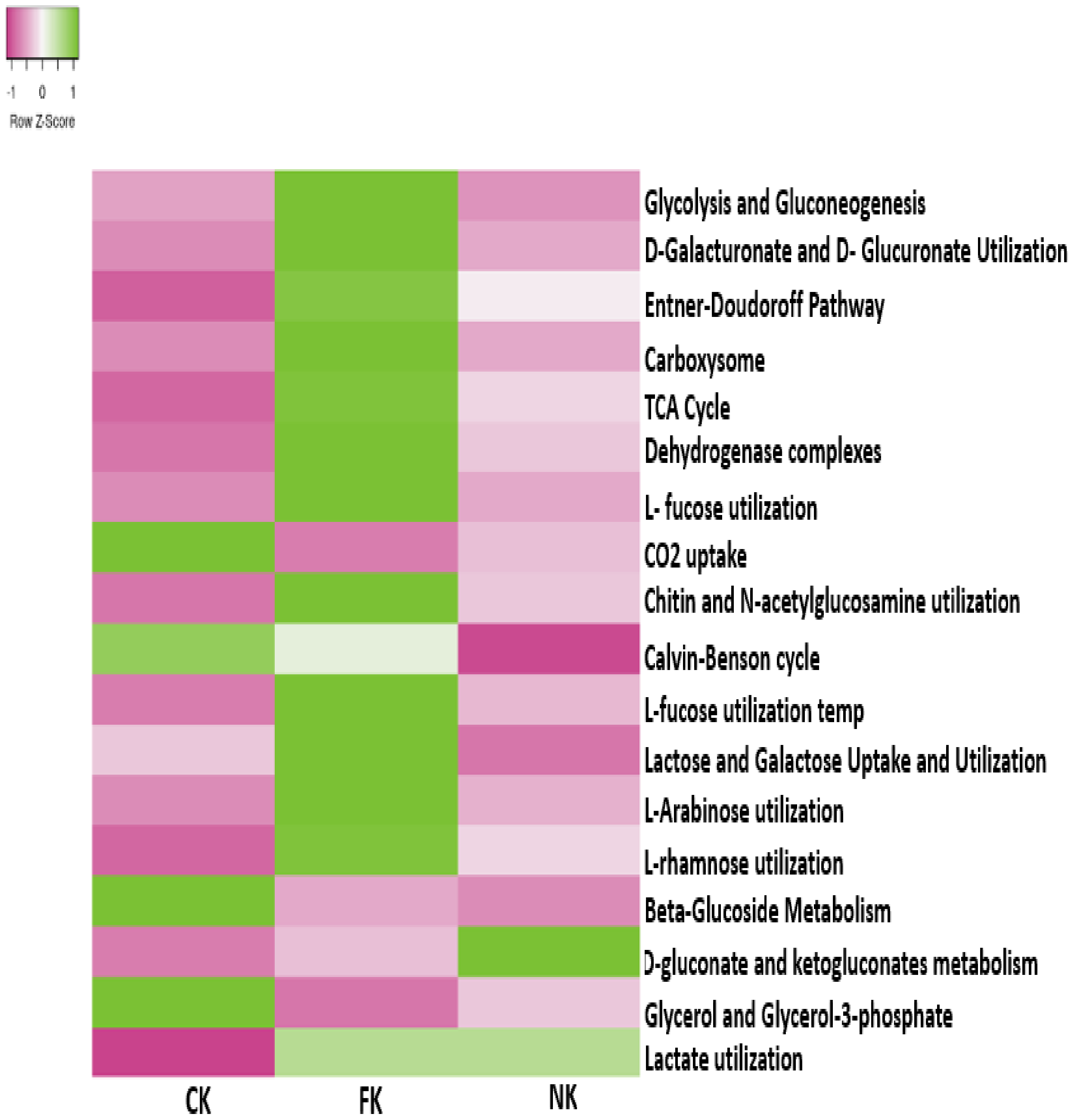

Figure S2A: Heat map showing the relative abundance of pathways involved in carbohydrates metabolism in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

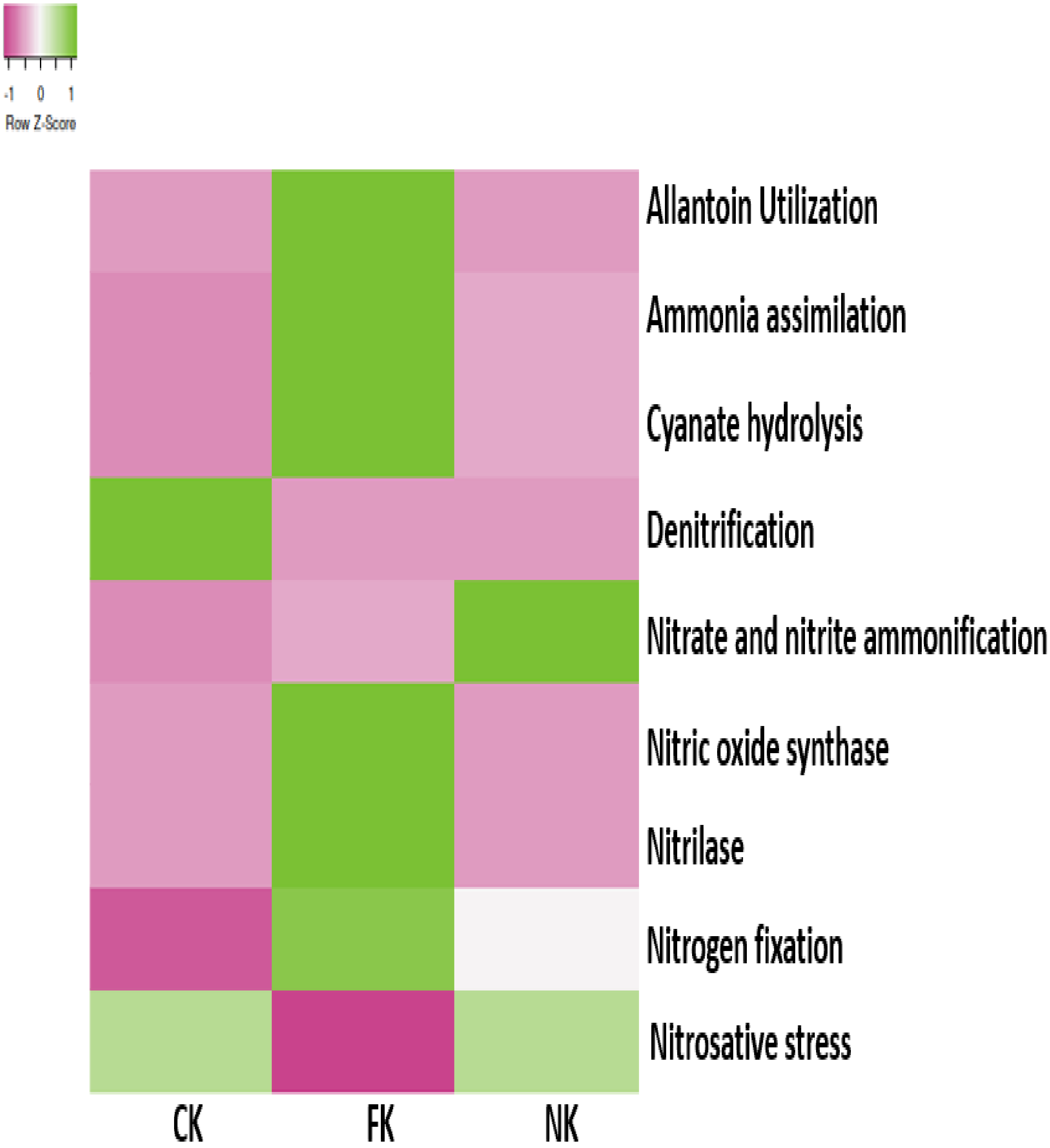

Figure S2B: Heat map showing the relative abundance of pathways involved in nitrogen metabolism in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

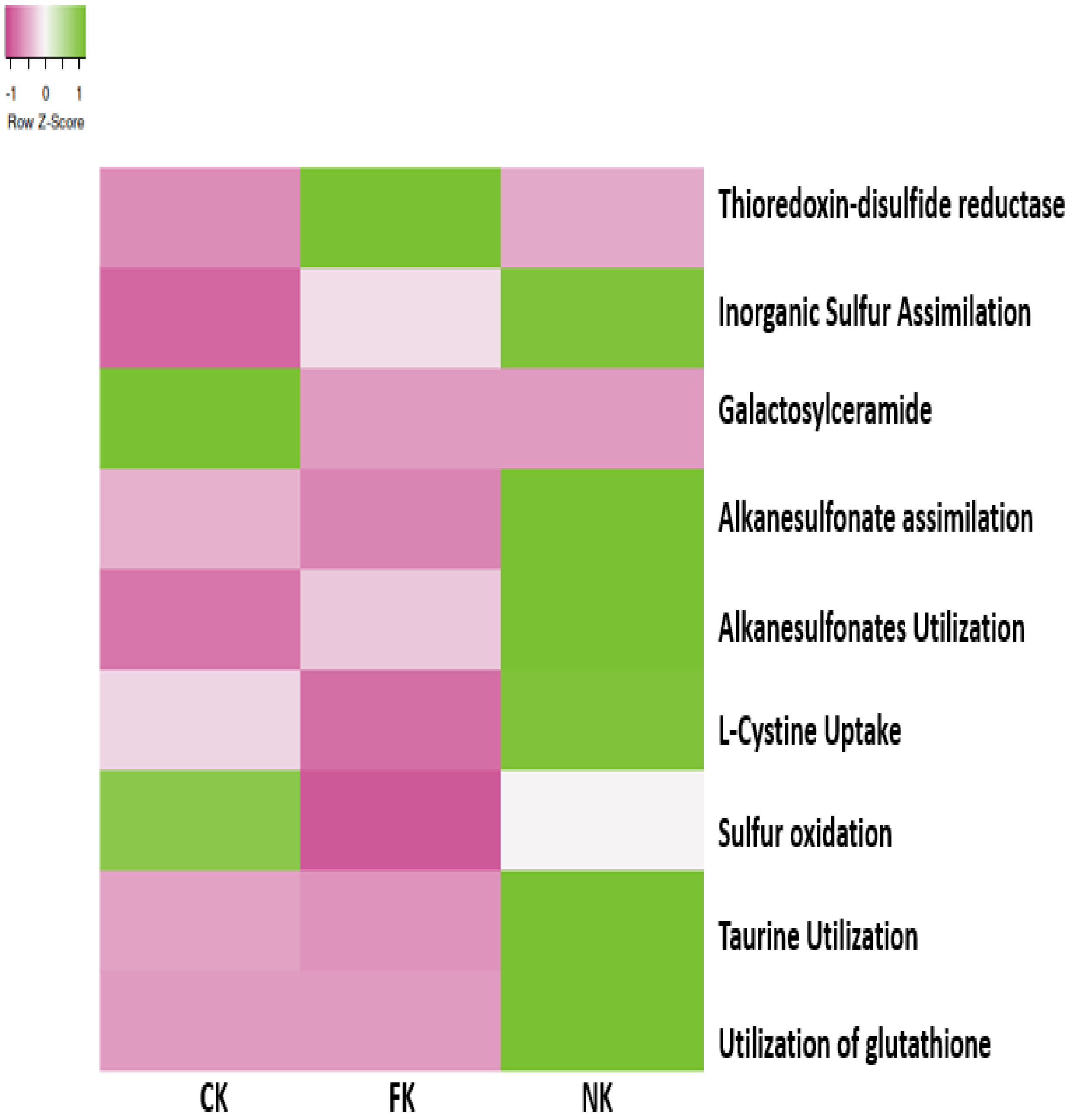

Figure S2C: Heat map showing the relative abundance of pathways involved in sulfur metabolism in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

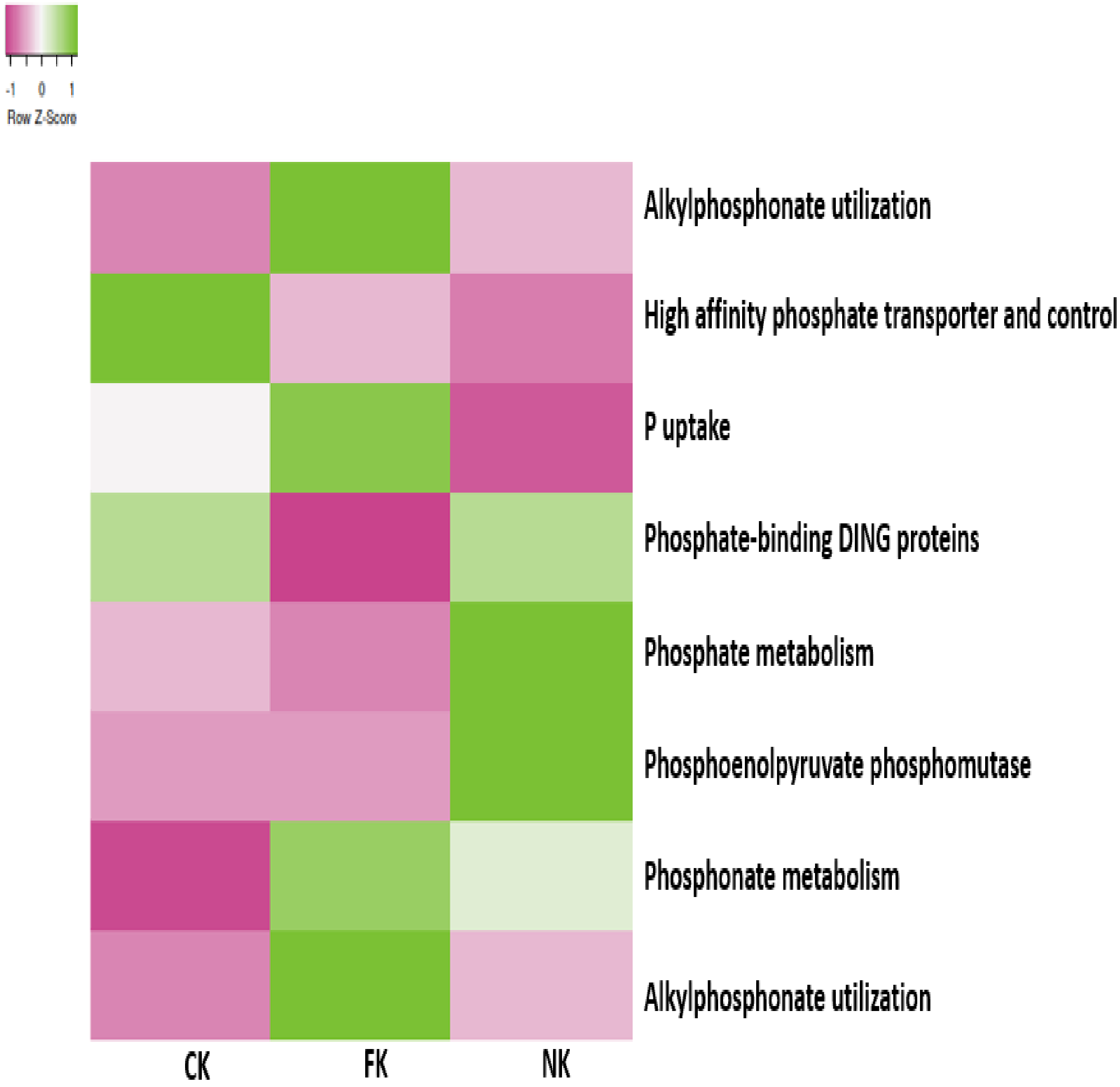

Figure S2D: Heat map showing the relative abundance of pathways involved in phosphorus metabolism in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

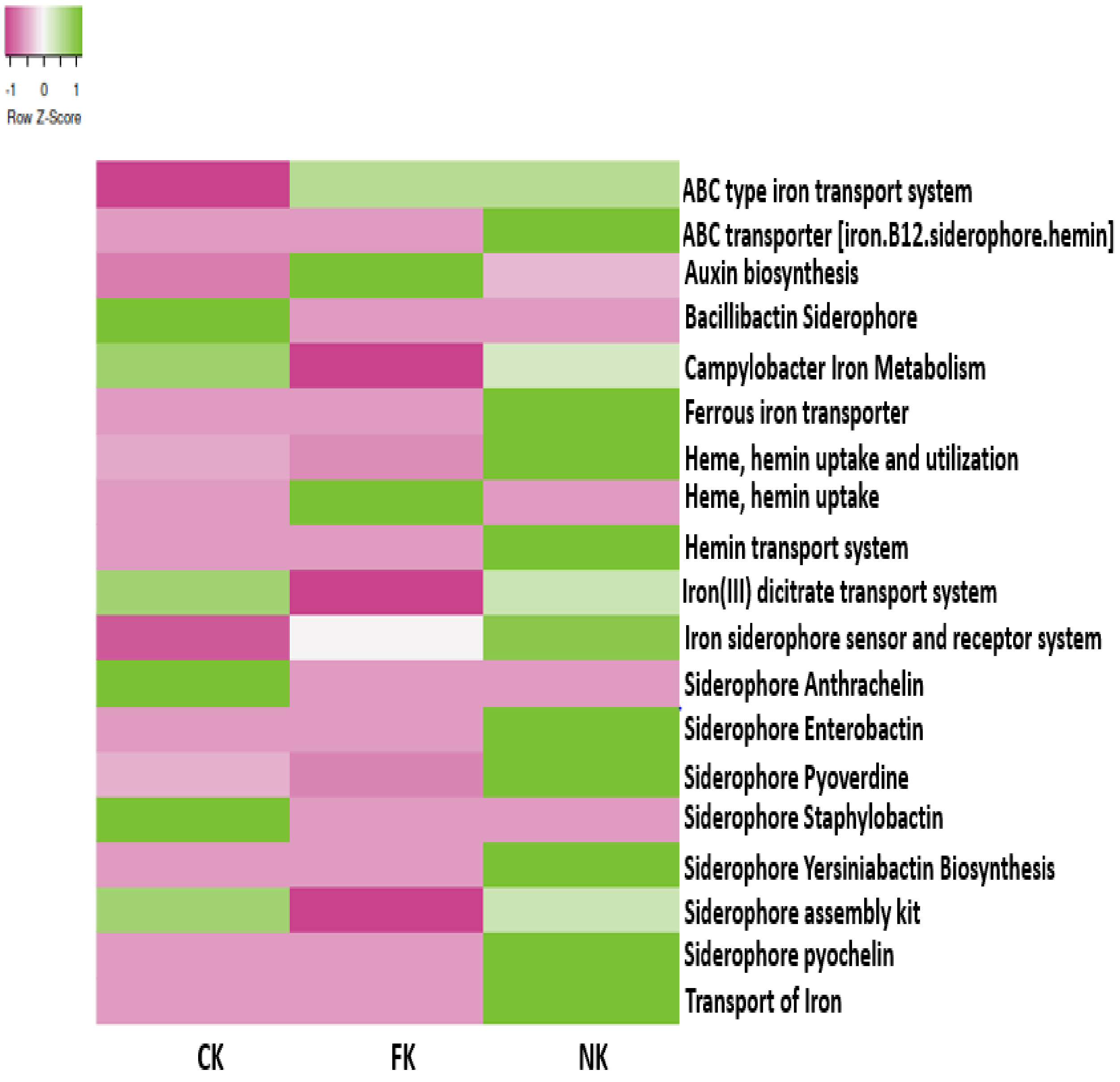

Figure S2E: Heat map showing the relative abundance of pathways involved in secondary and iron metabolisms in samples across the sites. The scale bar represents the color saturation gradient based on the relative abundances with a z-score transformed relative abundance of the endophytic bacteria taxa. NK= samples from inorganic fertilizer site, FK= samples from organic fertilizer site, CK= samples from no fertilizer site

OTHER PUBLICATION

Shotgun Metagenomic Data of Root Endophytic Microbiome of Maize (*Zea mays* L.)

Olubukola Oluranti Babalola, **Ayomide Emmanuel Fadiji** and Ayansina Segun Ayangbenro

Published in *Data in Brief* 2020; 31:105893. https://doi.org/10.1016/j.dib.2020.105893

Abstract

This dataset represents the root endophytic microbial community profile of maize (*Zea mays* L.), one of the largest food crops in South Africa, using a shotgun metagenomic approach. To the best of our understanding, this is the first account showcasing the endophytic microbial diversity in maize plants via the shotgun metagenomics approach. High throughput sequencing of the whole DNA from the community was carried out using NovaSeq 6000 system **(**Illumina). The data obtained consists of 10,915,268 sequences accounting for 261,906,948 bps with an average length of 153 base pairs and 43% Guanine+Cytosine content. The metagenome data can be accessed at the National Centre for Biotechnology Information SRA registered with the accession number PRJNA607664. Community analysis was done using an online server called MG-RAST, which showed that 0.12% of the sequences were archaeal associated, eukaryotes were 15.06%, while 84.77% were classified as bacteria. A sum of 28 bacterial, 22 eukaryotic and 3 archaeal phyla were identified. The predominant genera were *Bacillus* (16%), *Chitinophaga* (12%), *Flavobacterium* (4%), *Chryseobacterium* (4%), *Paenibacillus* (4%), *Pedobacter* (3%) and *Alphaproteobacteria* (3%). Annotation using Cluster of Orthologous Group (COG) revealed that 41.47% of the sequenced data were for metabolic function, 24.10% for chemical process and signaling, while 17.43% of the sequences were in the poorly characterized group. Annotation using the subsystem method showed that 18% of the sequences were associated with carbohydrates, 9% were for clustering-based subsystems, and 9% contain genes coding for amino acids and derivatives, which might be beneficial in plant growth and health improvement.

Keywords: BLAT, Illumina NovaSeq, Maize metagenome, MG-RAST, Plant microbial community, Novel genes.

Table S10: Specification Table

Subject	**Microbiology**
Specific subject data	Environmental Microbiology
Type of data	Raw NGS data
How data were acquired	Shotgun sequencing using NovaSeq 6000 system (Illumina), then structural analysis and annotation of the metagenome through MG-RAST
Data format	Raw data (fastq.gz.file)
Parameters for data collection	Samples from the environment, plant root metagenomes and maize plants.
Description of data collection	Metagenomic DNA extraction from the roots of maize plants from North-West University Farm, Molewane using DNeasy Plant Mini kit (Qiagen), NGS on NovaSeq 6000 system (Illumina) and analysis carried out using MG-RAST
Data location/source	North-West University, Mafikeng, NorthWest, South Africa (S25º47'25.24056", E25º37'8.17464").
Data Accessibility	National Centre for Biotechnology Information SRA DIN: PRJNA607664 URL: https://www.ncbi.nlm.nih.gov/sra/PRJNA607664

Value of the data

- Endophytic microbial communities’ resident in maize plant could serve as a reservoir of plant growth-promoting compounds and novel genes which can help in the growth and health improvement of crops.
- They could serve as an alternative to synthetic fertilizers via the discovery of eco-friendly biofertilizers and potential biocontrol agents in the management of crop diseases.
- Future studies should explore the application and contribution of the novel microbial species and gene discovered in this study for improved agricultural practices.

Data description

This dataset contains raw NGS data obtained via shotgun sequencing of maize plant metagenome from South Africa. All datasets obtained in fastq.gz file were deposited at the National Centre for Biotechnology Information SRA database (PRJNA607664). Details of the microbial community and functional structure using SEED subsystem of endophytic microbial communities in maize plants are shown in Figure 1 and Figure 2 correspondingly.

Experimental design, materials and methods

Fresh roots of maize plants were collected from the North-West University school farm (S25^{o}47'23", E25^{o}37'15"), Molelwane, Northwest, South Africa. Surface sterilization of the maize roots was carried out using standard methods as described by (Correa-Galeote et al., 2018), the whole community DNA was extracted from maize plant using Qiagen DNeasy Plant Mini Kit, following guidelines as described by the manufacturer. Shotgun metagenomic sequencing was done using NovaSeq 6000 system (Illumina, USA) following standard methods as provided by the manufacturer. Structural analysis and functional annotation of sequenced data were carried out using an online server called Metagenomics rapid annotation subsystem (MG-RAST) (Meyer et al., 2008) using default specifications. After quality assessment, sequenced data were annotated using a BLAST-like alignment algorithm called BLAT (Kent, 2002), against M5NR database (Wilke et al., 2012) which offers a concise alliance with other numerous databases.

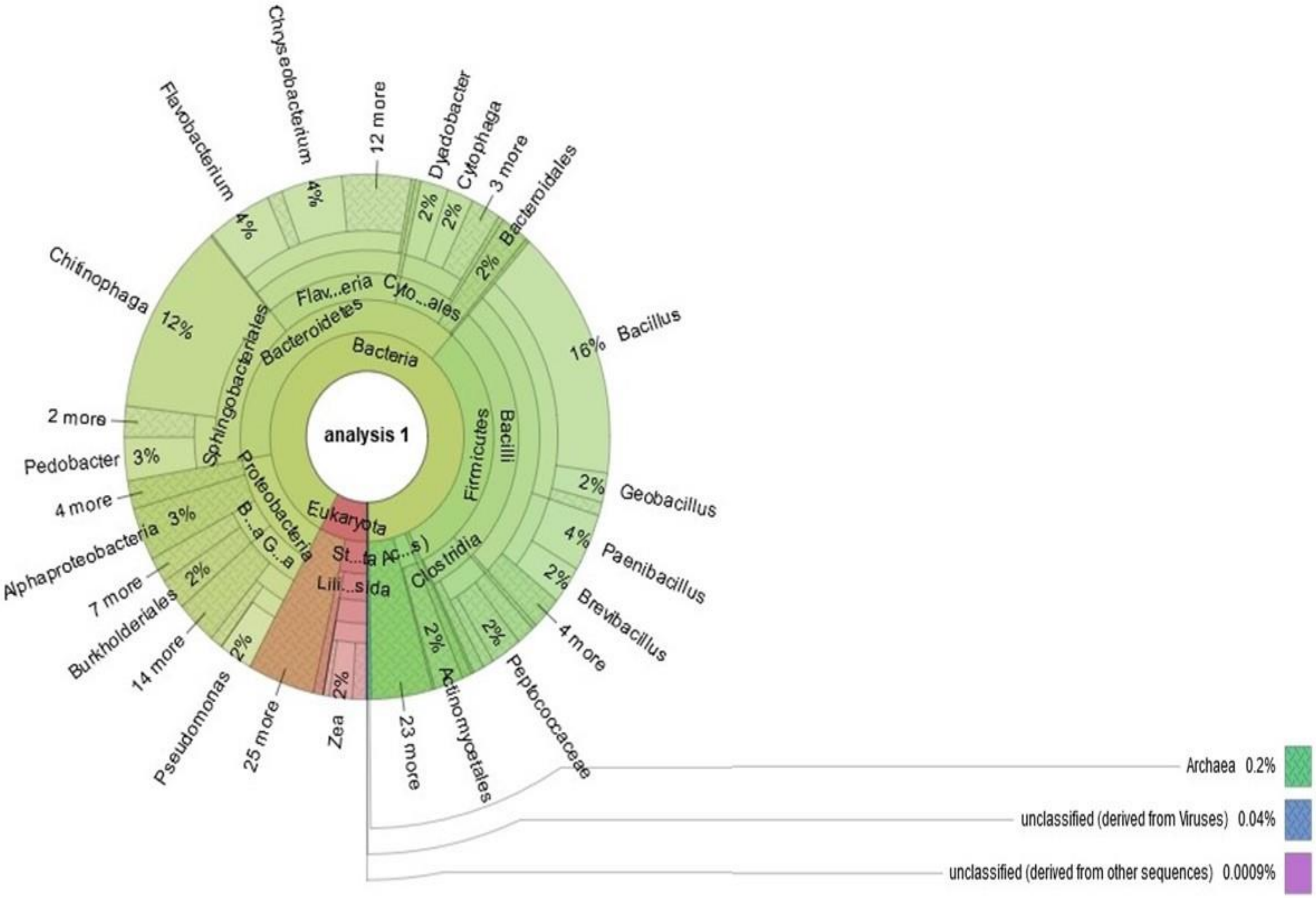

Figure S3: Structure of endophytic microbial communities inhabiting maize plant

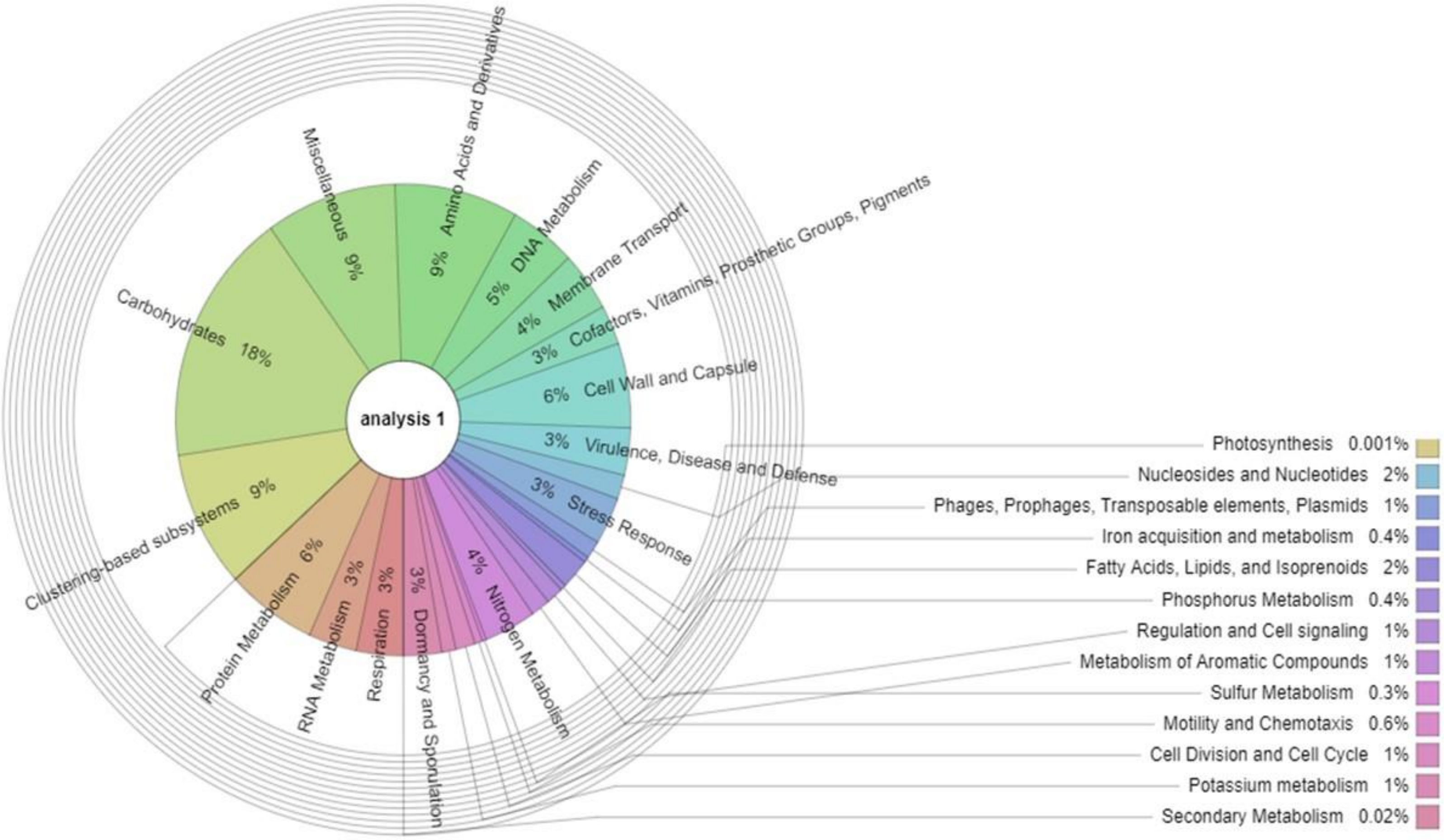

Figure S4: Functional structure of endophytic microbial communities inhabiting maize plants using SEED subsystem

APPENDICES

Appendix1: List of pipeline/tools used for the downstream analysis through MG-RAST

Pipeline	Function	Available code
SolexaQA	trim low-quality reads and to dereplicate the metagenomic data	module load SolexaQA++ module load R mkdir dynamictrim SolexaQA++ dynamictrim read_R1.ad.fastq -h 22 -b -d dynamictrim/
DRISEE	Assessment of the sample sequencing error based on artificial duplicate read	Usage: drisee.py [options] input_seq_file output_stat_file_pattern Input/Output summary: Input: fasta/fastq file (input_seq_file) Output: error matrix file(s) (output_stat_file_pattern) STDOUT: Runtime summary stats
Bowtie aligner	Screening of reads for unwanted genomes related to model organisms such as mouse, human, cow, and other animals	-Aligning unpaired reads bowtie2 -x example/index/metagenome sequence -U example/reads/longreads.fq
BLAT	It is used for the annotation of sequences against the M5NR database, which provides nonredundant incorporation of different databases.	run "Blat -install smtp.yoursite.tld youruserid@yoursite.tld"

Appendix 2: Some of the annotation sources presently available on MG-RAST through the M5NR mechanism

Database	Sources	Type
KEGG	KEGGhttp://www.genome.jp/kegg	Protein
SEED	SEEDhttp://www.theseed.org/	Protein
SwissProt	UniProthttp://www.uniprot.org/	Protein
RefSeq	NCBIhttp://www.ncbi.nlm.nih.gov/	Protein
COG	eggNOGhttp://eggnog.embl.de/	Functional Hierarchy
KO	KEGGhttp://www.genome.jp/kegg	Functional Hierarchy
Subsystems	SEEDhttp://www.theseed.org/	Functional Hierarchy
NOG	eggNOGhttp://eggnog.embl.de/	Functional Hierarchy

Appendix 3: Details of the analysis pipelines used in MG-RAST