Maize microbial community modulates plant health in North West Province, South Africa

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DECLARATION

I, **Dlamini Siphiwe Prudence**, 40417018 a student registered for the degree of Master of Science in Biology in the 2022 academic year.

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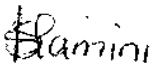
SIGNATURE: ……  **……**

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Declaration by supervisor

I confirm that the candidate worked on the project detailed in her dissertation while I was supervising her.

Professor Olubukola Oluranti BABALOLA

SIGNATURE…….

DATE…….

DEDICATION

This work is dedicated to the person of the Holy Spirit who is the very reason for my being and every good thing.

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GENERAL ABSTRACT

Food sustainability is a serious agricultural issue that needs to be watched. The effective functioning of the rhizosphere microbiome significantly contributes to plant development, disease resistance, and agricultural sustainability. Hence, it is a significant predictor of plant health. Northern Corn Leaf Blight (NCLB) is the most prominent leaf disease of maize caused by the ascomycete *Setosphaeria turcica* and anamorph *Exserohilum turcicum* and is affecting maize production in South Africa. It is interesting to note that little is known about how this disease impacts the functional diversity and community structure of rhizosphere microbial communities associated with maize plants. Therefore, using a shotgun metagenomics method, we investigated the community structure and functional diversity of the microbiome living in the rhizosphere of NCLB diseased and healthy maize plants at selected farms in South Africa's North West Province. The study was carried out from NCLB diseased and healthy maize plant rhizosphere at the North West University Farm (Mafikeng) and a Farm in Lichtenburg. The rhizosphere soil samples tightly bound to maize roots were collected in replicates from different healthy and NCLB diseased maize plants from Lichtenberg and Mafikeng, North West Province, South Africa. The DNA was extracted and libraries were prepared and sequenced conducted with 300 cycles on an Illumina NovaSeq 6000 system. Each metagenome sequence was annotated against the M5NR database, and MG-RAST v4.0.3 server was used at default settings, to execute downstream analytical processing. Sequences were submitted to NCBI SRA with bioproject number PRJNA763110. With the aid of the SEED subsystem database, a total of 12 bacteria, 4 archaea, and 2 fungus phyla were discovered as being predominant across the fields. *Proteobacteria, Dienococcus-Thermus, Gemmatimonadetes, Chlorobi, Cyanobacteria, Planctomycetes, Verrucomicrobia, Acidobacteria, Firmicutes, Chloroflexi,* and *Bacteroidetes* were the most prevalent phyla of bacteria. *Basidiomycota* and *Ascomycota* were the two most prevalent fungal phyla, and Archaea was made up of *Euryarchaeota,*

Thaumarchaeota, Crenarchaeota, and *Korachaeota*. In the order LI > MA > LID > MAD, microbial abundance and diversity were higher in the rhizosphere of healthy maize (LI and MA) compared to the NCLB diseased (LID and MAD). We also found that the rhizosphere of the healthy maize plant was dominated by 24 functional categories, whereas the rhizosphere of the infected maize plant was dominated by 4 functional categories. Alpha diversity analysis revealed no difference between the healthy and diseased maize rhizospheres ($p>0.05$). The examination of beta diversity, however, revealed a substantial distinction. The significant abundance of functional groups found, particularly in LI, suggests that the presence of plant diseases changed how the soil microbiome works. The shotgun metagenomics study unveiled higher microbial abundance and diversity in the healthy rhizosphere. This positively influence the rhizosphere microbiome in modulating the microbial functions towards the management of NCLB and plant health.

Keywords: Microbial interaction; metagenomics; shotgun sequencing; food safety

RESEARCH OUTPUTS

1. **Chapter 2:** Dlamini, S. P., Akanmu, A. O., & Babalola, O. O. (2022). Rhizospheric microorganisms: The gateway to a sustainable plant health. *Frontiers in Sustainable Food Systems*, 6, 925802 (Published).
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3. **Chapter 4:** Dlamini, S. P., Akanmu, A. O., Fadiji A.E., & Babalola, O. O. (2022) Maize rhizosphere modulates the microbiome diversity and community structure to enhance plant health. *Saudi Journal of Biological Science*, *Elsevier.* (Published)
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4. **Chapter 5:** Dlamini, S. P., Akanmu, A. O., & Babalola, O. O. (2022) Variations in the Functional Diversity of Root Microbiome of Healthy and Northern Corn Leaf Blight Infected Maize (Zea mays L.). This manuscript was submitted to *Spanish Journal of Soil Science, Frontiers Journal*
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Poster presentation

Akinlolu Olalekan Akanmu, **Siphiwe Prudence Dlamini**, Ayomide Emmanuel Fadiji, Olubukola Oluranti Babalola. Maize rhizosphere modulates the microbial community structure and functions to enhance plant health. Poster presented at the 2nd Africa Food Safety Workshop 2022, dated 27th of June- 1st of July 2022, Emperor's Palace, Johannesburg, South Africa.

ABBREVIATIONS

NCBL	Northern Corn Leaf Blight
LI	Healthy samples from Lichtenburg site
LID	Diseased samples from Lichtenburg site
MA	Healthy samples from North West University farm (Mafikeng) site
MAD	Diseased samples from North West University farm (Mafikeng) 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
DNA	Deoxyribonucleic Acid
e^H/S	Evenness
α	Alpha
β	Beta

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Maize is an important food crop in the world, and the most significant grain crop in South Africa (Mangani *et al.*, 2019). In addition, this crop, which produces the most starch among cereals (5.9 t/ha), is crucial for indirect food security. The quantity of external input required in agriculture to produce this amount of crop may not be long-term sustainable (Jat et al., 2021a). Despite the management systems in place, maize health and productivity are often impaired by diseases, with the most commonly reported in South Africa including northern corn leaf blight, charcoal-rot, bacteria stalk rot, common rust, black bundle disease and late wilt and downy mildews among others (Okoth *et al*., 2017; Schoeman *et al*., 2018). These diseases, particularly severe critical diseases like northern corn leaf blight (NCLB), can result in over 30% productivity loss, which might pose a serious threat to the corn harvest (Xia *et al*., 2020). Since, many of the causative pathogens of the disease are soilborne, and with the array of reported beneficial soilborne microbes (Omomowo and Babalola, 2019), the structure and function of soil microbial communities are frequently used as indicators defining the soil fertility and quality, because of the fast response of organisms to abiotic and biotic modifications (Enagbonma *et al*., 2020).

A dynamic ecological niche where microbial interactions and structures are formed is the rhizosphere of maize. These are formed by physicochemical properties of the soil, the presence of exudates from the plant roots, and other environmental conditions (Lladó *et al.*, 2017). In the rhizosphere, there are more active microorganisms than in the bulk of the soil, including saprotrophs, plant diseases, and beneficial microbes (Benitez *et al.*, 2017). Numerous research studies have proved for more than a decade that plant–microbe interactions are critical not only

for understanding plant health and growth, but also for understanding agricultural sustainability and nature conservation (Fadiji *et al*., 2021b). Therefore, to be fully aware of the predicted benefits of plant microbiomes in agriculture, it requires comprehensive knowledge of the mechanistic and functional aspects of interactions between microorganisms, plants, agricultural management techniques and the farm environment (Busby *et al*., 2017). The natural compounds released by plant roots in exudates enhance the abundance of microbial and functional diversity, rather than in the rhizosphere, causing the formation of highly active and distinctive microbial communities linked with plant roots (Chukwuneme *et al.*, 2021).

Rhizosphere microbes protect against pathogens, produce plant hormones to improve growth, and may help adaptation of plants to environmental stimuli such as an abnormal change in temperature, salinity, and drought (Lu *et al*., 2018). Securing soil integrity is vital because climate stability, biodiversity services, energy, water, and food security rely on its functioning (Enagbonma *et al*., 2020). The interaction between soil microbial diversity and the food web has received more attention over the years due to its considerable effect on food safety (Akinola *et al*., 2021b). Previously, microbial analysis included routine culturing and characterization of organisms (Akinola *et al*., 2021a). Despite the fact that more than 95% of soil microbial species cannot be grown in culture, metagenomic analysis is revolutionizing our understanding of the variety and breadth of soil microbial communities and their interactions' abilities to function (Kellog, 2021). Since there exists a pausity of knowledge on the diversities, functions, and roles of the unassessed rhizospheric microbiomes of maize, there is therefore a need for further investigation to explore the microbial diversity and functions associated with rhizosphere of diseased and healthy maize, towards their productive deployment in plant health management.

1.2 Problem Statement

Maize production is affected by various factors including insects, environmental stressors such as drought and sunburn, and diseases which can frequently cause up to a 40% direct loss in production yield (Back *et al*., 2021). Despite its importance, maize yields are expected to declines by more than 10% by 2050 (Murdia *et al*., 2016), a situation which is set to be magnified by a projected increase in demand for maize in the developing world (Zunjare *et al*., 2014). Many of the diseases associated with maize-based crop decline are caused by soilborne microorganisms which in turn may have a significant impact on grain yield and quality (Schoeman *et al*., 2018). In the instance of rhizoshphere soil which are also noted to habour microbiome with plant growth promoting traits an assessment of these microorganisms can at times present a challenge as the majority of the organisms are non-culturable. There is an abundance of unknown functions associated with maize rhizospheric microbiomes, which indicates the need for further investigation to explore their productive deployment in plant health management. Such an investigation should employ metagenomics instruments to explore the community structure and functional diversity of these organisms at plant's proximity.

1.3 Justification

Maize is one of the most important economic crops, it is imperative to gain a better understanding of the individual roles of the native microbial community in plant growth. The rhizosphere plays the host to a wide range of biological activities which predetermine the health status of plants (Glick and Gamalero, 2021). It harbours vital microorganisms of agricultural importance, and their functional uniqueness remained relatively untapped. Taking into account that the functional uniqueness and application of soil microorganisms are under investigated given their relative importance in crop growth, there is therefore the need to employ metagenomics instruments to explore the community structure and functional diversity of these

organisms at plant's proximity. These investigations will explore such benefits towards plant health management and show difference between the taxonomic and functional diversity of the maize rhizosphere of NCLB healthy plants compared to the diseased. To the best of our knowledge, no research has been done utilizing shotgun metagenomic analysis to compare the structure, functions, and diversity of rhizosphere microbiomes in the roots of NCLB affected and healthy maize plants. Therefore, using a shotgun metagenomic method, this study gives the first report assessing the structure and roles of rhizosphere microbiomes in the roots of NCLB diseased and healthy maize plants. Using this method, which is culture independent, we can analyze how the NCLB has affected the variety, abundance, and functions of the rhizosphere microbiomes that are present in the maize plant and are known to be extremely important for the development, growth, and health of their host. Also, to enhance the development and growth of maize plants, the exudates released by plant roots, including aromatic acids, enzymes, organic acids, and sugars, form interactions with soil microbial communities and soil properties such as texture, pH, soil moisture, organic matter, and available nutrients. Therefore, it is assumed that due to variations in soil qualities seen at the study sites, microbial functions in our study samples will vary.

1.4 Research aims and objectives

1.4.1 Specific Aims

Aim: To investigate the diversities and functions of maize plant's native microbial community towards plant health management, using the shotgun metagenomics approach.

1.4.2 Specific Objectives

Objective:

(i) To determine the diversity and taxonomic abundance of microbial community associated with maize rhizosphere of diseased and healthy sites, at two selected maize farms in the North West province.

(ii) To investigate the functional diversity of maize rhizosphere microbiome using metagenomics and bioinformatic tools.

(iii) To determine the physicochemical properties of soil samples obtained from each location.

CHAPTER TWO

2.0 RHIZOSPHERE MICROBIOME: THE GATEWAY TO A SUSTAINABLE PLANT HEALTH

Abstract

Plant health is essential for food security, and constitutes a major predictor of safe and sustainable food systems. Over 40% of the global crops' productions are lost to pests, insects, diseases, and weeds, while the routinely used chemical-based pesticides to manage the menace also have detrimental effects on the microbial communities and ecosystem functioning. The rhizosphere serves as the microbial seed bank where microorganisms transform organic and inorganic substances in the rhizosphere into accessible plant nutrients as plants harbor diverse microorganisms such as fungi, bacteria, nematodes, viruses, and protists among others. Although, the pathogenic microbes initiate diseases by infiltrating the protective microbial barrier and plants' natural defense systems in the rhizosphere, whereas the process is often circumvented by the beneficial microorganisms which antagonize the pathogens to instill disease resistance. The management of plant health through approaches focused on disease prevention is instrumental to attaining sustainable food security, and safety. Therefore, an in-depth understanding of the root microbiomes in response to crop development as discussed in this review opens up more opportunities of reaping the profit of beneficial root–microbiomes' interactions towards attaining sustainable plant health.

Keywords: microbial community, food security, food safety, plant-soil-microbe association, rhizosphere engineering, plant disease management.

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2.1 Introduction

Agricultural management practices, such as crop rotation, fertilizer application, and land tilling modify soil physical and chemical properties, amidst diverse interacting forces which shape soil microbial populations (Schmidt *et al*., 2019). The important plant microbiomes can be categorized into aboveground and belowground. The phyllosphere/ phyllobiome characterized by the aboveground is made up of leaves, stems, and reproductive organs, while the rhizosphere/ rhizobiome which consists of the belowground is the roots' part and the associated soil (Lucaciu *et al.*, 2019). The interactions between plant roots and microorganisms have a direct impact on the soil, and the rhizosphere region is the home to diverse microorganisms (Adedeji and Babalola, 2020), and a "hot spot" of intensive microbial activity (Dai *et al*., 2019). The plant microbiome consists of a variety of microbes such as bacteria, fungi, protists, archaea, and viruses that reside on or inside their host plants (Zhang *et al*., 2021). Rhizosphere-associated microbes have an extensive variety of metabolic abilities and play an important role in the rhizosphere environment, such as organic matter breakdown and nutrient cycling, both of which benefit plant growth and health. More importantly, the microbial population aids plant development and responds to a range of environmental conditions (Dai *et al.*, 2019).

Plants are key sources of energy input in a plant-soil subsystem, providing nutrients and carbon for heterotrophic microbial communities in the form of root exudates and litter deposits (Shanmugam and Kingery, 2018). Through rhizodeposition which is a major phenomenon that occurs in the rhizosphere, high- and low-molecular-weight compounds including secondary metabolites, organic acids, amino acids, sugars, vitamins, and polysaccharides are released into the surrounding soil in the form of exudates. This increases the richness of microbiological and functional diversity, leading to the formation of extremely lively and unique microbial communities linked with plant roots (Chukwuneme *et al.*, 2021). Although the microbiome is

made up of a variety of organisms, most research on microbial function in disease prevention and plant health has concentrated on fungi and bacteria (Xiong *et al*., 2020).

As reported in a recent study, soil and climatic characteristics are significant determinants of microbial community composition, variety, and metabolic potential in a range of the surroundings (Lladó *et al*., 2018). This is a result of the swift response of the microbes to abiotic and biotic changes. Hence, the structure and function of soil microbial communities are frequently utilized as pointers for determining soil fertility and quality (Enagbonma *et al*., 2020). Further insights into the essential functions of rhizosphere microbiomes and plant genetic characteristics under various situations have been accessed through the advent of OMICS technology which is based on Next-Generation Sequencing (NGS) (Pramanik *et al*., 2020), and provides the gateway to attaining sustainable plant health.

The plant rhizospheric zone is a niche of microbial activity based on the resistance of the root system against phytopathogens. The region provides a variety of benefits to plant health both directly and indirectly (Gupta *et al*., 2019). It is generally recognized that soil microbes serve as a primary nutrient reservoir for plants through their active participation in nutrient cycle processes such as nitrogen cycling, organic matter decomposition and mineral weathering, all of which influence plant growth (Jilling *et al*., 2018). Thus, plant roots and microbe interactions are critical for plant participation in the ecosystem's functioning. Understanding ecosystem responses to the changing environments require knowledge of microbial diversity distribution and functions. More so, root exudates contain an extensive variety of chemicals, including amino acids, carbohydrates, siderophores, and enzymes, that can reinforce the activities of the microbial community and significantly change the structure of the rhizospheric microbial community, contingent on the plant species and genotype (Wei *et al*., 2017). Plant-associated microbial communities promote plant development and resilience to diverse abiotic and biotic stressors. Soil microbial species through their roles in carbon sequestration and biogeochemical

cycling play important roles in the mitigation of climate change that presently constitutes a global concern (Dubey *et al*., 2019). Improved ability to predict functionality from dynamic aberrations in root microbial composition is critical for building 'designer' microbiome interventions and enhancing agricultural production and sustainability. This will necessitate a more comprehensive genomic and functional classification of plant-linked microbes, their genetic factor, and plant-microbe interactions for their exact useful roles (Richardson *et al.*, 2021).

Beneficial, commensal, or pathogenic relationships exist between specific microorganisms and their plant hosts. Plant microbiomes, in many instances, show a beneficial role at the community level, protecting plants from pathogens, while also boosting growth, health, and providing plants with an adaptive edge (Zhang *et al*., 2021). Therefore, the interactions between plants and their microbiota are intricate and constantly changing (Fausto *et al*., 2018). This interaction and interdependence on the abiotic and biotic components of the soil environment can be explained by the plant-soil feedback mechanism, as shown by Shanmugam and Kingery (Shanmugam and Kingery, 2018).

2.1 Plant defense system: Abating the invasion of pathogens by the rhizospheric microbiome

Plant diseases have caused serious economic and nutritional crises in the past and are still responsible for significant losses in global crop production today (Chun and Chandrasekaran, 2019). Among the factors that contribute to production loss, pests and diseases constitute major global damages (Ab Rahman *et al*., 2018). As a result of the unrestrained and widespread use of artificial agrochemicals for plant protection and growth, environmental pollution has become a serious problem. Agrochemical use indefinitely has various negative consequences, including increasing resistance in plant pathogenic microorganisms, detrimental impacts on non-target organisms, and deterioration of soil health (Choudhary *et al*., 2017). When it comes

to plant disease, there is a complex interplay between the host plant and the pathogen, and the resistance/susceptibility response might include a variety of factors (Kumar and Dubey, 2020). Disease suppressive soils are the most visible example of microbe-mediated plant defense against pathogen infection in agricultural soils. Structures such as the physical and chemical barriers, quick active defenses, passive and delayed active defenses including the non-host resistance, all cumulate to plant defense systems. However, the rapid active defenses entail changes in cell wall reinforcement, membrane function, the hypersensitive reaction, and the initial oxidative burst which results in phytoalexins, and programmed cell death.

Systemic acquired resistance (SAR), pathogenesis-related gene expression, pathogen containment, and wound repair are all examples of delayed active defenses. Salicylic acid, which is required for defense against SAR and biotrophic pathogens, along with ethylene and jasmonic acid are both involved in defense against necrotrophic pathogens in addition to beneficial plant-microbe interactions, such as induced systemic resistance (ISR) and priming as examples of plant defense signaling molecules (Ab Rahman *et al*., 2018). Our ability to control and modify the rhizosphere microbiota is currently restricted. Inoculation is the most direct technique to change the microbiome (Kumar and Dubey, 2020). For decades, commercially accessible products comprising of one or more kinds of fungi or bacteria have been available. The majority of these species, however, were obtained using typical culturing methods that do not mimic the chemical milieu of soil. Other options include adding organic materials to soils that contain diverse but undefined microbial mixes in the hopes of improving function and pathogen resistance. In this case, the capacity of beneficial inoculants could be limited to spread (Alori *et al*., 2017). Plant breeding or engineering features are other techniques to engineer the rhizosphere. We will be able to engineer root architecture, specific root exudates, or other plant qualities that support beneficial microbiomes into crops using CRISPR and other gene-editing technologies as we identify root architecture, specific root

exudates, or other plant traits that support beneficial microbiomes. This technology has been effectively explored in safeguarding plants against both biotic and abiotic stress. An instance is the production of rice lines which has broad-spectrum resistance to *Xanthomonas. oryzae* pv. *oryzae* that resulted in the modification of the promoter region of *O. sativa* SWEET11, *O. sativa* SWEET13 and *O. sativa* SWEET14 (Oliva *et al*., 2019), while other studies have also investigated the plant – microbe interactions with the use of CRISPR-Cas (Shelake *et al.*, 2019). This method appears promising because it mimics the interactions that sustain beneficial microorganisms in natural systems and that have been designated through holobiont development (Thomashow *et al*., 2019; Wallenstein, 2017). Chemical pesticides are now widely employed to ensure long-term productivity and harvest, stressing the widespread usage of phytochemical pesticides. Nonetheless, chemical pesticides have negative consequences for crops, the environment, and the health of both farming communities and consumer populations. As a result, alternative and long-term disease methods are required. An alternative pest control strategy using bio-pesticides instead of synthetic or chemical pesticides poses a positive phenomenon in reducing pest threat with prevention of chemical residue deposition, reduced toxicity, combinatorial optimal attributes on efficacy, and reduced environmental impacts in the realm of humans and the environment affecting only the pests will positively have an impact (Chun and Chandrasekaran, 2019). In some recent studies, the success of biopesticde formulations from varying *Trichoderma* species were reported effective in the management of tomoto root rot diseases (Olowe *et al*., 2022), while (Mishra *et al*., 2022) found that the use of 2,4- DAPG-producing *P. fluorescens* JM-1 suppressd *F. monoliforme*-causing ear rot diseases in maize and the PGP features of *P. fluorescens* JM-1 (IAA generation and solubilization phosphate and Zn) also aided in the enhancement of maize growth indices.

Given the importance of microorganisms in nutrient mineralization and solubilization, soil organic matter creation, and pathogen pressure, rhizosphere engineering would benefit from methodologies and tools that permit us to modify the microbiome (Wallenstein, 2017). The interaction between plants and rhizospheric bacteria has been thoroughly investigated for biogeochemical cycling activities, plant growth promotion, and biocontrol, all of which are critical for plant health. Hence the need for in-depth knowledge of microbial diversities in the plant rhizosphere to enable adequate deployment of plant beneficial microbes towards plant growth promotion (Olanrewaju *et al.*, 2019). Variations in the physical environmental condition and structure of the host-associated rhizosphere microorganisms are frequently linked to plant pathogen invasion. Thus, understanding plant-pathogen infections in highly varied field conditions constitutes a critical challenge for food security and crop protection in the future (Wei *et al*., 2018). The pathogen's introduction has the potential to disrupt the plant's finely regulated relationships with its microbiome. Changes in the native microbial community structure and interactions that provide favorable conditions for the pathogen virulence led to fast declines in host health and productivity (Zhang *et al*., 2021). For that reason, the pathogen must compete with other bacterial species for colonization of the plant rhizosphere, which is the initial stage in infection. When a pathogen reaches a certain density, it activates its virulence genes and invades plant roots (Wei *et al.*, 2018), which may infect host tissue and successfully exit the rhizosphere war zone, most soil-borne pathogens must grow saprophytically in the rhizosphere to influence their host or to attain adequate numbers on their host. The microbial population of the soil in which infection occurs has an impact on the pathogen's success. To some extent, typical agricultural soils have the ability to restrict a pathogen. This can be determined by comparing the severity of disease after pathogen inoculation in sterilized and non-sterilized soils (Berendsen *et al*., 2012).

Recent research has found that microbiome functioning, including disease resistance, may result through the interaction of species instead of the existence of a specific feature or species. This has been shown as an emerging approach to overcoming the laboratory to field hurdles, since the use of microbial consortia entails the deployment of microbes with similar mode of action that is able to tolerate different environmental conditions and effective on the plant genotypes, thereby complementing each other. This has been effectively explored in tomato, maize and potato among other crops (Compant *et al*., 2019). Each stage of ecological succession is connected with certain features in the main species, putting the existing community arrangement in the context of a dynamic temporal procedure. When applied to the rhizosphere microbiome, ecological succession can offer a novel prognostic outline for species and trait occurrence, offer new entry points to engineer its ability to prevent diseases, and elucidate how a suppressive microbiome assembles (Hu *et al*., 2020).

2.3 The plant-soil feedback: Essential tools in microbiome management

The past investigations of plant-soil feedback have revealed the varying effect of different plant species on the biotic and abiotic soil conditions, which in turn affects the growths of other heterospecific and conspecific individuals in that soil (Kulmatiski and Kardol, 2008). Either positively or negatively, the plant-soil feedbacks can drive patterns of plant diversity, succession, and invasion, which can shape the community composition and ecosystem functioning. While the environmental forces that occur over the landscape include stress, disturbance, and competition, plants allocate resources to growth, reproduction, and maintenance in diverse ways to cope with these constraints (Beals *et al*., 2020).

One of the basic structures that allow plant stability against both biotic and abiotic pressures is the root system. Although scientists have given less attention to the study of plants root, roots have been found essential for plant productivity and adaptations. The root architecture is made up of structural features such as root length, length of lateral roots, and spread number that

enable plants to exhibit plasticity in response to environmental changes, and the optimum performance of the root (Khan *et al.*, 2016). This three-dimensional spatial organization of root structures is especially important for the function of roots in difficult situations. A deep root structure, for example, is required for plants to use water and nitrate in deeper soil layers, and hence useful for drought resistance, mostly in nitrogen-scarce circumstances. A shallow root structure with increased adventitious roots to promote foraging on the topsoil, on the other hand, is critical for crops to acquire comparatively steady nutrients like phosphorus. Developing cultivars with root architectures that are appropriate for the field conditions promises to be a long-term and cost-effective way to improve crop nutrient efficiency and stress tolerance (Yang *et al*., 2017). The advent of more detailed molecular and analytical tools which allow full explanation of the dynamics of rhizosphere and endosphere communities has sparked transformed interest in plant-microbe interactions.

Recent data has shown that root type and location influence the composition and diversity of the communities of bacterial, fungal, and actinobacterial, including specialized functional groups like arbuscular mycorrhizal fungi, across a wide range of plant species. Differences in root assembly and the quantity and composition of root exudates, as well as plant-microbe, microbe-microbe, and microbe–fauna interactions, all contribute to these variances (Richardson *et al.*, 2021). The importance of interactions between plants and their phyllosphere microbes is gaining attention, as they stimulate plant development and contribute to pathogen defense, these may later alter the suitability of natural plant inhabitants along with the quality and production of agricultural products. Meanwhile, endophytic bacteria have been recognized for their ability to defend their hosts from pests, insects, and microbiological diseases, thereby acting as biocontrol agents (Fausto *et al*., 2018). Crop rotation may also improve microbial diversity in soil because various plant species favor distinct bacteria in the soil due to changes in root exudation. Intercropping, where diverse crops are planted together and the roots of

different plant species interact directly, has a good impact on soil fertility in addition to microbial variety and activity (Mitter *et al.*, 2019).

A greater understanding of the root microbiome succession, such as how the microbes vary in response to crop development, opens up more opportunities of reaping the advantages of beneficial root–microbial interactions (Richardson *et al*., 2021). Metagenomics has a large potential to equip with vital information on plant-microbe interactions, which is required for new technologies to boost agricultural output in the long run (Bramhachari *et al*., 2017).

Soil microbial communities play a contributory role in determining plant growth and soil fertility. The changes that occur in their structure and dynamics in response to various soil management approaches can provide information regarding soil quality and biological complexity (Fausto *et al.*, 2018). Also, microbes such as *Azotobacter* spp*., Azoarcus* spp*., Burkholderia* sp*., Actinorhizobium* spp., *Rhizobium* spp*., Gluconacetobacter* sp*., Herbaspirillum* sp*., Bacillus polymyxa,* and especially *Azospirillum* sp., *Enterobacter,* and *Pseudomonas* spp., that stimulate nutrient acquisition and plant growth have traditionally been utilized in agriculture as single strains to counter nitrogen and phosphorus fertilizer inputs Thus, a variety of parameters, including tissue, genotype, physiological status, growth stage, soil ecological conditions, and agricultural practices influence the plant microbial colonization. Natural population studies, on the other hand, reveal that clusters of bacteria with specific functions play crucial roles in adhering and releasing inorganic nutrients to physical surfaces, along with breaking down organic wastes and integrating them into the soil (Arif *et al*., 2020; Backer *et al.*, 2018; Ray *et al*., 2020). Soil microbes are regarded as a significant constituent of the complex interplay of elements that contribute to the environmental quality required for long-term, healthy food production (Barea, 2015).

Soil organic matter stabilization, decomposition, bioremediation, nutrient cycling, and soil aggregate production are all significant tasks carried out by soil microorganisms. This reaction

with organic matter and inorganic fertilizers consequently determines the fitness of soil health for crop production (Dangi *et al*., 2020). Furthermore, plant beneficial microbes are essential in the development of plant growth stimulators, bio-pesticides, and bio-fertilizers to decrease agrochemical use. These microbes improve soil fertility, soil health, and plant growth in a variety of ways, including improving nutrition (recycling, nutrient mobilization, nitrogen fixation), regulating phytohormone levels (cytokinins, gibberellic acid, ethylene, and indole acetic acid, among others), suppressing soil-borne pathogens (via antibiotic production, cyanides, and siderophores), and environmental symbiosis (via siderophores). Microbes may improve environmental conditions and soil biochemicals, while generating plant tolerance to environmental challenges, as a result of these qualities (Sahib *et al*., 2020). Organic fertilizers have been proven to recover soil microbial biomass by providing carbon-rich chemicals to the normally carbon-limited microbial populations in agricultural soils. Change of practices in soil management, for example, organic fertilizer treatment, has been found to increase biochemical properties and soil microbial community quickly after application. Although there are benefits to employing organic nitrogen in traditional farming where mineral manures are widely employed, this area has been scarcely investigated (Dangi *et al*., 2020). Breeding for increased plant response for enhanced interactions and colonization of the plant environment is a crucial feature for the more efficient and effective use of microorganisms in agriculture. Plant breeding has mostly focused on increasing harvest yield and developing pathogen resistance; however, these procedures have brought about a decrease in genetic diversity in modern crops (Mitter *et al*., 2019).

2.4 Interplay of the environment and species-specific microbiome on plant health.

The potential of some soil microbes to promote plants' health and performance has been harnessed to boost pest management and agricultural yield by altering the soil microbiome community (Howard *et al*., 2020). Since the era of next-generation sequencing, new interest

has sparked in the composition and role of microbial species diversity towards controlling plant productivity and soil health in broader ecological contexts (Sahib *et al*., 2020). As a result, a wide range of bacteria, fungi, and archaea components of the soil microbiome have received significantly greater attention in soil microbiology research (Barea, 2015). In line with some earlier studies, plant species in different successional phases possess different eco-physiological properties that could significantly impact the soil biological features, as in the case during early succession when fast-growing plant species produce a labile carbon substrate that favors bacteria-dominated communities. On the other hand, slow-growing plant species that control late-successional phases produce complex substrates (high in lignin) which favor the efficient lignin decomposing fungi (Shanmugam and Kingery, 2018).

There is growing knowledge of the interactions between any consortium or inoculant strain and the receiving environment, such as abiotic soil conditions, the target plant and soil, their associated micro-and macrobiota, and more broadly ecological conditions that provide knowledge-based approaches for microbiome applications towards developing more targeted and-effective microbial applications (Mitter *et al*., 2019) (Figure 2.1). Plant-associated microbiomes can benefit their hosts, however, the rhizosphere's microbial makeup, along with colonization efficiency, are influenced by factors such as ecological, host's genetics, and physiological status (Rodriguez *et al.*, 2019). To a greater extent, plant properties including biomass production, disease suppression, growth response, and flowering phenotype are all linked to specific plant microbiota. As a result, plant microbiota modification or the effects of farming management will influence plant characteristics and performance (Compant *et al.*, 2019). The functional and mechanistic elements of the interactions between microorganisms, plants, the environment, and agricultural management methods are essential to realizing the predicted benefits of plant microbes in agriculture (Busby *et al*., 2017) (Figure 2.1).

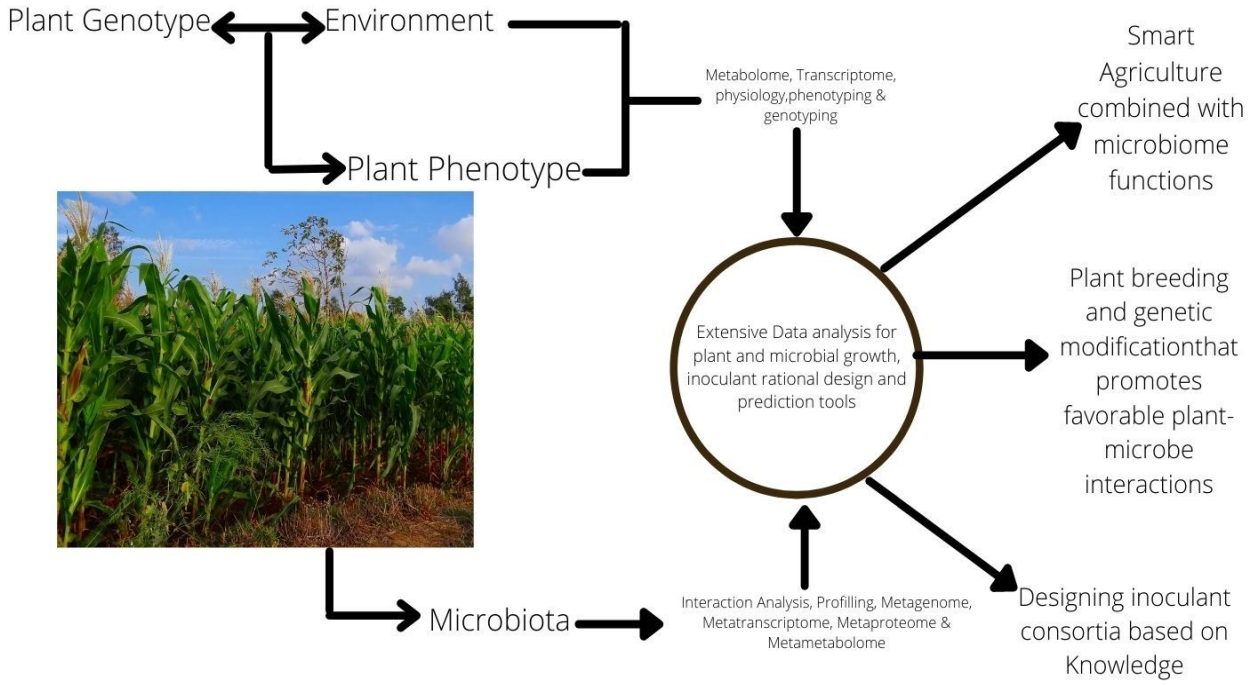

Figure 2.1: In-depth knowledge of the interactions of holobiont and their function leads to knowledge-based approaches for microbiome applications. This includes harnessing the potentials of the Plant – Soil – Microbes interaction in i) the selections of plant type or varieties that favors beneficial plant-microbe interactions, ii) microbial inoculant complementation and iii) agricultural management practices that enrich microbiota with desirable bacteria.

2.5 Mechanism of action of beneficial microbes

Microbial biological control agents work in a variety of ways to protect crops against diseases. The many processes used by biocontrol agents to control plant diseases can be broadly characterized as direct antagonism and indirect antagonism.

2.5.1 Direct antagonism

Physical contact and/or a high degree of selectivity for infections by the biocontrol agent cause direct antagonism. It contains the following items:

a) Hyperparasitism- this interaction entails the tropic development of a biocontrol agent toward the target organism, coiling, and eventual attack and disintegration of the target

pathogen's cell wall or membrane by enzyme activity. Fungi frequently engage in this type of interaction e.g. *Trichoderma.* (Junaid *et al*., 2013; Köhl *et al*., 2019a; Köhl *et al*., 2019b).

b) Direct Antibiosis - Antimicrobial metabolites are secondary metabolites that belong to a diverse category of organic, low-molecular-weight chemicals produced by bacteria and are toxic to other microorganisms' growth or metabolic processes (Köhl *et al.*, 2019b). However, because of the modest quantities produced in comparison to other, less hazardous organic chemicals in the phytosphere, the effective quantities are difficult to quantify. A biocontrol agent that produces adequate amounts of antibiotics in the area of the plant pathogen is effective (Junaid *et al.*, 2013). Biocontrol bacteria from the genera *Agrobacterium, Bacillus, Pantoea, Pseudomonas, Serratia, Stenotrophomonas, Streptomyces*, and others have been found to produce antimicrobial compounds, most of which have broad-spectrum activity (Köhl *et al*., 2019b).

2.5.2 Indirect antagonism

The capacity of biocontrol bacteria to boost plant development indirectly has piqued attention, in terms of getting a better understanding of some of the underlying mechanisms used by biocontrol bacteria and commercializing these bacteria instead of chemical pesticides (Admassie *et al.*, 2020). It includes the following:

a) Competition – Soils and living plant surfaces are frequently nutrient-limited environments from a microbiological standpoint. As a result, a microorganism colonizing the phytosphere must efficiently compete for the available nutrients. Both biocontrol agents and infections battle for nutrients and space in the environment in order to develop themselves. This rivalry is thought to be an indirect interaction between the pathogen and the biocontrol agent, in which the pathogens are kept out of the site by depletion of the food supply and physical occupation (Junaid *et al*., 2013).

For example, *Bacillus* spp. can control fungal pathogens by competition for space at the root surface and nutrients, particularly those produced as seed or root exudates, occurring in the rhizosphere (Pertot *et al.*, 2015).

b) Induced Resistance and Priming - Plants defend themselves against infections through a number of physical and chemical processes. One of the most promising agronomic strategies for preventing biotic losses in crops is to improve resistance. Inducible resistance mechanisms supplement constitutive mechanisms like cuticles. Stimuli identified by particular recognition receptors activate induced plant defense mechanisms. Microbe-Associated Molecular Patterns (MAMPs) are microorganism-produced resistance-inducing stimuli. MAMPs with recognized receptors include glucan, chitin, and xylan, which are generated by *Phytophthora megasperma* and *Trichoderma viride,* respectively (Köhl *et al*., 2019b).

2.6 Bioprospecting rhizospheric microorganisms as biocontrol agents

Microbial-based solutions are currently one of the rapidly-growing agricultural biopesticides which are potential alternatives or complement the use of synthetic fertilizers and pesticides (Malusà *et al*., 2021). Microbial pesticides are biopesticides created from beneficial microorganisms that promote plant development, and nutrient utilization efficiency which results in higher agricultural yields (Asad, 2022). Furthermore, microbial pesticides have the potential to suppress pests and are one of the choice alternatives for plant disease management in recent times (Patil and Solanki, 2016). Since only about 1% of the candidate isolates retrieved from the studied samples could eventually become commercial products, many scientists currently explore approaches to improve the search and identification of suitable microbial strain that qualifies as potential biopesticides.

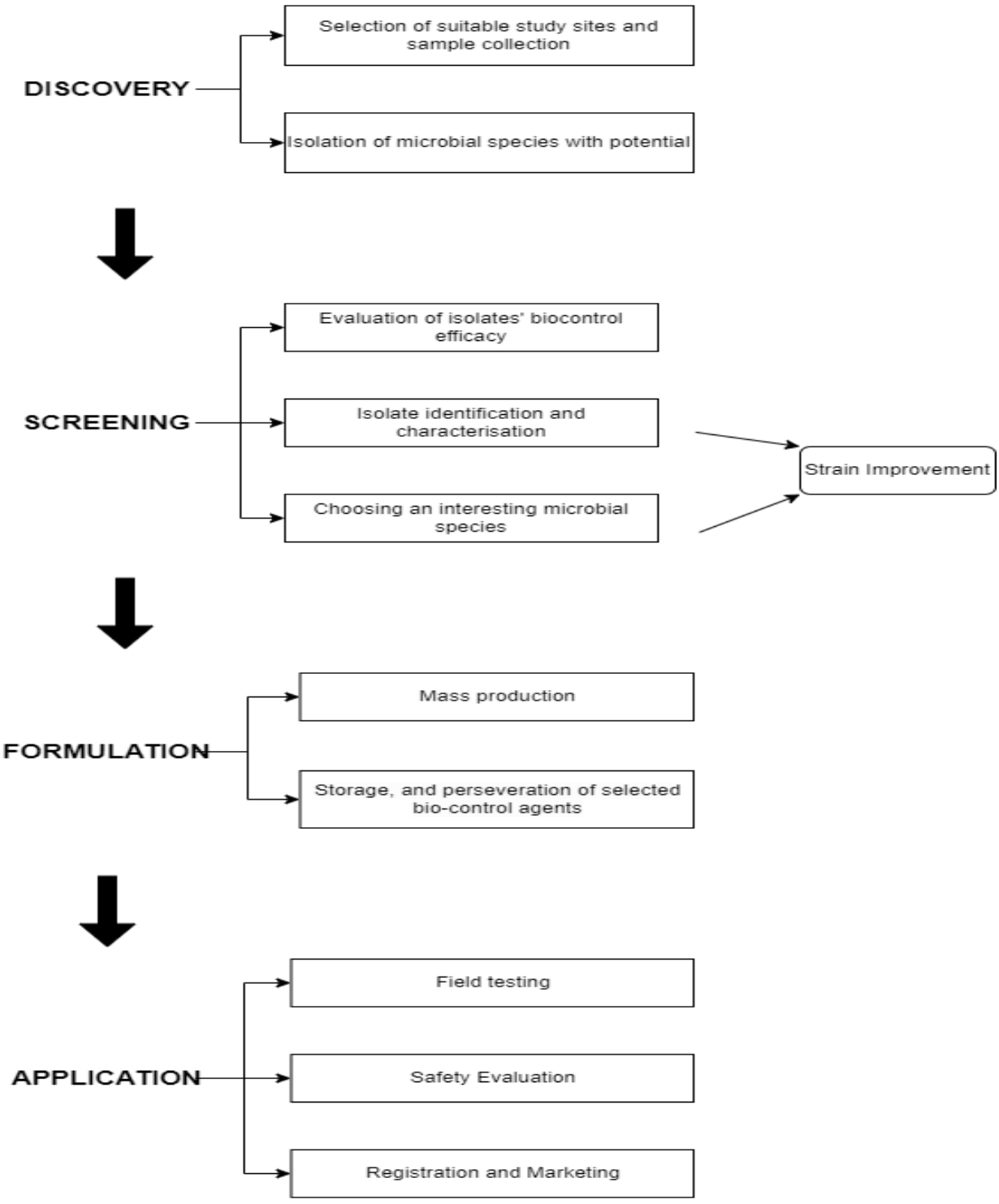

Figure 2.2: Illustration of the process involved from discovery to application of potential microbial.

However, recent approaches have embraced the use of DNA sequencing of representative samples to directly target activity-related genes (Glare *et al*., 2016). This method has been promising in unveiling the diversities of microbes associated with the rhizosphere for possible

exploration of those with biocontrol functions. Hence, discovering, screening, identifying, and characterization of microorganisms with biocontrol potentials and the ability to colonize, persist, and adapt to a complex environment like the rhizosphere is an intriguing disease management method (Gomez-Lama Cabanas *et al*., 2018) (Figure 2.2). Identification and selection of a suitable sampling environment where prospective microbial candidates could be isolated is the first step in the development of microbial-based biopesticides. It is important to assess the biology and nature of the prevailing pest problem in the area where a microbial biopesticide is planned to be used.

This assessment considers the biological principles of diseases and pests such as the host range, nature of the symptoms, the most vulnerable phases of the host, and the most suitable period of intervention (Mandakini and Manamgoda, 2021). Isolation using culture-dependent approaches have traditionally been used to exploit ambient microbiomes. High-throughput sequencing techniques that have been developed in recent decades have shown considerable promise in probing the intricate microbial networks found in plant microbiomes. The efficacy of the sequencing method in the identification of new and highly efficient microbial strains has been proved (Malusà *et al.*, 2021).

Microorganisms exert biocontrol influence through a variety of processes, including antibiosis, parasitism, competition, and generation of plant systemic resistance. These properties promote the employment of biocontrol bioassays in screening for members with biopesticide potentials among a large number of microbial communities (Mandakini and Manamgoda, 2021). Microbiome modification has also recently been revealed as an effective method for screening microbial strains (Malusà *et al*., 2021), while the incorporation of molecular approaches into the screening process has also been equally beneficial in determining the full potential of the promising strains.

2.7 Formulation and application of microbial biopesticides

The first and most important step in the formulation of biocontrol agents is biomass generation. Culturing conditions affect the fitness and viability of microorganisms throughout formulation, storage period, application, as well as population densities upon harvest. A procedure that is peculiar to each microbial strain must be properly screened to enhance the performance of microbes as bioagents in the field (Bejarano and Puopolo, 2020). Once the biopesticide inoculum is generated, it must be kept stable during storage, distribution, and use at the target site. The formulation enables technical product stability, as well as persistence, safety, high efficacy, and user-friendliness. As a result, it is regarded as an important stage in the development of a high-quality and practical biopesticide. The active component (generated inoculum) is blended with inert ingredients like transporters, surface active ingredients (stickers, spreaders), and other additives during the formulation process (stabilizers, coloring agents, etc.) (Bejarano and Puopolo, 2020).

Biopesticides formulation becomes more efficient as production technology improves in the last few decades, this has therefore resulted in some breakthroughs recorded in the field of biopesticides. In the recent times, the application, persistence, and efficacy of biopesticides are enhanced with the use of prills and emulsions in its formulation. More so, the application through seed coating is appreciated for its appealing means of delivering biopesticides particularly in the soil. This method increasingly gains relevance as more agents are discovered to be rhizosphere colonizers or even capable of endophytic colonization (Glare *et al.*, 2016). However, one of the difficult aspects of biopesticides use is that most application procedures were designed for synthetic pesticides rather than living organisms. Whereas, to ascertain the efficacy of the formulation produced, it must be successfully delivered to the target plant diseases under a favorable field condition suitable for the disease expression (Mishra *et al*., 2019). The application should ideally bring the active chemicals in contact with the pest/

disease and keep them active for several weeks. The aboveground application has traditionally been carried out with spray applicators, while the below ground application of microbial agents with the use of specialized equipment with appropriate droplet size, and focused treatment, are receiving more attention (Glare *et al*., 2016). The roles of biological formulations as biofertilizer which entails the improvement of resource acquisition and efficient use, and biopesticide which reduces losses due to pests and pathogens, have been embraced by the legislation and regulatory agencies (Malusà *et al*., 2021). However, the current legislative framework in place in many countries as regards the development and marketing of microbial-based goods makes it difficult to vigorously explore and take advantage of the multifunctionality of these beneficial microbes.

2.8 The beneficial microbes and soil health: Impacts on food safety

Soil health refers to the ability of soil to continue to operate as a vital living ecosystem that supports humans, animals, and plants, and it links agricultural and soil research to stakeholder demands, policy, and long-term supply chain management (Lehmann *et al*., 2020). Currently, safe and agricultural practices emphasizes need for the reduction in agrochemical use in favor of various eco-friendly crop management techniques (Vida *et al.*, 2020). Furthermore, the biotic stressors which consist of the fungal pathogens of maize includes *Exerohillum turcicum* causing Northern corn leaf blight (NCLB), *Bipolaris maydis* (Southern corn leaf blight) *Rhizoctonia solani* (Banded leaf and sheath blight), *Colletotrichum graminicola* (*Colletotrichum graminicola*) and *Macrophomina phaseolina* (Charcoal rot) (Table 2.1).

Table 2.1: Fungal and bacteria pathogens, diseases caused, and biological agents for managing agricultural crops

Fungal pathogens	Hosts	Disease caused	Antimicrobial and growth-promoting microbes	References
Exerohillum turcicum (Teleomorph *Setosphaeria turcica*), *Helminthosporium turcicum* (Pass.), Drechslera turcica (Pass.) Subramanian & Jain, Xanthomonas axonopodis pv. coracanae.	Maize, Sorghum, Millet	Northern Corn Leaf Blight (NCLB)	*Rhizophagus intraradices*	(Begum *et al*., 2019; Degani *et al*., 2020; Ogolla *et al*., 2019; Zhu *et al*., 2021)
Bipolaris maydis (Teleomorph *Cochliobolus heterostrophus*)	Maize	Southern corn leaf blight (SCLB)	*Bacillus subtilis, Fenneliformis mossease*	(Begum *et al*., 2019; Zhu *et al*., 2021)
Rhizoctonia solani (Teleomorph *Thanatephorus cucumeris*)	Maize, Soybean	Banded leaf and sheath blight (BLSB)	*Pseudomonas aeruginosa, Trichoderma, Gliocladium, Pseudomonas, Bacillus subtilis, Paenibacillus polymyxa, P. fluorescens, Chryseobacterium gleum, Streptomyces* spp., Binucleate Rhizoctonia spp. and hypovirulent R. solani	(Ajayi-Oyetunde and Bradley, 2018; Begum *et al*., 2019; Fiers *et al*., 2012; Herr, 1995; Rai and Singh, 2018; Singh *et al*., 2020)
Cercospora zeina , Cercospora zeae-maydis, Curvularia lunata (Teleomorph *Cochliobolus lunata*)	*Urdbean or Black gram (Vigna Mungo L.), Arachis hypogea, Vigna sinensi, Pennisetum typhoides, Vigna rediata, Glycene max, Vigna mungo, Oryza sativa, Zea mays*	Gray leaf spot (GLS)/ Curvularia leaf spot (CLS),	*Bacillus subtilis*	(Lal *et al*., 2013; Xie *et al*., 2020; Zhu *et al*., 2021)

Colletotrichum graminicola (teleomorph *Glomerella graminicola*)	Maize, wheat, sorghum, bluegrass, barley, ryegrass	*Anthracnose* stalk rot, Anthracnose leaf blight	*Streptomyces ganmycicus, Sporobolomyces roseus*	(Back *et al*., 1999; Elizabeth and Mordue, 1967; Zhu *et al*., 2021)
Puccinia polysora	Maize	Southern rust	*Trichoderma* spp.	(Veenstra *et al.*, 2019; Zhu *et al*., 2021)
Fusarium verticillioides, Fusarium graminearum (Teleomorph *Gibberella zeae*), *Aspergillus flavus, Stenocarpella maydis*	Maize, wheat (*Tricitcum aestivum & Tricitum durum)*), Oat (*Avena sativa*), Barley (Hordeum vulgare)	Maize ear and stalk rot, *Fusarium* head blight	*Trichoderma asperellum, Bacillus velezensis, Bacillus subtilis, Trichoderma harzianum, Trichoderma viride, Pseudomonas fluorescens*	(Arici and Tuncel, 2020; Ramesha, 2018; Tom and Patel, 2021; YE *et al*., 2012; Zhu *et al.*, 2021)
Macrophomina phaseolina	Sunflower, *Medicago littoralis, Vigna mungo, Lens culinaris, maize, and Medicago scutellata*	Charcoal rot	*Pseudomonas* spp*., Trichoderma*	(Al-Sa'di *et al*., 2008; Ali and Dennis, 1992; Degani *et al*., 2020; Emayavarman *et al*., 2019; Xavier and Kaushik, 2021)
Pythium aphanidermatum and *Pythium inflatum*	Pawpaw, Cucumber, Soybean, pepper, cotton, cucurbits, turf-grasses, beets, and chrysanthemum	*Pythium stalk rot*	*Bacillus subtilis, Streptomyces spp.*	(Abd-Elsalam, 2020; Hassanisaadi *et al.*, 2021; Olawuyi *et al*., 2014; Zhu *et al*., 2021)
Bacteria pathogens				

Dickeya zeae (Syns. *Erwinia chrysanthemi* pv. *Zeae*)	Maize, rice, banana,	Bacterial stalk rot, soft rot, footrot	*Pseudomonas aureofaciens, P. chlororaphis, P. putida, P. fluorescens, P. kilonensis, P. Protegens*	(Begum *et al*., 2019; dos Santos *et al*., 2020; Hu *et al*., 2018; Zhu *et al*., 2021)
Clavibacter michiganensis subsp. *Nebraskensis*	Maize, Sorghum (*Sorghum halepense*), Large Crabgrass (*Digitaria sanguinalis*), Ryegrass (*Lolium multiflorum*)	Goss's bacterial wilt and blight	*Pseudomonas* sp*., Bacillus* sp.	(Goyal and Anvitha, 2021; Ikley *et al*., 2015; Zhu *et al*., 2021)
Xanthomonas vasicola pv. *vasculorum* (*Xvv*), *Xanthomonas campestris*	Maize, Banana, Sorghum, sugarcane	Bacterial leaf streak, bacterial leaf spot	*Rhizobium, Azotobacter, Azospirillum*	(Coutinho *et al.*, 2015; Damicone *et al*., 2018; Lalitha, 2017; Ocimati *et al*., 2018)
Pantoea stewartii subsp. *stewartii* (*Pss*), *Pantoea ananatis, Erwinia amylovora*	Maize, Onion, Apple (Malus domestica), Bermuda grass, soybean, cowpea	Stewart's wilt, Fire blight, die-back, leaf blotches and spots, stalk, fruit, and bulb rot.		(Baek *et al*., 2018; Goszczynska *et al*., 2007; Stumpf *et al*., 2018)

Also, the bacterial diseases caused by the pathogens *Dickeya zeae* (Bacterial stalk rot), *Clavibacter nebraskensis* (Goss's bacterial wilt and blight), *Xanthomonas vasicola* (Bacterial leaf streak/spot) among many others reduce the grain quality and yield produced (Begum *et al*., 2019; Damicone *et al.*, 2018; Zhu *et al*., 2021).

Recent research has shown that using a beneficial microbe can improve agricultural output and plant health when produced under constrained environments. Varying antimicrobial and growth-promoting microorganisms have been investigated and developed from beneficial microbes such as *Pseudomonas aureofaciens, Pseudomonas chlororaphis, Bacillus* sp*., Rhizobium, Azotobacter,* and *Azospirillum* among several others, and were effective in mitigating the debilitating effect of the pathogens (Begum *et al.*, 2019; dos Santos *et al*., 2020; Lalitha, 2017) (Table 2.1).

The diversity of microorganisms in the rhizosphere zone aids plant growth by providing defense against pests and pathogens, facilitating nutrition uptake, and assisting plant tolerance to a variety of abiotic and biotic stimuli. Various types of abiotic stress include salinity, high temperature, and drought which results in negative impacts, for example, losses in crop productivity by disease susceptibility, nutrient acquisition, reducing water absorption and disturbing hormonal balance, and by affecting the photosynthetic capacity of the plant. However, because only around 1–5% of the bacteria on the planet are cultivable, the remaining 95–99% of germs are uncultivable; these helpful microbes are not fully utilized. For numerous years, understanding plant-microbe interactions have been a major emphasis of study (Kumar and Dubey, 2020). The employment of beneficial microorganisms (which improve the plant's health and quality, as well as assist in the reprocessing of crop leftovers with less ecological impact) is one of the most important techniques for long-term energy and food production (Dubey *et al.*, 2019). Numerous soil microorganisms interact with each other and with plants in a variety of ways that aid in the maintenance and molding of various ecosystem components.

Healthy soils are the base of productive and sustainable agroecosystems, and they can be maintained by following basic soil health principles such as increasing plant diversity through crop rotation, protecting surface soils by growing more plants, minimal soil disturbance, polycultures, and cover crops, and enhancing soil microbiome, among others. Microbes aid to maintain soil health, improving root growth, water retention capacity, vital nutrient availability, carbon storage, cycling, filter contaminants, and conserving biodiversity (Dubey *et al*., 2019). Beneficial microbes are opening up new avenues for long-term disease (pathogen) control, emphasizing the relevance of the plant microbes for crop productivity (Bonanomi *et al.*, 2018). Since the 1980s, organic amendments have been used to manipulate the soil microbiota to minimize pathogen inoculum or virulence in favorable soil, albeit the efficiency of these methods varies depending on the pathogen/host system (De Corato, 2020). Compost, crop residues, peat, organic wastes, and biochar are examples of such organic soil additives (Akanmu *et al.*, 2020a; Chukwuka *et al*., 2020) although biofumigation is also used, for example, to reduce disease through soil fungistatic (Bonanomi *et al.*, 2018). Organic manure (both animal dung and biomass) applied as a soil amendment, in contrast to issues linked with the use of agrochemicals, plays a vital role in the control of plant health and the recycling of soil nutrients (Akanmu *et al*., 2021). Organically altered soil has a potentially superior operational microbiome, with more positive microbial activities and functionally connected species than soils that have been fertilized with chemical fertilizers.

To better understand soil health, it will be required to characterize the functional groupings of microorganisms that are connected with specific organic amendments. Organic amendments including materials produced from composts, farmyard manure, crop residue and biochar among others, have been reported to modify soil structure and enables the efficient absorption and both nutrient and water retention in the soil (Akanmu *et al*., 2021). Organic amendments fine-tuned and optimized for specific soil/crop combinations, would create long-term microbial

ecosystem services and soil health, resulting in yields that are equivalent to or greater than chemical fertilizers alone (Arif *et al.*, 2020). Since the early 2000s, supplementing beneficial external microbiota with Composts, organic amendments, and biological control agents has appeared to be a potential strategy for boosting the suppression of favorable soils (De Corato, 2020). As a result, soil organic matter can be conserved with the help of organic amendments in agricultural systems, which is a recognized technique for reducing soil deterioration. Organic additions, on the other hand, have the potential to change the soil microbiome. The quantity and quality of organic matter input not only affect the soil's physicochemical properties, but also affects biotic factors related to the soil microbiota, such as microbial biomass and diversity, community structure, and soil activities, all of which have a direct impact on disease biological control (Vida *et al*., 2020).

Plants contribute to the stability of the rhizobiome by producing fixed carbon resources. Plant beneficial microbes, in turn, aid plants' growth by root modification, acquiring nutrients and protecting plants from infections, among other things. As a result, the rhizobiome plays a crucial role in sustaining the plant's health. Disassembling plant microbiomes and building synthetic microbial communities for reconstitution studies to examine interspecies and intraspecies interactions is a potential way to understand the reciprocal impacts of plants and their microbiota (Lucaciu *et al*., 2019). Fast-growing species were gradually substituted by stress-tolerant and hostile species as the plants grow. Using synthetic consortia derived from different plant growth phases shows that the high functional diversity of climax microbiomes contributes to increased resistance to pathogenic microbial invasion. Hence, root-associated microbes have the potential of improving plant efficiency and output in cropping systems, according to a growing body of studies (Ab Rahman *et al*., 2018; Hu *et al*., 2020).

2.9 Current trends in the use of rhizospheric microorganisms

The use of rhizospheric microbes has been explored in the formulation of biopesticides, and the efficacy of the products against pest and diseases in a diverse type of crops have been proved. Newly discovered microbial species and strains are analyzed on a regular basis for their potential to serve as a biocontrol agent. Despite several typical drawbacks, the use of biocontrol agents still holds a lot of potential (Köhl *et al*., 2019a). In recent times, many biopesticide companies are entering into licensing agreements to market and sell microbial biocontrol products, while many international companies have ventured into the formulation and marketing of microbial biocontrol products (Marian and Shimizu, 2019). However, despite the large corpus of research on the bioagents' ability to protect crops, only a few microbes are recognized as active substances at the time of writing, and the majority of which are *Trichoderma* and *Bacillus* spp. strains (Table 2.2).

Table 2.2: Commercialized microbial biocontrol formulations

S/N	Trade name and company	Type	Microorganism	Reference
1	AQ 10 WG - CBC EUROPE	Fungicides	*Ampelomyces quisqualis* AQ10a	(Benuzzi and Baldoni, 2000; Pertot *et al.*, 2015)
2	Blossom-Protect®	Bactericides and fungicides	*Aureobasidium pullulans* DSM 14940 and DSM 14941	(Chen *et al.*, 2009; Freimoser *et al*., 2019; Sellitto *et al*., 2021)
3	Serenade ASO - Bayer CropScience	Bactericides and fungicides	*Bacillus amyloliquefaciens* QST 713	(Bejarano and Puopolo, 2020; Chen *et al*., 2009; Matzen *et al*., 2019)
4	Basf - Serifel, Histick N/T, Integral, Subtilex	Fungicides	*Bacillus amyloliquefaciens* MBI 600	(Dimopoulou *et al*., 2021; Samaras *et al*., 2021a; Samaras *et al*., 2021b)
5	FZB24 - AbiTEP, Novozymes	Fungicides	*Bacillus amyloliquefaciens* FZB24	(Bejarano and Puopolo, 2020; Chowdhury *et al*., 2015; Dimopoulou *et al.*, 2021; Gül *et al*., 2008; Pertot *et al*., 2015)
6	Amylo-X; CBC Europe S.r.l.- Biogard Division, Nova Milanese (MB), Italy & Certis USA L.L.C	Fungicides	*Bacillus amyloliquefaciens* subsp. *plantarum* D747	(Bejarano and Puopolo, 2020; Dimopoulou *et al*., 2021; European *et al.*, 2014; Pertot *et al*., 2015; Rotolo *et al.*, 2016)
7	VOTiVO® - (Bayer CropScience)	Nematicides	*Bacillus firmus* I-1582	(Bejarano and Puopolo, 2020; d'Errico *et al.*, 2019; Ghahremani *et al.*, 2020; Mendis *et al*., 2018; Pertot *et al.*, 2015)
8	GB34 Concentrate Biological Fungicide - Bayer Crop Science	Fungicides	*Bacillus pumilus* QST 2808	(Dimopoulou *et al*., 2021; Lazzari *et al*., 2018; Pertot *et al*., 2015; Wang *et al*., 2015)
9	Avogreen – Next	Fungicides	*Candida oleophila* O	(Bejarano and Puopolo, 2020; Lahlali *et al*., 2011; Pertot *et al.*, 2015)
10	Prestop – Biobest	Fungicides	*Clonostachys rosea* J1446	(Bejarano and Puopolo, 2020; Chatterton and Punja, 2010; Gimeno *et al.*, 2019)
11	Contans WG - Bayer CropScience	Fungicides	*Coniothyrium minitans* CON/M/91-08 (DSM 9660)	(Bejarano and Puopolo, 2020; European and Authority, 2016; Pertot *et al.*, 2015)
12	MeloCon WG	Nematicides	*Paecilomyces lilacinus* 251	(Bejarano and Puopolo, 2020; Dahlin *et al*., 2019; Kiewnick and Sikora, 2006; Pertot *et al*., 2015)

13	Cerall - BioAgri	Fungicides	*Pseudomonas chlororaphis* MA342	(Anderson *et al*., 2018; Bejarano and Puopolo, 2020; Pertot *et al*., 2015; Products *et al*., 2020)
14	Proradix® - Sourcon Padena	Fungicides	*Pseudomonas* sp. DSMZ 13134	(Buddrus-Schiemann *et al.*, 2010; Pellicciaro *et al.*, 2021; Pertot *et al*., 2015)
15	Biopreparáty, Ltd (Únětice, Czech Republic)	Fungicides	*Pythium oligandrum* M1	(Bejarano and Puopolo, 2020; Gabrielová *et al*., 2018; Pertot *et al*., 2015; Pisarčik *et al*., 2021)
16	Mycostop - Vedera Oy	Fungicides	*Streptomyces griseoviridis* K61	(Bejarano and Puopolo, 2020; Pertot *et al.*, 2015)
17	Actinovate AG – Novozymes	Bactericides and fungicides	*Streptomyces lydicus* WYEC 108	(Chen *et al*., 2016; Dimopoulou *et al*., 2021; Evangelista-Martínez *et al.*, 2020)
18	Remedier WP	Fungicides	*Trichoderma asperellum* ICC012, T25 and TV1	(Bejarano and Puopolo, 2020; Pertot *et al*., 2015; Woo *et al*., 2014)
19	T34 biocontrol - Asperello Biobest, Biocontrol Technologies S.L., Biolchim	Fungicides	*Trichoderma asperellum* T34	(Bejarano and Puopolo, 2020; Estévez-Geffriaud *et al*., 2020; Gugliuzzo *et al*., 2022; Pertot *et al*., 2015)
20	Binab T - Bio-innovation AB	Fungicides	*Trichoderma atroviride* IMI 206040 and T11	(Bejarano and Puopolo, 2020; European *et al*., 2015; Morán-Diez *et al*., 2019; Pertot *et al*., 2015)
21	Tri-Soil®, Esquive WP - (Agrauxine)	Fungicides	*Trichoderma atroviride* I-1237	(Letousey *et al.*, 2017; Mounier *et al*., 2014; Woo *et al*., 2014)
22	VINTEC (Bi-Pa NV/SA, Belchim crop protection Italia)	Fungicides	*Trichoderma atroviride* SC1	(Bejarano and Puopolo, 2020; Gugliuzzo *et al.*, 2022; Pertot *et al*., 2015; Savazzini *et al.,* 2009)
23	Remedier WP (Isagro USA)	Fungicides	*Trichoderma gamsii* ICC080	(Bejarano and Puopolo, 2020; Celar and Kos, 2022; Pertot *et al*., 2015; Woo *et al.*, 2014)
24	TRIANUM-P (Koppert B.V., Koppert Italia), Agroguard WG™ Antagon WP™ ECO-T™, PlantShield™/RootShield™, Trichosav	Fungicides	*Trichoderma harzianum* T-22 and ITEM 908	(Gugliuzzo *et al*., 2022; Pertot *et al.*, 2015; Shahriar *et al*., 2022)

25	Binab T - Bio-innovation AB	Fungicides	*Trichoderma polysporum* IMI 206039	(Bejarano and Puopolo, 2020; European *et al.*, 2013; Pertot *et al*., 2015)

2.10 Conclusion and future prospects

Effective management of plant health is strategic to attaining sustainable food production and reducing hunger, thereby fulfilling an important Sustainable Development Goal (SDG) of 2030. To achieve this, an efficient disease and pest management approach focused on disease prevention need to be instituted. This could be achieved through the beneficial microbes' roles in the abatement of pathogenic invasion, support of plant-soil feedback, and modulating the species-specific rhizosphere microbiome interplay towards plants' wellness and productivity. Hence, the gateway to attaining safe and sustainable plant health is dependent on the efficient coordination of the rhizosphere microbiome in plant development and disease management, as well as their sustainable deployment as biofertilizers and biopesticides in managing plant health. Recently, the breakthroughs in system biology, molecular biology, and associated computational technologies has enabled the elucidation of the structural and functional characteristics of microorganisms with prospects in promoting growth promotion and biocontrol potentials. These have therefore enabled the in-depth understanding of the rhizospheric microbes, however, the functional dynamics among the antagonists opens up new possibilities for the design of microbial antagonist consortia which could be an effective broad spectrum microbial formulation against varying plant diseases. Although strain mixtures for the biocontrol of plant pathogens are already commercially available as described in the literature, employing taxonomically divergent but functionally complementary strains could be a promising approach to follow in the near future in the quest to design a standardized, multi-targeted, efficacious biocontrol strategy.

CHAPTER THREE

3.0 SHOTGUN METAGENOMIC SURVEY OF THE DISEASED AND HEALTHY MAIZE (*ZEA MAYS* L.) RHIZOBIOME

ABSTRACT

The effective functioning of rhizosphere microbiome significantly contributes to plant development, disease resistance, and agricultural sustainability. Hence, it is a major predictor of plant health. This study evaluated the microbial diversities and functions associated with healthy and diseased maize rhizosphere at selected farms in the North West Province, South Africa.

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3.1 Announcement

Despite the contribution of maize to global food security, the prevalence of soilborne diseases significantly reduces its quality and yield (Sibiya and Sumbwanyambe, 2019; Van Wyk *et al*., 2017). In this study, sample collection was carried out from the rhizospheric region of maize plants from Lichtenberg (25º59’40.2” S, 26°31'44.2" E) and Mafikeng (25°47'19.1"S 25°37'05.1"E), North West Province, South Africa. Each field was demarcated into two areas which were independently sampled. The respective samples from different heathy and Northern Corn Leaf Blight (NCLB) infected maize plants were pooled into two replicates. The samples were scraped from the soil tightly-bound to plant root. In all, four samples were collected from each farm site, and each sample was filtered through 2 mm sieve, collected in sterile bags, transferred to cooler

boxes containing ice packs, and later stored at -20°C in the laboratory until DNA was extracted for shotgun metagenomic sequencing.

A calibrated scale was used to weigh 5 g of stored rhizosphere soil from each sample. The extraction of DNA was carried out using NucleoSpin Soil kit (Macherey-Nagel, Germany). The quality of DNA obtained was evaluated using NanoDrop spectrophotometer.

The libraries were prepared with 50 ng DNA using Nextera DNA Flex kit (Illumina, San Diego, Ca, USA, catalog number: 20018705), while the samples were subjected to simultaneous fragmentation and addition of adapter sequences. The final concentration of the libraries was measured using the Qubit dsDNA HS Assay Kit (Life Technologies), and the average library size was determined using Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries were pooled and diluted to 0.6nM and sequenced pair end for 300 cycles using NovaSeq system. The quality control (QC) of raw data, reduction of low-quality reads and removal of replicate data were conducted by SolexaQA v1.6 (Cox *et al*., 2010). Duplicate Reads Inferred Sequencing Error Estimation (DRISEE), a package of MG-RAST v4.0.3 (Meyer *et al*., 2008) was employed to evaluate the error of sequenced samples caused by the artificial replicated sequenced data. To execute the analytical processing downstream and the taxonomic assignment with k-mers, we used the MG-RAST v4.0.3 (Meyer *et al.*, 2008) server at default settings (Table 3.1).

Table 3.1: Sequence reads for the rhizosphere soil samples analyzed

Status	Sample	LI R1	LI R2	LID R1	LID R2	MA R1	MA R2	MAD R1	MAD R2
Data Before QC	Size (Basepair)	1,615,034,280	896,730,992	37819340	1,014,288,894	1,416,428,319	34,607,850	934,504,478	1,275,590,445
	No of sequence reads	11,021,733	5,814,118	230808	6,815,631	9,358,543	258,899	6,189,474	9,025,681
	Mean Sequence length (bp)	147 ± 70	154 ± 71	164 ± 120	149 ± 72	151 ± 71	134 ± 118	151 ± 72	141 ± 71
	Mean GC content (%)	64 ± 12	64 ± 12	39 ± 34	65 ± 12	64 ± 12	30 ± 34	65 ± 13	65 ± 13
	No. of artificial duplicate read sequences	522,127	261,549	5657	329,713	470,311	4,650	269,079	446,229
Data after Processing	No. of known proteins predicted	8,121,617	4,475,247	119,523	5,054,384	6,897,633	103,708	4,590,770	6,271,956
	No. of RNA features predicted	14,059	9,015	215	9,845	17,342	284	11,588	15,275
Data After QC	Size (bp)	1,490,181,369	841,065,375	32,563,182	942,747,215	1,318,000,396	28,343,946	874,147,383	1,176,234,249
	Sequences count	9,618,671	5,214,826	125,593	5,984,443	8,287,108	110,541	5,481,595	7,735,499
	Mean sequence length (bp)	155± 67	161 ± 67	259 ± 61	158 ± 68	159 ± 67	256 ± 64	159 ± 68	152 ± 67
	Mean GC content (1%)	65 ± 9	65 ± 9	68 ± 9	66 ± 9	66 ± 9	67 ± 9	66 ± 9	67 ± 8
Data after alignment	No. of known proteins identified	2,910,995	1,657,022	45,046	2,009,779	2,646,340	37,768	1,794,264	2,401,895
	No. of RNA features identified	4,383	3,083	46	3,582	5,086	45	3,863	4,305

LI: Rhizosphere soil obtained from the healthy maize in Lichtenburg

MA: Rhizosphere soil obtained from the healthy maize in Mafikeng.

LID: Rhizosphere soil obtained from the diseased maize in Lichtenburg

MAD: Rhizosphere soil obtained from the diseased maize in Mafikeng.

R = Replicate

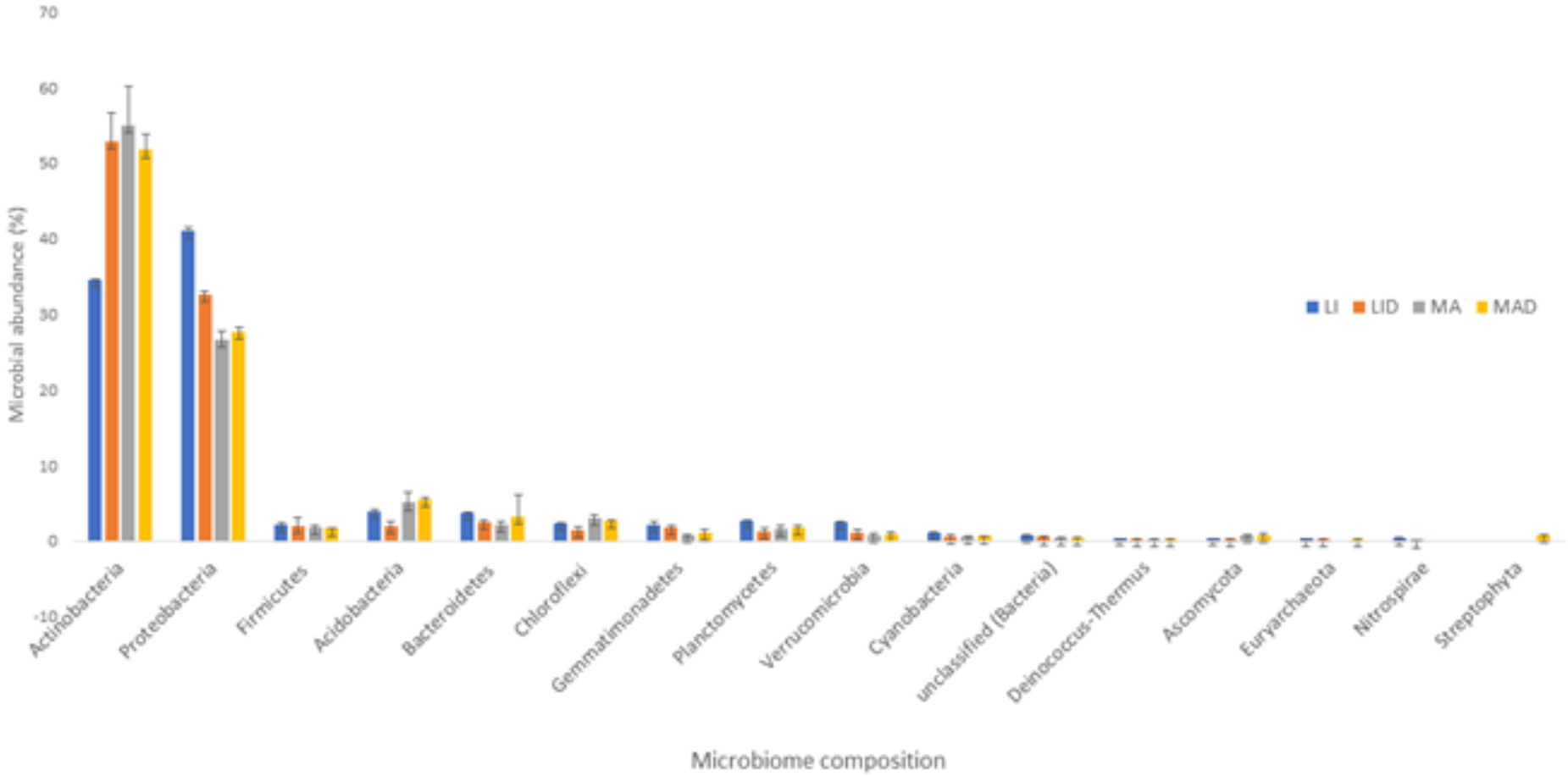

Figure 3.1: Abundant phyla obtained according to the taxonomic system

Each bar represents the mean ± standard error of each phyla component recovered

Rhizosphere soil obtained from the healthy maize in Lichtenburg (LI) and Mafikeng (MA).
Rhizosphere soil obtained from the diseased maize in Lichtenburg (LID) and Mafikeng MAD).
R = Replicate

Bacteria, Eukaryota, and Archaea are the domains or kingdoms derived from the taxonomy system. The bacteria domain had the most abundant phyla. *Actinobacteria* (34.53 to 55.01%) and *Proteobacteria* (26.6 to 40.98%) were the most abundant, and others, such as *Acidobacteria* (1.95 to 5.52%), *Bacteroidetes* (2.23 to 3.75%), *Firmicutes* (1.75 to 2.21%), *Planctomycetes* (1.29 to 2.71%), *Verrucomicrobia* (0.92 to 2.5%), and *Gemmatimonadetes* (0.82 to 2.14%), were also significant. Additionally, readings for fungi (*Ascomycota*) and archaea (*Euryarchaeota*) were found, albeit at (< 1%) relative abundance (Fig 3.1).

Functional annotation after mapping with SEED subsystems revealed the essential properties as, carbohydrate processing enzymes (15.27 to 16.05%), clustering-based systems (13.78 to 13.91%), amino acids and derivatives (11.48 to 11.96%), protein metabolism (6.67 to 6.88%), RNA metabolism (4.41 to 4.61%), fatty acids, lipids, and isoprenoids (4.33 to 4.78%), DNA metabolism (3.71 to 3.84%), cell wall and capsule (3.40 to 3.72%), virulence, disease and defense (2.26 to 2.51%), stress response (2.13 to 2.22%) among others.

3.2 Data availability

The metagenomes of rhizosphere soil sequence reads were submitted to the NCBI with BioProject accession number PRJNA821718 and Sequence Read Archive (SRA) accession numbers SRX14775303 and SRX14775302 (MAD), SRX14775301 and SRX14775300 (MA), SRX14775299 and SRX14775298 (LID), SRX14775297 and SRX14775296 (LI).

CHAPTER FOUR

4.0 MAIZE RHIZOSPHERE MODULATES THE MICROBIOME DIVERSITY AND COMMUNITY STRUCTURE TO ENHANCE PLANT HEALTH

ABSTRACT

The use of metagenomics methods has been explored in investigating the rhizosphere microbiome diversity. However, there is limited available information on its application towards securing plant health. Hence, this study adopts the metagenomic approach to unravel the microbiome diversity associated with healthy (LI and MA) and Northern corn leaf blight (NCLB) infected (LID and MAD) maize rhizosphere in the maize growing field at Lichtenburg and Mafikeng, North West province of South Africa. The extraction of whole DNA from the respective healthy and diseased rhizosphere soils was conducted and sequenced using shotgun metagenomics. A total of 12 bacteria, 4 archaea and 2 fungal phyla were found as predominant across the fields with the use of the SEED subsystem database. The most predominant bacteria phyla included *Proteobacteria, Dienococcus-Thermus, Gemmatimonadetes, Chlorobi, Cyanobacteria, Planctomycetes, Verrucomicrobia, Acidobacteria, Firmicutes, Chloroflexi* and *Bacteroidetes*. Archaea consisted of *Euryarchaeota, Thaumarchaeota, Crenarchaeota* and *Korachaeota*, while *Ascomycota* and *Basidiomycota* were the dominant fungal phyla. Microbial abundance and diversity were higher in the rhizosphere of healthy maize (LI and MA) rhizosphere as compared to the NCLB diseased (LID and MAD), in the order LI > MA> LID > MAD. At phylum and genus level, alpha diversity index showed no significant ($p > 0.05$) difference in the abundance of the microbial community of healthy and NCLB infected maize rhizosphere, while beta analysis produced a significant ($p=0.01$) difference in the microbial diversity in the soil. Taken together, the study revealed that the

abundance of microbial diversity in the maize rhizosphere influences the efficacy of the rhizosphere microbiome to modulate microbial functions towards managing and sustaining plant health.

Keywords: Metagenomics, shotgun approach, community structure, microbial diversity, sustainable agriculture, plant health management

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4.1 INTRODUCTION

Maize is a food and industrial crop that is cultivated in more than 160 countries and on every continent except for Antarctica (Rahmawati *et al*., 2021). Its global relevance is hinged on its economic, nutritional, environmental, and cultural impacts (Tanumihardjo *et al.*, 2020). It is a cereal of choice in many households and is especially preferred in Southern and Eastern Africa, Central America, and Mexico (Mangani *et al*., 2019; Ranum *et al*., 2014). As a result of the population expansion, maize consumption in the developing countries has been predicted to double by 2050, while its output is projected to peak by 2025. Therefore, considering its position as the basis of food security in some of the world's poorest regions of Africa, Asia, and Latin America, there arises the need to investigate the factors limiting the production of this important cereal as to ensure the sustainability of its productions.

Despite the management systems in place, diseases impairing maize health, productivity and contamination of grains have been recorded across the globe (Ahmad *et al*., 2012; Akanmu *et al.*, 2020b; Enebe and Babalola, 2021). In South Africa, bacterial stalk rot, black bundle disease, late

wilt, charcoal-rot, common rust, downy mildew and northern corn leaf blight among others, have been reported (Okoth *et al*., 2017; Schoeman *et al*., 2018). Northern corn leaf blight (NCLB) is a disease characterised by gray-green borders surrounding the canoe-shaped lesions which range between 3 to 15 cm long and form large-blighted areas at advanced stage till the entire leaves becomes blighted. It is an important foliar maize disease caused by the ascomycete *Setosphaeria* turcica and anamorph *Exserohilum turcicum* [(Pass.) K.J. Leonard & Suggs] (Vieira *et al*., 2014; Welz and Geiger, 2000). NCLB has been reported as the most prominent leaf diseases of maize in South Africa which is more prevalent under irrigation farming. An average yield loss of 15 to 30% has been reported, and up to 50% yield loss has been documented while for every 10% increase in disease severity, 2-8% potential yield reduction exist (Craven *et al.*, 2020).

Since plants are sessile creatures with roots that anchor them in the earth, soil thereby plays an important role in the growth, development, and health of maize plants. Securing the integrity of soil is thereby vital and strategic to attaining the optimum level of health and yield. This is because biodiversity services including climate stability, water, energy, and food security rely on its functioning (Babalola *et al*., 2020; Enagbonma *et al*., 2020; Odelade and Babalola, 2019). As in other microbes, the causative pathogens of plant diseases have also been recovered from the plant phyllosphere, endosphere, and rhizosphere (Dong *et al*., 2019; Igiehon *et al*., 2019). However, the vast majority of microbes are found in the rhizosphere which is the major site of interaction between the plants (via the roots) and the microbes. The rhizosphere thus plays the host to a wide range of biological activities which predetermine the health status of plants. This array of microorganisms includes both the beneficial and the potentially pathogenic ones. Therefore, it is essential to understand the dynamics of the criteria for plants recruitment of beneficial microbes which to a large extent restricts the pathogens (Glick and Gamalero, 2021). Studies have shown

that microbe-microbe and plant-microbe communication which occur in the rhizosphere via the exchange of numerous signal molecules has a significant effect on microbial behavior (Akanmu *et al*., 2021). The disease suppressive soils, therefore, operate on a natural principle of competition for dominance and survival. This leads to stiff microbial competition that eventually determines the chances for development or sustenance of plants' health or diseased conditions (Enebe and Babalola, 2021; Omomowo and Babalola, 2019).

The naturally occurring rhizosphere microbiomes have been estimated to contain hundreds to thousands of different microbes with plant growth promoting bacteria (PGPB), and plant Growth promoting fungi (PGPF) as the most abundant (Akanmu *et al*., 2021; Dlamini *et al*., 2022). Both PGPB and PGPF benefits the growth and development of plants in diverse ways, including the enhancement of plant growth, facilitating the provision of mineral and fixed nitrogen for plants' use, including the enhancement of seed germination, root and shoot length, production of secondary metabolites, increasing plant tolerance to environmental stresses, and protection of plants from a wide range of phytopathogenic microbes (Glick and Gamalero, 2021; Olowe *et al*., 2020). Investigation into the microbiome since the last decade have to an extent unravelled the complexity and structure of microbial communities, although the knowledge of the organization of such complex communities and their interdependency among themselves and with the biotic and abiotic environment is still at its infancy (Sergaki *et al*., 2018). Some molecular tools including; Phospholipid Fatty Acid (PLFA) (Liu *et al*., 2017), Fatty Acid Methyl Ester (FAME) (Cavigelli *et al*., 1995), Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Marschner, 2007) have earlier been applied to the study of microbial communities in soil. However, the advent of metagenomics enables the sequencing of the whole environmental DNA using 454 pyrosequencing and 16S-

rRNA sequencing (Amplicon) in the diversity study of microbiome. While these methods lack the temerity of deeper insights into the functions of the soil organisms, the culture-independent high-throughput sequencing (HTS) and shotgun metagenomics have improved over this limitation (Fadiji *et al.*, 2021b; Prigigallo *et al*., 2016). With the shotgun metagenomics, it is reported to unravel the functional diversity of microbiomes in a specified environment (Fadiji *et al.*, 2021a; Fadiji and Babalola, 2020).

The structural profile of microbial communities is often an indicator for defining soil quality and fertility. It aids the response of the microbes to both biotic and abiotic modifications (Babalola *et al.*, 2020; Enagbonma *et al.*, 2020; Ojuederie *et al*., 2019). Interestingly, the abundance and diversity of microbes in the maize rhizosphere of both healthy and NCLB infected maize have not been fully explored, thereby indicating the possibility of other useful but undiscovered organisms or microbial roles that could be productively engaged towards crop improvement. To the best of our knowledge, this is one of the foremost reports in which shotgun metagenomic approach was used to investigate the community structure of rhizosphere microbial community in maize plant infected with Northern Corn Leaf Blight (NCLB). Therefore, the understanding of the abundance and diversity of microbial profiles associated with the rhizosphere soils of healthy and NCLB diseased maize plants is strategic to ensure sustainable and secured maize productions. Hence, we hypothesized that abundance and diversity of rhizosphere microbiome positively impacts disease management in healthy maize compared to the NCLB infected maize.

4.1 MATERIALS AND METHODS

4.2.1 Field history

The maize rhizosphere soil samples used in this study were collected from the maize cultivated sites at Lichtenberg (25º59’40.2” S, 26°31'44.2" E) and Mafikeng (25°47'19.1"S 25°37'05.1"E)

which are two different locations within the North West Province of South Africa. The province during summer has the temperature range of 17 ° to 31 °C (62° to 88 °F) and in winter 3° to 21 °C (37 ° to 70 °F). The annual total rainfall is estimated at an average of 360 mm (about 14 in) which majorly occur during summer (October and April). In the two farms sampled, maize cultivar WE 3127 was cultivated. Agricultural management systems employed include application of NPK fertilizers (150 N, 75 P2O5, and 75 K2O all in kg ha−1), artificial irrigation, mechanized land tilling systems and machinery-assisted weed control measures. Based on the documented records and information gathered from the farmers, the field in Mafikeng (MA), which is the agricultural farm of the North West University occupies approximate 20 hectares and has been under rotational cultivation of maize and cowpea since 1989. Also, the farm sampled at Lichtenberg (LI) has been under continuous maize cultivation for about 10 years. The choice of the locations was influenced by the occurrence of Northern corn leaf blight (NCLB) disease in some parts of the farms. Identification of NCLB diseased plants was based on the symptoms occurrence on the leaves, such as elliptical, gray-green lesions of 3 – 15 cm long, tan with distinct dark zones of fungal sporulation, which turns to a blight that becomes severe in its advanced stage (Vieira *et al*., 2014). From each of the farms, soil samples were collected from the rhizosphere of both the healthy and the NCLB infected plants. This was carried out about 8 weeks after planting the maize to allow adequate interaction of plant root and soil microoganisms within the rhizosphere.

4.2.2 Sample collection

The sample collection was conducted in January 2021. Each of the two farms (MA & LI) were demarcated into two regions. In each region, about 50g of rhizosphere soil samples considered sufficient for DNA extraction and physicochemical analysis (Akinola *et al*., 2021a; Bai *et al*., 2014) were collected between 5 – 20 cm in-depth from each healthy (15 samples) and NCLB

infected (15 samples) maize plants rhizosphere. The respective soil samples collected per region were pooled into two replicates and labelled accordingly as either sample from healthy or NCLB diseased maize plants. This makes a total of four samples obtained from each farm site. The maize plants sampled were carefully uprooted and shaken to remove loose soils around the roots, while soils that were tightly bound to the root were collected in sterile bags, transferred to cooler boxes containing ice (4°C) and immediately transported to the laboratory. All the samples were stored at -20°C until analysis. Representative samples were prepared for DNA extraction and high throughput sequencing. Analysis of the soil samples collected showed higher values of soil pH (LI - 6.95, LID- 6.76) and Organic Carbon (LI - 1.02%, LID – 0.89%) in both the rhizosphere of healthy and diseased maize plants at Lichtenburg compared with those collected at Mafikeng pH (MA – 5.17, MAD – 5.37), Organic carbon (MA – 0.26, MAD – 0.25) (Supplementary 1, Table 1). These were carried out according to the procedure described by (Muwawa *et al.*, 2010; Walkley and Black, 1934).

4.2.3 Extraction of DNA and shotgun sequencing

The rhizosphere soils collected were subjected to the DNA extraction protocol of the Qiagen DNeasy PowerMax Soil Kit (USA). Shotgun metagenomic sequencing was conducted on the extracted DNA at the Molecular Research LP, Texas, USA. Nextera DNA library preparation Flex kit (Illumina) was employed in the library preparation, following standard procedure. Evaluation of the initial DNA concentrations in the prepared samples was carried out using the Life Technologies Qubit® dsDNA HS Assay Kit produced by Carlsbad, USA. The samples were further cleaned with DNEasy PowerClean Pro Cleanup Kit (Qiagen) and the concentrations of the samples after the clean-up were again checked using the Qubit® dsDNA HS Assay Kit (Life Technologies, the USA). The library was then prepared using 20–25 ng of DNA, after which the

samples were concurrently fragmented and an adapter sequence added. After that, the adapters were employed in a limited-cycle PCR to introduce unique indices into each sample. The final library concentrations were determined using the Qubit® dsDNA HS Assay Kit (Life Technologies) after the libraries were prepared, and the average size of the library was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries were pooled and diluted to 0.6 nM before being sequenced for 300 cycles paired-end on the NovaSeq system (Illumina) 6000 system.

4.2.4 Metagenome annotation and Data analysis

Each metagenome sequences were uploaded to the metagenomics rapid annotation online service (MG-RAST) (Meyer *et al.*, 2008), which performed quality checking on the raw data. This included the use of Trimmomatic v 0.33 software with default parameters to remove adaptor and low-quality reads from the sequenced data (Bolger *et al*., 2014). The artificial sequences were removed, ambiguous bases filtered, minimum read size specified, and length filtering was part of the quality control process. Following quality control, the generated sequences were annotated using BLAT (BLAST-like alignment tool) (Kent, 2002) against the M5NR database (Wilke *et al*., 2012), which permits non-redundant integration of several datasets. The SEED database was used to do taxonomic profiling, with criteria such as a 10^{5}% e-value cut-off and a minimum 60% sequence similarity to a subsystem. On the MG-RAST, the data normalization option was selected to reduce the impact of experimental error. The identified microbiome table was grouped by taxon, and the unclassified sequence reads were saved for statistical analysis. After an independent examination of the 8 sequences using MG-RAST, the relative abundance of the taxa in percentages was determined. For statistical analysis, the average values of the relative abundance of the two replicates for each sample site (LI, LID, MA & MAD) were utilized. The accession number for

these standard sequences may be obtained on the NCBI SRA collection bio project number PRJNA763110.

The Shiny heatmap was used to plot the relative abundance graph of rhizosphere microbiome communities at the phylum level after the dataset was adjusted (Khomtchouk *et al*., 2017). The Shannon diversity and Pielou Evenness indices for each of the sampling sites were assessed using PAST version 3.20, and the indices between the sites were compared using the Kruskal–Wallis test. The beta diversity was defined using primary coordinate analysis based on a Euclidean distance matrix, and the variations in community structure among the locations were assessed using one-way analysis of similarities (ANOSIM) (Carrell and Frank, 2015).

4.3 RESULTS

4.3.1 Metagenomic sequencing and processing

The sequences uploaded for analysis on MG-RAST server showed the mean values of samples from rhizosphere of healthy maize in Lichtenburg (LI) (1,615,034,280) and Mafikeng (MA) (725,518,084,5), while the sequence count for rhizosphere soils from NCLB diseased plants in Lichtenburg (LID) and Mafikeng (MAD) were 526,054,117 and 1,105,047,462 respectively. However, the quality of retained mean sequences after the Quality Control (QC) assessment recorded LI = 1,165,623,372 and MA = 673,172,171 for samples from healthy rhizosphere, and LID = 487,655,198,5 and MAD= 1,025,190,816 for those of NCLB diseased plant rhizosphere soils (Supplementary 1, Table 2). The rarefaction generated in the MG-RAST analysis depicts the richness and diversity of the microbial communities in each rhizospheric soil samples (Figure 4.1).

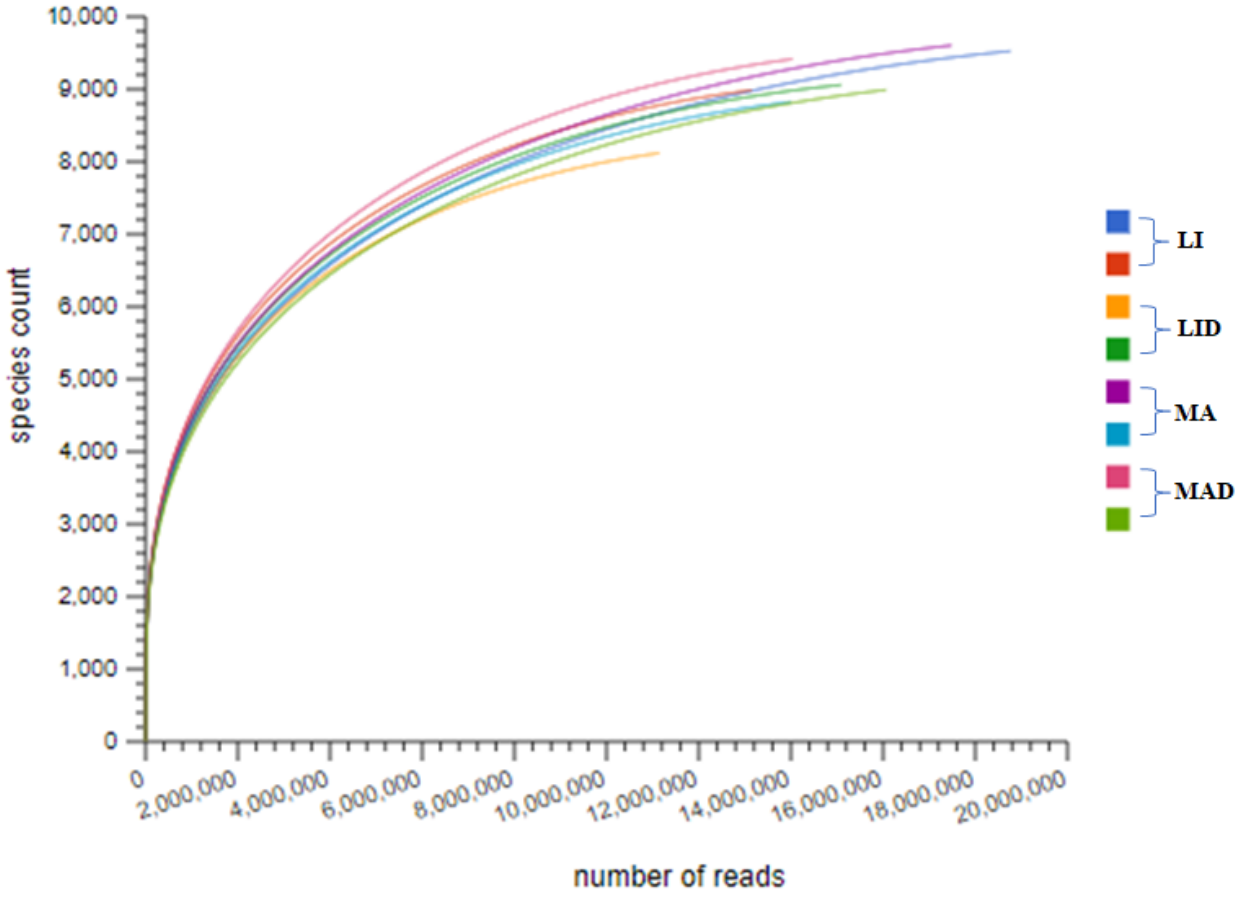

Figure 1.1: Rarefaction curve showing the species richness sequence in the studied site. LI- rhizosphere soil from healthy maize in Lichtenburg field, LID- rhizosphere soil from diseased maize in Lichtenburg field, MA- rhizosphere soil from healthy maize in Mafikeng field, MAD- Mafikeng.

4.3.2 Composition of the major microbiome phyla across the fields

A total of 12 bacteria, 4 archaea and 2 fungal phyla were found as predominant across the fields with the use of the Subsystem database. Except in the cases of *Actinobacteria* and *Crenarachaeota*, all other phyla were more abundant in the rhizosphere soils from healthy maize (LI and MA), than those of diseased maize (LID and MAD).

The most predominant bacteria phyla included *Proteobacteria, Dienococcus-Thermus, Gemmatimonadetes, Chlorobi, Cyanobacteria, Planctomycetes, Verrucomicrobia, Acidobacteria,*

Firmicutes, Chloroflexi and *Bacteroidetes.* Archaea consisted of; *Euryarchaeota, Thaumarchaeota, Crenarchaeota* and *Korachaeota* while *Ascomycota* and *Basidiomycota* were the major fungal phyla observed. The microbial community was found to be more abundant in soil samples from healthy plants than diseased in both locations, with LI, MA, LID and MAD as the decreasing order recorded (Figure 4.2), although no significant $p>0.05$ difference was recorded in the microbial variations across the site (Supplementary 1, Table 3). Meanwhile, the principal component analysis distribution of the identified microbial phyla, as indicated in Figure 4.2, showed LI as the treatment with the largest dispersion (Figure 4.3).

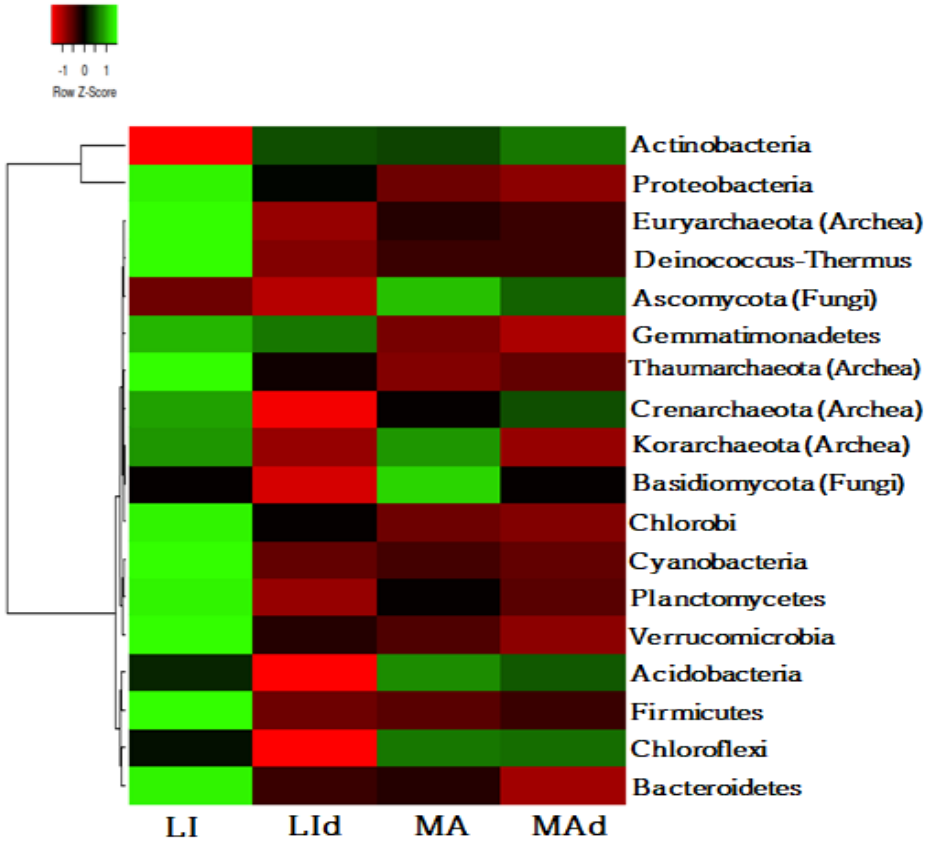

Figure 4.2: The heatmap shows the major phyla of soil microbial communities associated with maize plants. LI, rhizosphere soil from the healthy plant at Lichtenburg; LID rhizosphere soil from the diseased

plant at Lichtenburg site, MA, rhizosphere soil from the healthy plant at Mafikeng; LID rhizosphere soil from the diseased plant at Mafikeng site.

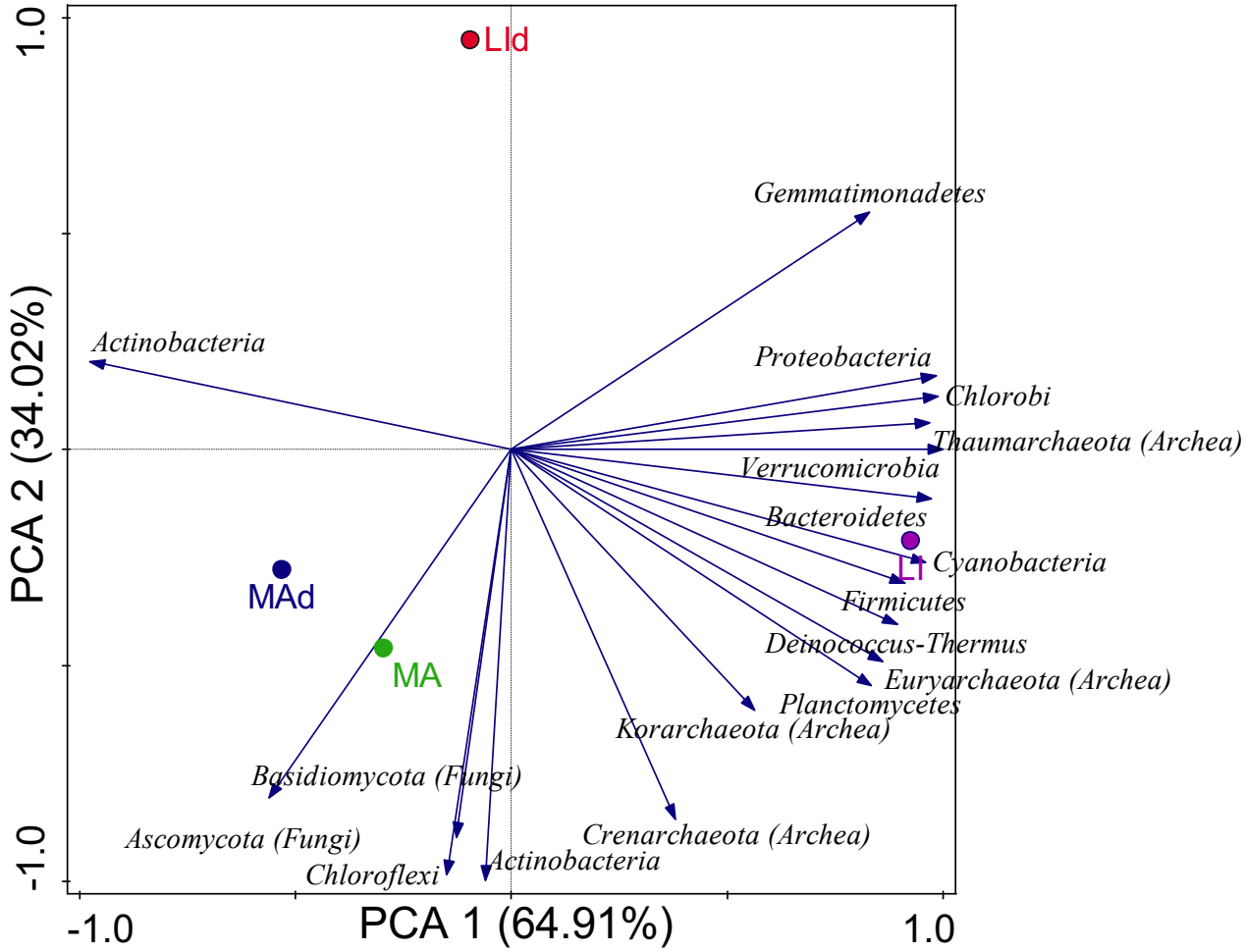

Figure 4.3: The PCA graph of average soil microbial community phyla

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

4.3.3 Composition and abundance of microbial communities

A higher proportion of bacterial phyla were recorded from the rhizosphere of healthy maize samples than those of diseased maize, especially for the samples recovered from Lichtenburg (LI) consisting of *Proteobacteria* (41.36%), *Bacteroidetes* (4.20%), *Firmicutes* (4.82%), *Cyanobacteria* (2.34%), *Planctomycetes* (2.68%), *Verrucomicrobia* (2.17%), *Gemmatimonadetes* (1.21%), *Deinococcus-Thermus* (0.77%) and *Chlorobi* (0.41%), while *Acidobacteria* (4.32%), *Actinobacteria* (MAD = 47.28%), *Chloroflexi* (MA= 2.98%) were more abundant in Mafikeng (MA) samples. *Ascomycota* (LI=0.36%, LID=0.26%) and *Basidiomycota* (0.04%, 0.03%) were the most predominant fungal phyla which showed respective abundance in healthy than diseased samples recovered from Lichtenburg. A similar observation was recorded in the phyla *Ascomycota* (MA=0.76%, MAD=0.68%) and *Basidiomycota* (MA = 0.05%, MAD=0.04%) from Mafikeng samples. The most abundant archaea phyla recorded across the sites were *Euryarchaeota, Crenarchaeota, Thaumarchaeota,* and *Korarchaeota,* which were mostly more abundant in the rhizosphere of the healthy samples (LI and MAD), except *Crenarchaeota* in Mafikeng (MA) (Supplementary 1, Table 3).

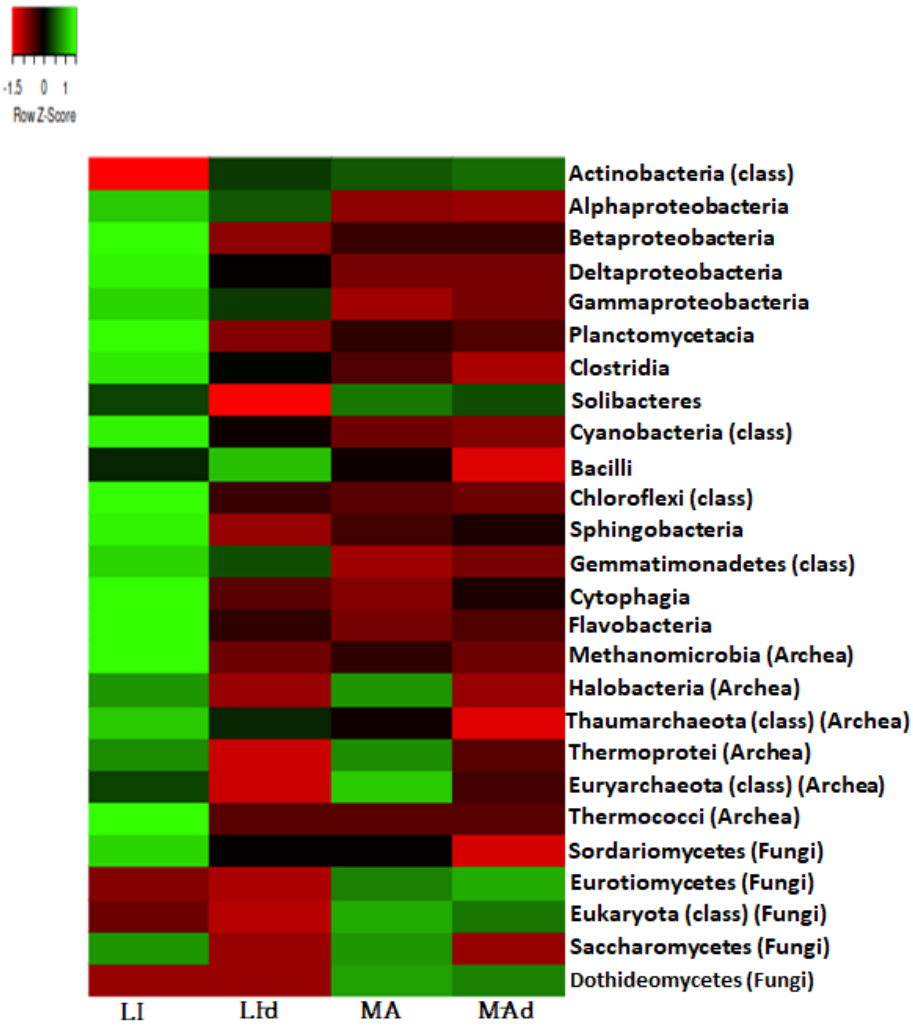

Figure 4.4a: The heatmap shows the major class of soil microbial communities associated with maize plants. LI, rhizosphere soil from the healthy plant at Lichtenburg; LID rhizosphere soil from the diseased plant at Lichtenburg site, MA, rhizosphere soil from the healthy plant at Mafikeng; LID rhizosphere soil from the diseased plant at Mafikeng site.

The class level showed higher abundance of bacteria in LI than MA, except in *Actinobacteria* (class) (44.07%) and *Solibacteres* (2.44%). Similarly, the predominant bacteria class from LID include *Alphaproteobacteria* (16.57), Deltaproteobacteria (4.83%), *Gammaproteobacteria* (5.45%) while the most predominant in MAD are Actinobacteria (class) (44.81%),

Betaproteobacteria (8.27%), and *Planctomycetacia* (1.85%). The fungi class *Sordariomycetes* (MA= 0.31%, MAD = 0.34%), *Eurotiomycetes* (MA= 0.25%, MAD = 0.23%), *Dothideomycetes* (MA= 0.13%, MAD = 0.12%) were more prevalent in Mafikeng. The dominant archea class *Methanomicrobia* (0.28%), *Thaumarchaeota* (class) (0.14%) and *Thermococci* (0.08%) were recorded in LI, while *Halobacteria* expressed similar level of abundance in the two locations (Figure 4.4a).

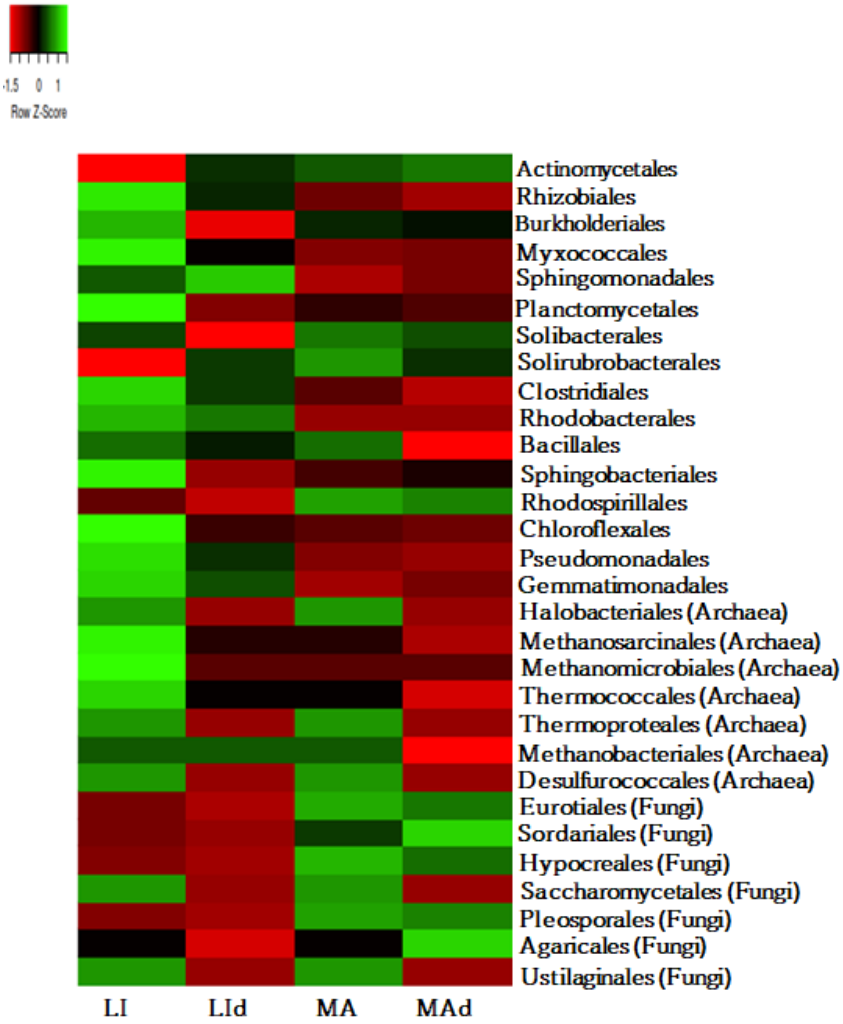

Figure 4.4b: The heatmap shows the major order of soil microbial communities associated with maize plants. LI, rhizosphere soil from the healthy plant at Lichtenburg; LID rhizosphere soil from the diseased

plant at Lichtenburg site, MA, rhizosphere soil from the healthy plant at Mafikeng; LID rhizosphere soil from the diseased plant at Mafikeng site.

Generally, there were more dominant bacteria order recorded in LI and MA than LID and MAD. Also, Lichtenburg samples (LI and LID) showed higher abundance except in *Actinomycetales* (MA= 39.15%, MAD = 40.29%), *Solibacterales* (MA = 2.44%%, MAD = 2.34%), *Rhodospirillales* (MA = 2.04%, MAD = 1.99). Archea order; *Methanosarcinales* (LI = 0.15%, LID 0.13%), *Methanomicrobiales* (LI = 0.11%), and *Thermococcales* (LI = 0.08%, LID = 0.07%) were the most dominant while there was no significant variation in the abundance of *Halobacteriales*, *Thermoproteales*, *Methanobacteriales* and *Desulfurococcales* across the respective healthy and diseased plants rhizosphere from the two locations. The fungal order expressed higher abundance in MA and MAD as shown in *Eurotiales* (0.22%, 0.20%), *Sordariales* (0.12%, 0.17%), Hypocreales (0.15%, 0.13), and *Pleosporales* (0.13%, 0.12%) respectively, while *Desulfurococcales*, *Saccharomycetales*, *Agaricales* and *Ustilaginales* recorded similar level of abundance in the two locations (Figure 4.4b).

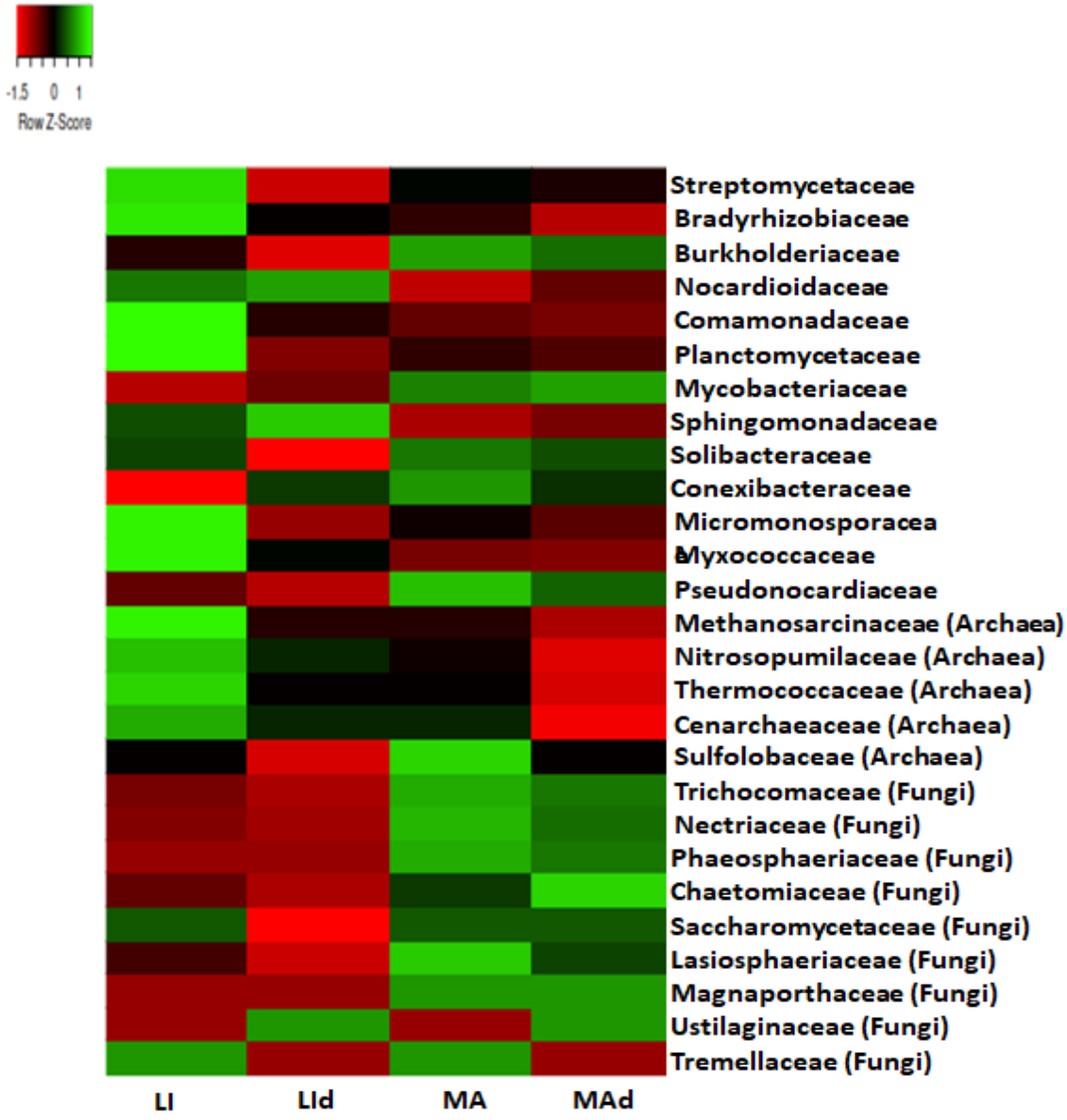

Figure 4.4c: The heatmap shows the major family of soil microbial communities associated with maize plants.

LI, rhizosphere soil from the healthy plant at Lichtenburg; LID rhizosphere soil from the diseased plant at Lichtenburg site, MA, rhizosphere soil from the healthy plant at Mafikeng; LID rhizosphere soil from the diseased plant at Mafikeng site.

At family level, the most dominant bacteria family associated with LI include *Streptomycetaceae* (10.16%), and *Bradyrhizobiaceae* (4.20%), LID; *Nocardioidaceae* (3.23%), *Sphingomonadaceae*

(2.74%), MA; *Solibacteraceae* (2.44%), *Conexibacteraceae* (3.60%) and *Pseudonocardiaceae* (3.37%), MAD; *Burkholderiaceae* (3.59%) and *Mycobacteriaceae* (4.42%). The archea family; *Methanosarcinaceae* (0.13%), *Nitrosopumilaceae* (0.10%), *Thermococcaceae* (0.08%), *Cenarchaeaceae* (0.04%) were dominant in LI and *Sulfolobaceae* (0.05%) in MA. The dominant fungal families were; *Trichocomaceae* (MA= 0.22%, MAD = 0.20%), *Nectriaceae* (MA = 0.15%, MAD = 0.13%), and *Phaeosphaeriaceae* (MA- 0.08%, 0.07%) (Figure 4.4c).

Figure 4.4d: The heatmap shows the major genus of soil microbial communities associated with maize plants. LI, rhizosphere soil from the healthy plant at Lichtenburg; LID rhizosphere soil from the diseased plant at Lichtenburg site, MA, rhizosphere soil from the healthy plant at Mafikeng; LID rhizosphere soil from the diseased plant at Mafikeng site.

At genus level, the bacteria found dominant in the rhizosphere samples from health maize than those of diseased maize include *Streptomyces* (LI = 10.16%, MA = 7.50%), *Candidatus Solibacter* (LI = 2.31%, MA = 3.60%), *Conexibacter* (LI = 3.26%, MA = 3.60%), *Bradyrhizobium* (LI = 1.96%, MA =1.86%). The general *Nocardioides* (LI = 2.10%, LID = 1.95%), *Rhodopseudomonas* (LI = 1.39%, LID = 1.21%), *Gemmatimonas* (LI = 1.26, LID = 1.00) and *Pseudomonas* (LI = 1.04, LID = 0.93) were more dominant in Lichtenburg while Mycobacterium (MA = 4.27, MAD = 4.42), *Candidatus Solibacter* (MA= 2.44%, MAD = 2.34%), *Conexibacter* (MA = 3.60%), *Burkholderia* (MA = 2.69%, MAD = 2.43%), Frankia (MA = 3.35%, MAD = 3.33%), *Methylobacterium* (MA = 1.27%, MAD = 1.34%) and *Rhodococcus* (MA = 1.64%, MAD = 1.56%) dominated the Mafikeng field. Furthermore, except for *Sulfolobus* (MA = 0.04%), the archea genus consisting of *Methanosarcina* (0.10%), *Nitrosopumilus* (0.10%), *Cenarchaeum* (0.04%) and *Pyrococcus* (0.04%) were more abundant in LI. However, the fungi genus showed higher prevalence in MA; *Gibberella* (0.10%), *Neosartorya* (0.09%), *Penicillium* (0.03%), *Phaeosphaeria* (0.08%) and *Nectria* (0.05%), while general *Aspergillus* (0.07%), *Neurospora* (0.07%) and *Chaetomium* (0.06 %) were more associated with diseased maize plant in Mafikeng field (MA) (Figure 4.4d).

4.3.4 The diversity study of the microbial communities of the healthy and diseased rhizosphere samples

The alpha and beta diversity indexes measured by Simpson, Shannon, and Evenness at both phylum and genus level showed no significant ($p > 0.05$) differences among the healthy and diseased rhizosphere samples collected in two farm sites (Table 4.3). As revealed in the PCoA analysis, the composition of soil microbial community across the treatments and sites showed a more significant difference between samples LI and LID, compared to MA and MAD (Figure 4.3).

Analysis of similarity (ANOSIM) revealed that variations and diversity of the soil microbial communities across the sampled sites differed significantly ($p = 0.01$).

Table 4.1: Evaluation of the evenness and diversity of rhizosphere microbial community from each sample

		LI	LID	MA	MAD	p-value
Phylum	Simpson_1-D	0,72±.06	0,66 ± 0.06	0,67±0.06	0,67±0.06	0.93
	Shannon_H	1,71 ± 0.23	1,47 ± 0.22	1,52±0.21	1,57±0.21	
	Evenness_e^H/S	0,31 ± 0.03	0,24 ± 0.10	0,25 ± 0.1	0,27±0.09	
Genus	Simpson_1-D	0,87±0,07	0,91±0,09	0,89±0,08	0,89±0,07	0.48
	Shannon_H	2,46±0,30	2,60±0,36	2,52±0,29	2,51±0,41	
	Evenness_e^H/S	0,36±0.14	0,42±0.23	0,39±0.13	0,38±0,14	

4.4 Discussion

For centuries, maize has remained a crop of choice across the globe owing to its nutritive values and its essential impacts on human and animal diets. To further harness the benefits of this important cereal and ensure its safe and secured consumption, there arises the need to understand the associated community structure and microbiome diversity which impacts its health and productivity (Tanumihardjo *et al*., 2020). Plant rhizosphere is considered the most complex ecosystem that inhabits various organisms including bacteria, fungi, nematodes, herbivores, and arthropods among others. It is, therefore, the most active section of the soil's frontier, where the plant-microbe activities affect a wide range of processes, including plant health (Akanmu *et al*., 2021). The current investigation employed a shotgun metagenomics approach to investigate the composition and diversity of the rhizosphere microbial communities of the healthy and Northern corn leaf blight (NCLB) diseased maize plants, to profile the associated microbes towards further exploration in enhancing plant health.

The higher abundance of the microbes recorded at the phylum level, in the rhizosphere of healthy maize compared to those of NCLB diseased suggests that the gap in the abundance could contribute to the differences in the resistance of the plants to diseases. This was verified in a report which considered the biological activities required for maintaining healthy soil and suppressing plant diseases to be controlled by the soil microbial population (An *et al*., 2011), while the reduction in soil microbial diversity is associated with the emergence of soil-borne plant disease (Zhao *et al*., 2021). More so, the diversity and abundance of microbes across the locations studied, especially dominance of bacteria phyla *Proteobacteria*, *Bacteriodetes, Firmicutes, Cyanobacteria, Planctomycetes, Verrucomicrobia, Gemmatimonadetes, Deinococcus-Thermus* and *Chlorobi* in Lichtenburg (LI) could be as a result of the variations in the factors influencing the microbial population and distributions in the soil horizons (Bhattarai *et al.*, 2015). Most members of the genera such as *Streptomyces, Candidatus Solibacter, Conexibacter* and *Bradyrhizobium* were prevalent in the rhizosphere of healthy compared to diseased maize, and they have been reported for their positive influence on maize growths (Enebe and Babalola, 2019; Fadiji *et al*., 2021b). Some of the specialised attributes of this group vary from the production of bioactive secondary metabolites, antimicrobial, promoting plant growths and most importantly antibiotics that suppressed the expression of pathogenic organisms as in the case of Streptomyces (Procópio *et al.*, 2012).

The shotgun metagenomic approach also enabled the observation of *Ascomycota* and *Basidiomycota* as the major fungal phyla observed in both locations though with higher abundance in Mafikeng and samples from healthy plants. This result is consistent with the earlier reports of fungal phyla associated with maize fields (Enebe and Babalola, 2020; Fadiji *et al*., 2021b). The fungal genus *Gibberella, Neosartorya, Penicillium, Phaeosphaeria, Nectria* dominated the healthy

rhizosphere samples (LI and MA), whereas *Aspergillus, Neurospora, Chaetomium* were more associated with diseased maize (LID and MAD) with higher abundance in MAD. Several species of *Aspergillus* are common soilborne pathogens of maize (Olawuyi *et al*., 2014), and their presence in the South African agricultural soil has been described as largely unexplored (Visagie and Houbraken, 2020). Studies showed that strains of *Neurospora crassa*, which is a species in the genus, have been collected in the tropical and subtropical geographical regions. Also, *N. crassa* has been documented as an endophyte of Scots pine (*Pinus sylvestris*), and the fungus possesses the ability to switch to a pathogenic state when its interaction with the host is disrupted (Kuo *et al*., 2014). Furthermore, despite *Chaetomium* being another most dominant fungal genera in the diseased maize field, some members of *Chaetomium* genera have been shown to demonstrate antimicrobial properties, and were found effective as biological control agents against *Phytophtora nicotianae* strain causing root rot in citrus (Hung *et al.*, 2015), wheat leaf rust (*Puccinia recondita*) and rice blast (*Magnaporthe grisea*) (Park *et al*., 2005), while it enhances the copper stress tolerance in maize seedling (Abou Alhamed and Shebany, 2012).

After the prokaryotes and eukaryotes, archaea have been classified as the third domain of life (Jung *et al*., 2020). Although at lower abundance in the rhizosphere compared to bacteria and fungi, but most of the predominant archaea phyla, *Euryarchaeota, Crenarchaeota, Thaumarchaeota,* and *Korarchaeota* recovered from this study have earlier been reported as members of rhizosphere microbiome of rice, maize, and Scot pine among others (Catão *et al*., 2013; Elkins *et al.*, 2008; Fadiji *et al*., 2021b). However, archaea genera *Methanosarcina, Sulfolobus, Nitrosopumilus, Cenarchaeum, Thermococcus* and *Pyrococcus* are microbes living in extreme environments and playing a vital role in exploring biological resources, some of which include mercury methylation (Ma *et al.*, 2019), indole acetic acid production (White, 1987), growth promotion and disease

resistance (Song *et al*., 2019). However, plants maintained a peculiar mode of interaction with archaea since the rhizosphere generates the natural habitats for both aerobic and anaerobic archaea (Lecomte *et al.*, 2018).

As revealed in the principal component analysis (PCA), PCA 1 contributed nearly 65% to the total variations indicating a higher abundance of up to two-third of the microbial phyla in location LI, while the principal coordinate analysis (PCoA) revealed more than 70% variation in the microbial communities of the healthy and NCLB diseased maize, with higher disparity recorded in Lichtenburg (LI and LID). It can be deduced from this observation that microbial diversity in soils evolves and plays an essential role in crop production sustainability by enriching the soil and reducing the biotic and abiotic stressors (Chaparro et al., 2012; Jat et al., 2021b). The measure of alpha diversity such as Simpson, Shannon and Evenness at phylum and genus level were not significant, revealing the close index of the microbial community at each level. Also, the microbial disparity across the rhizosphere soils of healthy and diseased maize suggests that the microorganisms could have developed specific-associated functions in plant ecosystems.

4.5 CONCLUSION

The shotgun metagenomic study of healthy and northern corn leaf blight diseased maize rhizosphere unveiled an interesting feature that revealed the participation of an array of microorganisms, especially the yet-uncultured in the soil-plant-microbe interaction. Microbial abundance and diversity were found as more associated with the healthy rhizosphere, and this was observed to positively influence the rhizosphere microbiome in modulating the microbial functions towards the management of plant health. Since securing the integrity of soil is essential towards attaining the optimum level of health and yield, it is therefore, opined that further investigation into the microbiome of healthy plant rhizosphere would provide novel insights into the microbe's

contributions to the stability of the microbiome, and this can be employed in sustainable management of northern corn leaf blight in maize.

CHAPTER 5

5.0 VARIATIONS IN THE FUNCTIONAL DIVERSITY OF ROOT MICROBIOME OF HEALTHY AND NORTHERN CORN LEAF BLIGHT INFECTED MAIZE (*Zea mays* L.)

ABSTRACT

Metagenomics is a scientific breakthrough that can reveal the variations in the microbial diversities and functions between the healthy and diseased plants, towards a productive deployment in diverse biotechnological processes and agricultural activities. This study investigated the possible functional diversity in the rhizosphere microbiome of both healthy and Northern Corn Leaf Blight (NCLB) infected maize growing farm at Lichtenburg (LI) and Mafikeng (MA) area of the North West Province, South Africa. We hypothesized variations in the abundance and diversities of microbial functions in the healthy (LI and MA) and diseased (LID and MAD) maize plants. Hence, we extracted DNA from the healthy and diseased maize rhizosphere in the two maize farms and sequenced using a shotgun approach. Using the SEED subsystem, we discovered that the healthy rhizosphere maize plant was dominated by 24 functional categories, while the NCLB infected rhizosphere maize plant was dominated by 4 functional categories. Alpha diversity analysis showed no significant ($p>0.05$) difference between healthy and diseased maize rhizosphere. However, the analysis of beta diversity showed a significant difference. The substantial abundance of functional groups detected especially in LI indicates that presence of plant diseases altered the functions of soil microbiomes. The significant abundance of the unknown role of rhizosphere microbiomes in the disease management suggests the presence of some undiscovered functional genes associated with the microbiome of healthy maize rhizosphere. Hence, further investigation

needs to explore the roles of these functional genes for their agricultural or biotechnological relevance.

Keynotes: rhizosphere, plant health, northern corn leaf blight, functional genes, rhizosphere micribiome.

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5.1 Introduction

Maize (*Zea mays* L.) is one of the world's most popular and fastest-growing economically important cereal crops. It is the most cultivated cereal across the globe that forms staple food for over 900 million people and one third of malnourished children across the globe. Despite its importance, the quality and quantity of maize is being reduced by a variety of diseases including viruses, bacteria, mycoplasma-like organisms, nematodes, and other higher parasitic plants (Peiffer *et al*., 2013; ur Rehman *et al*., 2021). This results in up to 9.4% annual losses across the world, which directly affects human health and income generation (Bandyopadhyay *et al*., 2019; Seyi-Amole and Onilude, 2021). Some of the most common diseases of maize limiting maize output worldwide include gray leaf spots, various types of rust diseases, northern corn leaf blight (NCLB), and southern corn leaf blight (SCLB) (Naeem *et al*., 2021). *Exserohilum turcicum*, which causes Northern Corn Leaf Blight, is a harmful foliar disease of maize distributed widely across the world and are more prevalent in humid weather with temperature between 20 – 28 °C. It has been reported to causing a range of 27.6 to 97.5% yield loss (Craven *et al*., 2020; Kumar *et al.*, 2011).

Several disease managements approaches that have been employed in ameliorating the impact of NCLB include improved management practices (crop rotation, conventional tillage), foliar fungicide use, biological control, and planting resistant cultivars (Galiano-Carneiro and Miedaner, 2017; Ranganatha *et al*., 2017). However, the proposed solution to the current threat to food security and safety, have been reported to lie in the sustainable utilization of beneficial microorganisms to achieve a for long-term agricultural production, with minimal or no adverse impact to the ecosystem, as a result of plants' selective recruitment of soil microorganisms to create a diverse, yet predictable, microbial community in their root region (Adedeji *et al*., 2020), which provide them with specific benefits, especially plant disease resistance (Akanmu *et al*., 2021; Fadiji *et al*., 2021a). The process entails the suppression of pathogens in the rhizosphere by secretion of antimicrobial chemicals, competition within an ecological niche (i.e., nutrition), or through induced systemic resistance (ISR). Thus, the microbiome can indirectly boost the capacity of resistant pathogens to regulate the plant immune system (Qu *et al*., 2020).

The first line of defense against soil-borne infections is the rhizosphere microbiome, and the ecological connections between soil and plant microbes are the primary determining factor of whether or not the pathogen can infect the roots (Chiaramonte *et al.*, 2021). Rhizosphere organisms that have been extensively investigated for their beneficial effects on plant development and health includes nitrogen-fixing bacteria, plant growth-promoting rhizobacteria (PGPR), mycorrhizal fungi, biocontrol microorganisms, protozoa, and mycoparasitic fungi (Mendes *et al*., 2013). The interaction between microbes and plants have been widely explored, however limited information exists on the impact of the vast majority of plant-associated microbes on plant health, development, and disease. As a result, understanding the functional roles of plant microbiome is essential in enhancing the plant health and productivity.

We hypothesize using a shotgun metagenomics method, that an invasive pathogen, either directly or indirectly through the plant, triggers stress responses in the microbial community, that lead to the activating antagonistic traits and shift in the microbiome composition that restricts pathogen invasion. Upon pathogenic attack, plants can use microbial assemblages from the soil for protection against infections (Mendes, 2018). Shotgun metagenomics enables researchers to decipher the functional variety of microbiomes in a given context (Fadiji *et al*., 2021b). Thus, knowledge of the relationship between microbial distribution and function could be harnessed to explore new strains of microbes useful for agricultural use while elucidating the role of rhizosphere microbiome in reducing disease impacts. Therefore, profiling the functional characteristics of microbial communities in the maize rhizosphere is critical to linking rhizosphere soils of healthy and NCLB diseased maize plants with varied functions that enhance plant and soil health thus ensuring sustainable maize production. Hence, this study aims at unveiling the functional diversity of microbes in the maize rhizosphere of both healthy and NCLB infected maize plants using shotgun metagenomics approach.

5.2 Materials and methods

5.2.1 History of the sampled fields

The maize rhizosphere soil samples used in this research was collected from two different locations within the North West Province in South Africa. The North West University farm in Mafikeng (25°47'19.1"S 25°37'05.1"E) and a commercial farm in Lichtenburg (25º59’40.2” S, 26°31'44.2" E) were sampled in this study. The province has summer season between October and March, the typical rainfall ranges from 300 to 700 mm per annum and temperatures from 22 to 34°C the average winter temperature as 16°C, with the hottest months being May and July. The maize

cultivar WE 3127 was planted in both sites at the period of sample collections. The Lichtenburg (LI) farm has been noted for maize monoculture practice since the past ten years, while the University farm (MA) has been exposed to the rotational cultivation of maize and cowpea since 1989. The two farms have a history of synthetic pesticides and NPK fertilizer use, mechanized land tilling systems, and artificial irrigation techniques. The choice of the locations was influenced by the occurrence of Northern corn leaf blight (NCLB) disease in some parts of the farms. Identification of NCLB diseased plants was based on the occurrence of symptoms on the leaves, such as elliptical, gray-green lesions of 3 – 15 cm long, tan with distinct dark zones of fungal sporulation, which turns to a blight that becomes severe in its advanced stage (Vieira *et al.*, 2014). From each of the farms, soil samples were taken from the rhizosphere of both healthy and NCLB-infected plants. This was done approximately 8 weeks after planting.

5.2.2 Sample collection

The sample collection from the two selected maize growing farms (MA and LI) was carried out in January 2021. To enable adequate representation of the field sampled, each field was demarcated into two areas, and each area was independently sampled. Each sampled plant was uprooted and gently agitated to remove the loose soils, while soil tightly bound to the root were carefully scrapped into the sterile bags for the healthy (15 samples) and NCLB infected (15 samples). The respective samples from heathy and diseased maize collected from each farm area were then pooled into two replicates and labelled accordingly. Hence, a total of four samples was collected from each farm sampled. These were transferred into iced-cooler boxes (4^{o}C) and transported to the laboratory where the samples were stored at -20°C until DNA extraction for high throughput sequencing were conducted. The analyses of soil which shows higher Organic Carbon (LI - 1.02%,

LID – 0.89%) and pH (LI - 6.95, LID- 6.76) in Lichtenburg, compared to Organic carbon (MA – 0.26, MAD – 0.25) and pH (MA – 5.17, MAD – 5.37) obtained from Mafikeng, in both healthy and diseased plant rhizosphere (Supplementary 2, Table 1).

5.2.3 Analysis of soil physicochemical properties

Prior to the use of each soil sample for analysis, they were separately ground and sieved with a 2 mm sieve. Determination of the total nitrogen content was conducted according to the method of Sáez-Plaza *et al*. (2013). pH was determined using a pH meter at soil: distilled water ratio of 1:2.5. Bray No. 1 solution was used as the extractant in the determining the phosphorous level of the soil (Bray and Kurtz, 1945), the phosphorus extracted was quantified colometrically, based on the development of the molybdenum blue colour with ammonium molybdate. The compound's absorbance, as measured by a spectrophotometer at 882 nm, was directly proportional to the quantity of phosphorus extracted from the soil. The exchangeable $N\text{-}NH_4$ and $N\text{-}NO_3$ were determined using 1 M KCl solution according to the method described by Kachurina *et al*. (2000). Spectrophotometric measurements of the absorbance were made at 260 nm and 220 nm, respectively. The dry combustion technique was used to calculate the total amounts of N and C (Wright and Bailey, 2001). The exchangeable potassium and sodium were measured by 1 mM ammonium acetate analysis at neutral pH, whereas the sulphur was identified by HCl extraction. The loss of ignition approach was used to calculate organic matter (Hoogsteen *et al*., 2015). Determination of soil organic carbon was conducted in line with the procedure explained by Walkley and Black (1934).

Metagenomics sequencing and processing was conducted as earlier discussed in section 4.3.1

5.2.4 DNA Extraction and shotgun sequencing

The samples of rhizosphere soils collected were subjected to DNA extraction protocol of the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). From each sample, 0.25 g was measured for DNA extraction. The shotgun approach of whole-metagenome sequencing method generated the datasets used in this investigation. This was carried out at the Molecular Research LP, Texas, USA. The DNA concentration of the samples were obtained using a life technologies assay kit-Qubit® dsDNA HS. The libraries were then prepared with 20–50 ng of DNA using the Illumina DNA Prep (M) segmentation library preparation kit in line with the manufacturer's instructions, and samples were fragmented and adapter sequences added. Next, a limited-cycle PCR was performed using the adapters, and the material was supplemented using the use of unique indices. The library preparation was followed by the ultimate concentration of the libraries, after which the final concentration of the prepared libraries was determined using the Qubit® dsDNA HS Assay Kit (Life Technologies) and the average size of the library was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The libraries were pooled and diluted to 0.6 nM equimolar ratios and sequenced at paired end for 300 cycles on the NovaSeq 6000 system (Illumina, USA).

5.2.5 Metagenome's annotation and Data analysis

The metagenome sequences were uploaded to the metagenomics rapid annotation online service (MG-RAST) (Meyer *et al*., 2008), which performed the quality control steps involve dereplication, which removes artificial sequences, ambiguous base-filtering, and host species-specific sequences. The annotation of the sequences was then carried out with BLAST (Basic local alignment search tool) algorithm (Altschul et al., 1990; Kent, 2002) against the M5NR database (Wilke *et al*., 2012) which permits non-redundant alignment of several databases. The RDP (Ribosomal Database

Project) database was used to classify organisms according to their taxonomy, and the SEED subsystem database's default settings were used to assign functional categories from level 1 to level 3 to the organisms. While constructing the functional table, the failed sequences during annotation were deleted, while the unclassified reads were retained for statistical analysis. The percentages were calculated and the functional table arranged for each functional level. The raw sequences associated with this study were deposited with NCBI under the BioProject accession number PRJNA821718. The variations in soil physicochemical properties between rhizosphere soil of the healthy and NCLB disease maize were calculated using a one-way ANOVA and Tukey's pairwise comparison test.

The Shannon diversity and Pielou Evenness indices of the samples were determined, and the indices between the sites were compared with Kruskal–Wallis test, and the test of significance was determined by Monte Carlo permutation test using 9999 random permutations. The variations in the community composition observed within the same sample group was determined by PAST version 3.20. The beta diversity was determined by using primary coordinate analysis based on the Euclidean distance matrix with using one-way analysis of similarities (ANOSIM) (Carrell and Frank, 2015). The Shiny heatmap was used to plot the heat maps (Khomtchouk *et al*., 2017). The PCA and PCoA were plotted using Canoco software (ter Braak and Šmilauer, 2012)

5.3 Results

5.3.1 Physicochemical analysis of NCLB diseased and healthy maize rhizosphere soil

The chemical properties of the soil samples show that pH in both LI and LID are close to neutral while MA and MAD are slightly acidic. All the samples expressed small quantity of total carbon, sulphur, organic carbon, organic matter and ammonium, while phosphorous and potassium

contents. Generally, a more significant ($p<0.05$) amounts were observed in LI and LID, compared to MA and MAD (Table 5.1).

Table 5.1: Chemical analysis of the NCLB diseased and healthy maize rhizosphere samples

Chemical Analysis	LI	LID	MA	MAD
pH	6.95 a	6.76 b	5.17 d	5.37 c
S (mg/kg)	2.86 a	2.71 b	0.34 c	0.00 d
Total % C (mg/kg)	1.16 a	1.04 b	0.445 d	0.45 c
P (mg/kg)	264.43 a	167.85 b	43.095 c	18.58 d
K (mg/kg)	159.50 a	148.50 b	115.50 c	102.50 d
N-NO3 (mg/kg)	6.38 a	9.67 b	1.44 d	1.45 c
N-NH4 (mg/kg)	3.03 d	7.96 b	9.31 a	4.86 c
Org. C %	1.015 a	0.89 b	0.26 c	0.25 d
OM %	3.11 a	2.96 b	1.46 d	1.54 c

Means with different letters along the row are significantly ($p\leq0.05$) different.

5.3.2 Taxonomy and Functional analyses of rhizosphere microbiomes associated with healthy and diseased maize plant.

The predominant phyla across the rhizosphere samples were *Proteobacteria, Dienococcus-Thermus, Gemmatimonadetes, Chlorobi, Cyanobacteria, Planctomycetes, Verrucomicrobia, Acidobacteria, Firmicutes, Chloroflexi* and *Bacteroidetes.* Archaea consisted of; *Euryarchaeota, Thaumarchaeota, Crenarchaeota* and *Korachaeota* while *Ascomycota* and *Basidiomycota* were the major fungal phyla observed. Higher abundance of the microbial community was found samples from the healthy (LI & MA) than diseased (LID & MAD) in both locations,

The functions of the microbial profile investigated at level 1 of the microbial profile through the SEED Subsystem hierarchical gene annotation showed 28 functions in both rhizosphere soil from healthy maize and rhizosphere soil from the diseased maize of the two-field sampled, which exhibited variations in their relative abundances. The functional categories dominated samples

from the LI site. Functions recorded in these samples including, Virulence, Stress response, disease and defense, RNA metabolism, membrane transport, cell wall and capsule, sulfur metabolism, regulation and cell signaling, phosphorus metabolism, cell division and cell cycle, nitrogen metabolism, iron acquisition and metabolism, potassium metabolism, miscellaneous and protein metabolism, were more predominant rhizosphere samples from the LI site while DNA metabolism, nucleosides and nucleotides, photosynthesis and clustering-based subsystems dominated the samples from the LID site. The dominant functional categories as obtained from the MA site were fatty acids, isoprenoids and lipids, respiration, motility and chemotaxis, metabolism of aromatic compounds, prophages, phages, plasmids, transposable elements, dormancy and sporulation, cofactors, prosthetic groups, vitamins, pigments, amino acids and derivatives, carbohydrates, as observed in samples from the MA sites while secondary metabolism, clustering-based subsystems, miscellaneous dominated the MAD samples (Figure 5.1).

Principal component analysis (PCA) was used to examine the distribution of different functional categories between samples from the healthy rhizosphere (LI and MA) and the diseased rhizosphere (LID and MAD) (Figure 5.2). No significant variation was observed in the functional categories observed across the study sites (Table 5.2). The findings showed that site LI was dominated by 16 functional categories, site MA by 10, the site LI by 4, and LID by 3 functional categories (Figure 5.3). The Lichtenburg location had 13 and 4 main functional categories dominated LI and LID, respectively, whereas the Mafikeng site had 8 and 4 major functional categories which dominated MA and MAD, respectively.

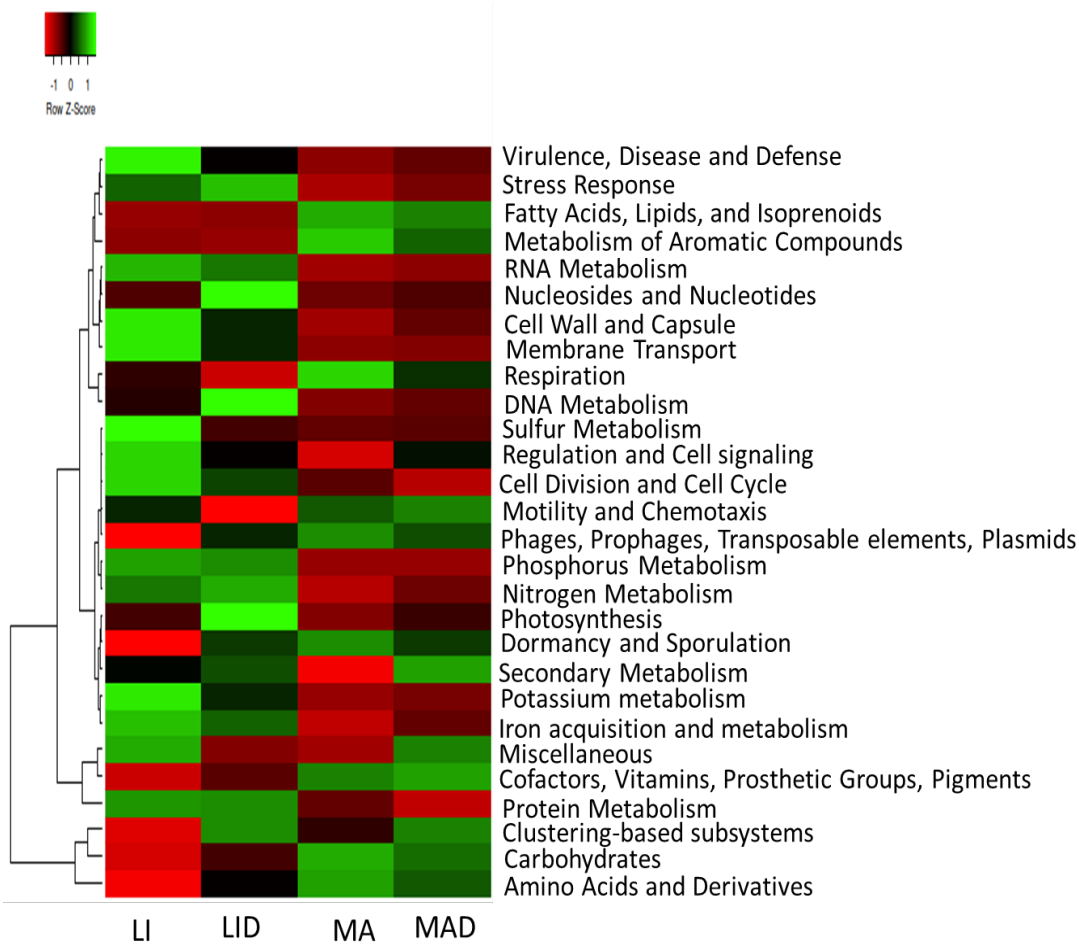

Figure 5.1: Heatmap of the subsystem level 1 showing the key function of the microbiome. The color saturation gradient of the scale bar represents the relative abundances with a z score. LI = healthy rhizosphere soil sample, LID = diseased rhizosphere soil from Lichtenburg Farm. MA= healthy rhizosphere soil sample, MAD = diseased rhizosphere soil sample from the North-West University farm.

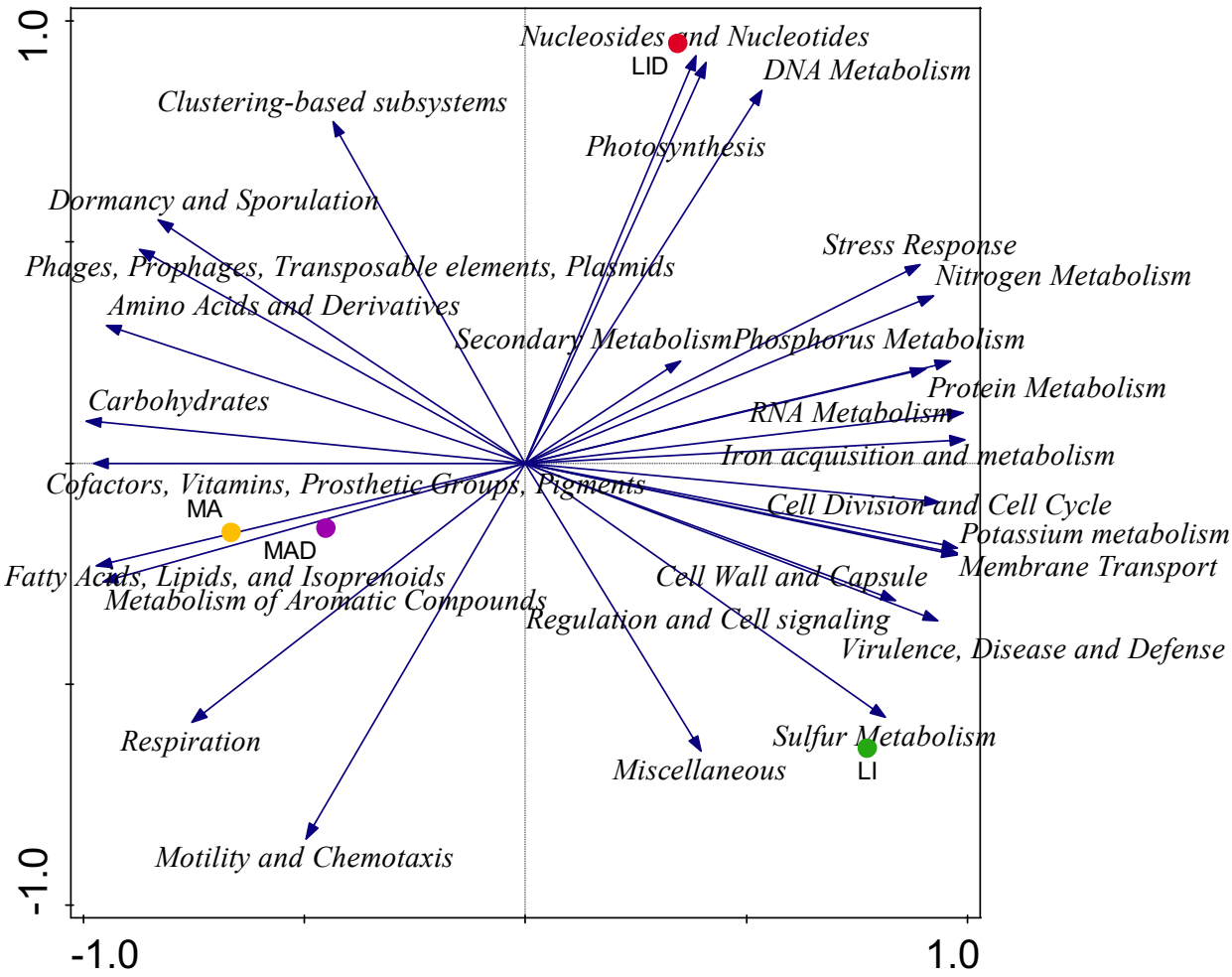

Figure 5.2: PCA analysis showing graph of the distribution of major microbial function of the healthy (LI and MA) and diseased (LID and MAD) rhizosphere soil samples. PC-Axis 1 (88.6%) and PC-Axis 2 (10.1%), the variations were described based Euclidean dissimilarities. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the North-West University farm.

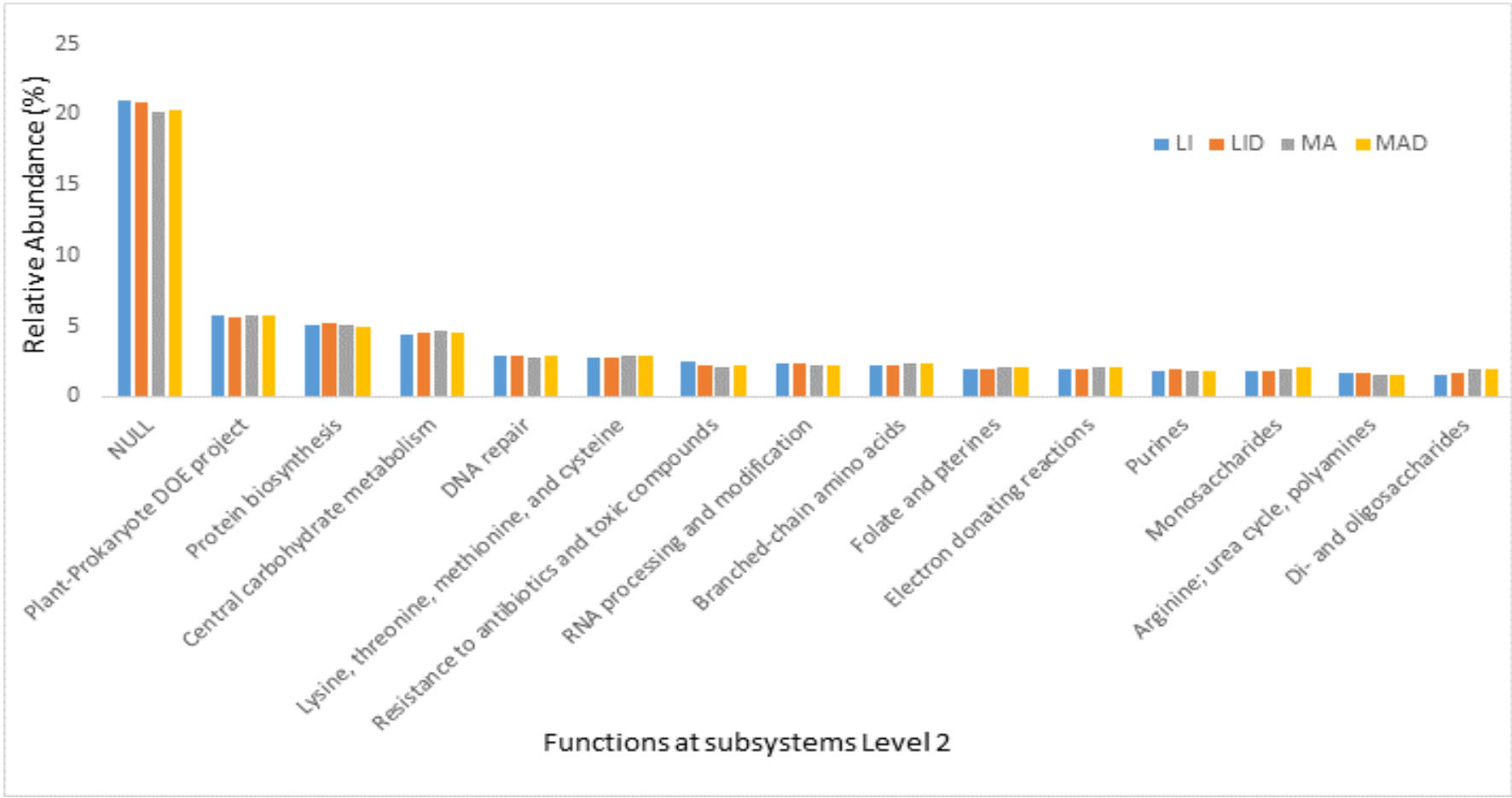

Figure 5.3: The bar char representing the functional groups obtained at level 2 of the SEED subsystems.

LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the North-West University farm.

Unknown functions were shown to be more prevalent at subsystem Level 2 hierarchy for gene annotation across all locations. The unknown protein had the highest relative abundance of 20.99% (LI), 20.8% (LID), 20.2% (MA), and 20.3% (MAD). This was followed by the plant-prokaryote DOE project with 5.71% (LI), 5.66% (LID), 5.7% (MA), and 5.76% (MAD). The abundance of protein biosynthesis was 5.08% (LI), 5.16% (LID), 5.02% (MA), and 4.95% (MAD) across the sites (Figure 5.3).

5.3.3 Assessment of the functional groups across the sampling sites based on their Alpha and beta diversity.

The variety of the functional categories at SEED subsystem level 1 was examined using the evenness, Simpson, and Shannon indexes. The findings showed no significant ($p>0,05$) variation. (Table 5.2). The amount of variation in functional diversity among the sites was analyzed using the Kruskal–Wallis method and found to be non-significant ($P = 0.99$). The PCoA graph revealed that the relative abundance of the 28 functional categories found at SEED Subsystems level 1 differed significantly across LI and LID sites and MA and MAD sites (Figure 5.4). To see if the locations were similar, one-way ANOSIM was utilized. The findings demonstrated a statistically significant ($p<0.05$) variance across all samples from the 28 functional categories ($R = 0.58$ and $p = 0.01$).

Table 5.2: Diversity evaluation of Evenness, Simpson and Shannon of microbial community at level 1 of the SEED subsystem across the sampled sites

Subsystem level 1	LI	LID	MA	MAD	p value
Simpson_1-D	0.93±0.02	0.93±0.02	0.92±0.02	0.92±0.02	0.9951
Shannon_H	2.87±0.15	2.86±0.15	2.85±0.16	2.85±0.15	
Evenness_e^H/S`	0.63±0.09	0.62±0.09	0.61±0.10	0.62±0.09	

Mean ± standard error (n = 2). P values based on Kruskal–Wallis test. LI Rhizosphere soil from healthy maize plants in Lichtenburg, LID Rhizosphere soil from the diseased maize plants in Lichtenburg. MA Rhizosphere soil from healthy maize plants in NWU Farm in Mafikeng, MAD Rhizosphere soil from diseased maize plants in NWU Farm in Mafikeng.

5.3.4 Pathways of the metabolic functions of the microbial communities in the soil samples

The Subsystems at level 3 expressed the sequences involved in the pathways present in the sulfur metabolism, carbohydrates metabolism, nitrogen metabolism, disease, and defense, virulence, secondary metabolism and phosphorus metabolism. From the sulfur metabolism pathways, there was an abundance of sulfur oxidation, sulfatide metabolism and galactosylceramide, thioredoxin-disulfide reductase, utilization of glutathione, taurine utilization, DMSP breakdown, sulfate reduction associated complexes, alkanesulfonates utilization in the LI site while the MA site obtained an abundance of release of Dimethyl sulfide (DMS) from Dimethylsulfoniopropionate (DMSP), inorganic sulfur assimilation and L-cystine uptake and metabolism. However, the same value was obtained as the relative abundance of alkanesulfonate assimilation in the two sites in F1 (LI and MAD). The abundance of utilization of glutathione, sulfur oxidation, DMSP breakdown and sulfate reduction associated complexes was higher in the LI rhizosphere than the LID, MA, and MAD rhizosphere samples (Figure S2.1 and Table S2.2). However, the relative abundances of alkanesulfonate assimilation and galactosylceramide and sulfatide metabolism in LID rhizosphere were higher than those obtained from LI, MA and MAD rhizosphere samples. In the

MA rhizosphere samples, the was higher abundance of Inorganic sulfur assimilation, release of Dimethyl sulfide (DMS) from Dimethylsulfoniopropionate (DMSP) and L-cystine uptake and metabolism obtained than LI, LID and MAD. Also, the same relative abundance of 0.02% alkanesulfonates utilization and 0.03% L-cystine uptake and metabolism was observed for this metabolic pathway across all the rhizosphere samples from all sites.

For the pathway involved in utilization of glutathione, its relative abundances were the same (0.07%) in all rhizosphere samples except in LID, where its relative abundance was 0.06% (Figure S2.1 and Table S2.2). For the metabolic pathways linked with carbohydrate metabolism, there was abundance in maltose and maltodextrin utilization, sugar utilization in thermotogales, trehalose biosynthesis, entner-Doudoroff pathway, pentose phosphate pathway, Pyruvate metabolism II: acetyl-CoA L-rhamnose utilization, inositol catabolism and photorespiration (oxidative C2 cycle) in the MA and MAD samples. The pathways including serine glyoxylate cycle, glycolysis and gluconeogenesis and dehydrogenase complexes were more dominant in the MA rhizosphere sample while calvin benson cycle was observed on the LID and MA samples. TCA cycle pathway was dominant in the LI rhizosphere sample, while Pyruvate metabolism I: PEP was only present in the LID rhizosphere sample. The same values were obtained for glycolysis and gluconeogenesis (0.46%) and Pyruvate metabolism II: acetyl-CoA L-rhamnose utilization (0.42%) for the diseased rhizosphere samples (LID and MAD). In TCA cycle the same value (0.57%) were obtained across all sites besides MAD which was (0.56%) (Figure S2.2 and Table S2.3). For sequences related to nitrogen metabolism were dominant in LI. Some metabolic pathways such as allantoin utilization, Nitrilase, Amidase clustered with urea and nitrile hydratase functions and Nitric oxide synthase were more dominant in the MA site. The dominance of major metabolic pathways such as ammonia assimilation, denitrification, nitrosative stress, cyanate hydrolysis, nitrogen fixation and

dissimilatory nitrite reductase was also observed in the LI site, more on the healthy maize plant rhizosphere sample (Figure S2.3 and Table S2.4). The same value (0.01%) Amidase clustered with urea and nitrile hydratase functions was obtained across all samples. In the results for virulence, disease, and defense, the metabolic pathways involved in beta-lactamase, arsenic resistance, copper homeostasis: copper tolerance, copper homeostasis, mercuric reductase, Methicillin resistance in *Staphylococcus* spp., *Streptococcus pyogenes* Virulome, resistance to fluoroquinolones, Multidrug efflux pump in *Campylobacter jejuni* (CmeABC operon), resistance to chromium compounds, The mdtABCD multidrug resistance cluster and multidrug resistance efflux pumps, were more dominant in the MA and MAD rhizosphere samples (Figure S2.2). Bacterial cyanide production and tolerance mechanisms, cobalt-zinc cadmium resistance, BlaR1 family regulatory sensor-transducer disambiguation, multilocus sequence typing (MLST), MexE-MexF-OprN multidrug efflux system, resistance to vancomycin, streptococcus pyogenes virulome and zinc resistance were dominant in the LI and LID site (Figure S2.4 and Table S2.5). Also, the multidrug resistance efflux pumps relative abundances were higher in the diseased rhizosphere samples (LID and MAD). However, the relative abundances observed in the metabolic pathways involved in Multidrug efflux pump in *Campylobacter jejuni* (CmeABC operon), *Streptococcus agalactiae* virulome and the mdtABCD multidrug resistance cluster were more dominant in the MA rhizosphere sample. We also observed that the pathway, Multidrug efflux pump in *Campylobacter jejuni* (CmeABC operon) and MexE-MexF-OprN multidrug efflux system, was the same (0.02%) in all rhizosphere samples (LI, LID, MA and MAD). With the proliferation of routes, it was also possible to establish the dominance of particular important metabolic pathways in phosphate metabolism such as alkylphosphonate utilization, phosphorus uptake, phosphonate metabolism, phosphoenolpyruvate phosphomutase, High affinity phosphate transporter and

control of PHO regulon and phosphate metabolism in the LI site (Figure S2.5 and Table S2.6). The abundance of Phosphate-binding_DING_proteins was observed in the MAD rhizosphere samples. The same value was obtained for phosphonate metabolism, phosphoenolpyruvate phosphomutase and phosphate metabolism (0.01%) across all samples from the two sites. Furthermore, for the pathways involved in secondary metabolism, we observed that the metabolic pathways, including steroid sulfates, alkaloid biosynthesis from L-lysine, cinnamic acid degradation and auxin degradation were relatively higher in LI and LID rhizosphere samples than those of the MA and MAD rhizosphere samples. The pathways of flavanone biosynthesis, Phenylpropionate degradation, Biflavanoid_biosynthesis, 2-isocapryloyl 3R hydroxymethyl-gamma butyrolactone and other bacterial morphogens, clavulanic acid biosynthesis, tannin biosynthesis, cannabinoid biosynthesis, auxin biosynthesis and nonribosomal peptide synthetases (NRPS) in Frankia sp. Ccl3 were more present in the MA and MAD rhizosphere samples. Also, the pathways of cinnamic acid degradation, phenazine biosynthesis and cannabinoid biosynthesis were present in the diseased rhizosphere samples (LID and MAD) (Figure S2.6 and Table S2.7). The same values were obtained for flavanone biosynthesis (0.02%), cinnamic acid degradation (0.02%), auxin degradation (0.03%), Nonribosomal peptide synthetases (NRPS) in Frankia sp. Ccl3 (0.01%) and auxin biosynthesis (0.10%) across all rhizosphere samples from both sites in the study (Figure S2.6 and Table S2.7).

5.4 Discussion

Maize is a significant food crop grown worldwide and is strategic in supporting the food security of the growing human population that is estimated to reach 9 billion people by 2050 (Fadiji *et al.*, 2021b; Sucher *et al.*, 2017; Tyczewska *et al*., 2018). However, the quantity and quality of maize are often compromised by plant diseases which cause significant economic damage, with additional cost implications on disease management (Sucher *et al.*, 2017). One of the most persistent and devastating foliar diseases of maize, Northern corn leaf blight caused by *Exserohilum turcicum* (Pass.) reduces yield by more than 30% (De Rossi *et al.*, 2021; Naeem *et al*., 2021). Hence, achieving improved plant health and yield necessitates the understanding of functions and nutrient pathways of the rhizosphere microbiome populations involved, particularly the beneficial ones engaged in agricultural sustainability, growth promotion, and ecosystem balance (Fadiji *et al.*, 2021b). We employed SEED subsystems for the hierarchical classification of the roles of microbial activities and metabolic pathways in enhancing plant health between the healthy and diseased maize plants.

The results of alpha diversity assessment across the two sites yielded 2.81 theoretical limits, this revealed the adequate representation of each subsystems in the two farms, as in line with the report of Dinsdale *et al*. (2008) (Table 5.2). Also, the low value of evenness obtained in this study indicates the presence of some dominant functions including carbohydrates metabolism, iron acquisition and metabolism, cell division and cell cycle, motility and chemotaxis as reflected in the subsystems at level 1 as confirmed by the Kruskal-Wallis test with a p-value 0.99 (Table 5.2). The cumulative variance of 93.09% recorded in the principal component analysis (PCA) explains the metabolic potentials of microbiome in the farms studied (Figure 5.2). Furthermore, the abundance of the unknown (null) function observed from all sites at subsystem level 2, combined

with clustering-based subsystems, indicate that there are several important functions connected with this rhizosphere microbiome that have yet to be discovered. According to the study, certain functional gene types are attributed to the cropping sites. Dominant sequences associated with carbohydrate, fatty acid, isoprenoids and lipid metabolism, phosphorus metabolism, nitrogen metabolism, sulfur metabolism, iron acquisition and metabolism, amino acid and derivatives, secondary metabolism and protein metabolism were recorded in the two sites.

In our samples, we found sequences relevant to the metabolism of important mineral nutrients as nitrogen, sulfur, phosphorus, and potassium (Figure 5.1). Mineral nutrition has a significant impact on the growth of plants and crop yields (Fageria and Moreira, 2011; White and Brown, 2010). The relative abundances of these functions were higher in LI samples than in MA samples. The findings show that the microbial communities in our samples are assisting maize plants in their development and growth by assisting in the availability of important nutrients. More so, the increased prevalence of these metabolism in the LI samples indicates that the microbial communities at the Lichtenburg farm are more supportive of nutrient availability in their environments, this could be validated with the presence of organic and inorganic sulfur metabolism, potassium metabolism, and ABC transporters in the subsystems at level 2 (Figure 5.3).

The level 3 metabolic pathways of sulfur metabolism also revealed that the samples were dominated with the pathways of L-cysteine uptake and metabolism, sulfur oxidation, galactosylceramide and sulfatide metabolism, thioredoxin-disulfide reductase, taurine utilization, alkanesulfonates utilization, utilization of glutathione, release of Dimethyl sulfide (DMS) from Dimethylsulfoniopropionate (DMSP), DMSP breakdown, sulfate reduction associated complexes,

alkanesulfonate assimilation and inorganic sulfur assimilation (Figure S2.1 and Table S2.2). Plants require sulphur biomolecules to develop effective defense mechanisms in response to a variety of stresses. Within the cell, Sulphur assimilates serve a critical role as structural components for essential cellular compounds. Furthermore, their function as a signaling agent for intracellular communication has been established (Ihsan *et al*., 2019). Our findings imply that microorganisms in the fields can create thioredoxin and use glutathione, a peptide that contains L-cysteine, as a cofactor to protect maize plants from oxidative stress (Chukwuneme *et al.*, 2021; Shahid et al., 2014; Takagi and Ohtsu, 2016). From our results on chemical soil properties (Table S2.1), the sulfur contents of our samples were lower at MA sites, especially in MAD soil, as a result, the presence of sulfur metabolic genes in our samples revealed that microorganisms -- maintains- a healthy sulfur metabolism in their environments. Studies have shown that sulfur absorption in plants is critical to the natural sulfur cycle. The sulfur absorption route in plants fixes inorganic sulfur in the environment, such as sulfate ions in soil and sulfur dioxide gas in the air (Saito, 2004). The presence of sulfur, nitrogen, potassium and phosphorus metabolism metabolic pathways in our samples implies that soil bacteria play an important role in plant growth promotion and nutrient cycling (Etesami and Adl, 2020).

Our findings also showed that sequences related to the metabolism of carbohydrates, including sugar utilization in thermotogales, serine glyoxylate cycle, maltose and maltodextrin utilization, trehalose biosynthesis, glycolysis and gluconeogenesis, Entener doudoroff pathway, dehydrogenase complexes, pentose phosphate pathway, pyruvate metabolism II: acetyl CoA acetogenesis from pyruvate, L-rhamnose utilization, inositol catabolism and photorespiration (oxidative C2 cycle) dominated the MA and MAD sites (Figure S2.2 and Table S2.3). Microbial survival is reliant on the ability to use a range of accessible carbohydrates as energy and carbon

sources in any environment. Carbon is the most significant medium component since it provides energy to microorganisms and aids in their both development and the generation of primary and secondary metabolites (Mekonnen *et al*., 2019). Our findings show that the microbial communities in our samples aid plants in collecting carbon through various metabolic pathways. The primary pathways for simple carbohydrate generation are gluconeogenesis and pentose phosphate shunt, and they have an important role as osmoregulatory chemicals in cells, particularly in stressful settings (Arnao *et al*., 2021). The glycolysis route allows glucose to be converted to pyruvate, which can be used as a respiratory substrate. If enzymes in the glycolysis route are suppressed, the amount of pyruvate used as a respiratory substrate decrease, and this produces an indirect effect on the electron transport chain through altering the TCA cycle. Furthermore, disruption to the TCA cycle has been linked to male sterility of the crop plant in the past (Geng *et al*., 2018).

In the results for virulence, disease, and defense, the metabolic pathways involved in beta-lactamase, arsenic resistance, copper homeostasis: copper tolerance, copper homeostasis, *C. jejuni* colonization of chick caeca, mercuric reductase, Multidrug efflux pump in *Campylobacter jejuni* (CmeABC operon), multidrug resistance efflux pumps, Methicillin resistance in *Staphylococci* spp., *Streptococcus pyogenes* Virulome, resistance to chromium compounds, resistance to fluoroquinolones and the mdtABCD multidrug resistance cluster were more dominant in the MA and MAD rhizosphere samples (Figure S2.4 and Table S2.5). Bacterial cyanide production and tolerance mechanisms, BlaR1 family regulatory sensor-transducer disambiguation, multilocus sequence typing (MLST), MexE-MexF-OprN multidrug efflux system, cobalt-zinc cadmium resistance, resistance to *vancomycin, Streptococcus pyogenes* virulome and zinc resistance were dominant in the LI and LID site.

In the LI site, nitrogen metabolism-related sequences predominated. At lower levels, major metabolic pathways such as nitrate and nitrite ammonification, ammonia assimilation, nitrogen fixation, allantoin utilization, cyanate hydrolysis, and nitrosative stress associated were distributed across the sites (Figure S2.3 and Table S2.4). The results showed lower levels of nitrogen fixing pathway on the MA site practicing legume crop rotation. Legumes are well-known for their contributions to nitrogen metabolism, particularly nitrogen fixing. Cereals, just like other plants, interact with a variety of bacteria and fungi, including nitrogen-fixing bacteria known as diazotrophs. Because the levels of nitrogen-fixation achieved with nitrogen-fixing bacteria in cereals are insufficient to meet the plant's needs and are never as good as those produced by rhizobium in association with legumes or artificial fertilizers, increasing nitrogen-fixation in cereals has been investigated in many studies (Rosenblueth *et al*., 2018). Microbial Interaction with roots have benefits where plant growth-promoting rhizobacteria plays a role in nitrogen-fixing that aid in plant growth stimulant or disease prevention and suppression (Franche *et al*., 2009), nutrient acquisition such as nitrogen, phosphorous, and promotes growth (Richardson *et al*., 2009). In Maize, some metabolites, such as phenolics and flavonoids, have been shown to attract plant-beneficial rhizobacteria, such as nitrogen-fixing and growth-promoting species (Zhao *et al*., 2019).

The dominance of some major metabolic pathways in phosphate metabolism was also established with the abundance of pathways in LI such as alkylphosphonate utilization, phosphonate metabolism phosphoenolpyruvate phosphomutase, phosphorus uptake, high affinity phosphate transporter and control of PHO regulon and phosphate metabolism (Figure S2.5 and Table S2.6). Additionally, the predominance of a few key metabolic pathways in secondary metabolisms, like flavanone biosynthesis, biflavanoid biosynthesis, phytoalexin biosynthesis, 2-isocapryloyl-3R

hydroxymethyl gamma butyrolactone and other bacterial morphogens, clavulanic acid biosynthesis, tannin biosynthesis, cannabinoid biosynthesis, nonribosomal peptide synthetases and auxin biosynthesis was also observed in the MAD samples (Figure S2.6 and Table S2.7). Polyketides (PKs) and Non-ribosomal peptides (NRPs) and are two of the most diverse families of secondary metabolites, encompassing a wide range of compounds with diverse functions such as iron scavenging siderophores, pigments that provide protection against a variety of stress factors, communication molecules, and antimicrobials. Microbes produce secondary metabolites which have antibacterial properties against the plant diseases. Plant defenses can also be induced by some microorganisms (Rat *et al*., 2021). Secondary metabolism, which involves the creation of many low-molecular-weight molecules known as metabolites by microorganisms as a representation of metabolic complexity, is an important feature of soil bacteria (Chukwuneme *et al*., 2021). The complex collection of plant secondary metabolites i.e. flavonoids that are found across the plant kingdom and have a wide range of biochemical actions, including estrogenic, antioxidant, antiviral, antibacterial, anti-obesity, and anticancer properties. and what they're up to in the rhizosphere microbiome (Fowler and Koffas, 2009). Despite widespread recognition of the importance of the rhizosphere microbiome for plant growth, the vast majority of rhizosphere bacteria are still unknown.

5.5 Conclusion

Using shotgun metagenomics, we conducted a comprehensive impact of diseases, notably known as northern corn leaf blight, on the functional diversity of the microbiome in the rhizosphere of healthy and infected maize plants. The study showed that the major functional groups were more prevalent in the rhizosphere microbial community of healthy than diseased maize samples obtained

from the two farms. Also, root-associated microbiomes are important for plant phenotypes, growth and health, according to plant microbiome research, hence, the rhizosphere microbiomes functions in agricultural production to boost plant growth and instill disease resistance. Furthermore, we identified some metabolic pathways sequences associated sulfur metabolism, phosphorus metabolism, carbohydrates metabolism, nitrogen metabolism, virulence, disease, and defense, and secondary metabolism. The study also discovered a large number of unknown functional groups, indicating that the maize rhizosphere microbial community contains several important functional genes.

5.6 Data Accessibility

The NCBI Sequence Read Archive (SRA) has received the standard sequence data obtained in this work and has placed it there under accession number PRJNA821718.

CHAPTER SIX

6.0 CONCLUDING REMARKS AND RECOMMENDATIONS

6.1 Concluding Remarks

Plant development, disease resistance, and agricultural sustainability are all greatly influenced by the optimum functioning of rhizosphere microbiome. As a result, it is a valuable predictor of plant health. This research therefore advances our understanding of the structure and function of rhizosphere microbiomes and provides information on the positive influence of the rhizosphere microbiome in modulating the microbial functions towards the management of plant health. Using shotgun metagenomic sequencing, this study examined the rhizosphere microbial populations and nutrient routes of healthy and diseased maize plants at two sites. Our findings showed that the healthy maize plant's rhizosphere recorded higher microbial profiles than that of NCLB diseased maize plant. They especially contain beneficial microbial taxa that stimulates plant's growth and the production of functional pathways, which enhance the beneficial interactions between the rhizosphere microbiota and the roots of maize plants. Thereby leading to improved plant health, and maize yield output. Also, our studies demonstrate how plant health status influences the functional diversity of microbial communities in both healthy and diseased maize plant rhizosphere. The broad functional categories, such as virulence, disease, and defense, sulfur metabolism, phosphorus metabolism, carbohydrates metabolism, and nitrogen metabolism, were more abundant in the healthy rhizosphere of maize plants, according to our findings. These functions are thought to help plant health, development, and growth, as well as ecosystem equilibrium.

6.2 Recommendations and Future Research Focus

The rhizosphere microbiome's functional responsibilities determine the plant phenotype's health status and disease. This translates to crop sustainability and innovative visualization and analytical tools that substantially close the current information gap. Details of plant-microbe interactions can be successfully deciphered to create sustainable agriculture practices with enhanced data processing and procedure standardization. Therefore, metabolomic analysis could be used to explore the metabolic capabilities of the microbial community in the rhizosphere of the maize plant in the future. We suggest more study be done on the potential of rhizosphere microbiomes to produce functional genes that contribute to the development of many crops in agricultural operations because there are numerous functions describing how the helpful rhizosphere microbes express significant genes. This study also shows that the microbial communities found in the healthy rhizosphere of maize plants were abundant in those functions that encourage plant growth. Therefore, by examining the key characteristics of the rhizosphere microorganisms to enhance sustainable agriculture, our study is relevant in enhancing maize plant health and productivity.

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6.4 SUPPLEMENTARY DATA

Table S1.1: Chemical analysis of the soil samples

Chemical Analysis	pH	S (mg/kg)	Total % C (mg/kg)	P (mg/kg)	K (mg/kg)	N-NO3 (mg/kg)	N-NH4 (mg/kg)	Org. C %	OM %
LI	6,945	2,86	1,16	264,43	159,5	6,37-5	3,03	1,015	3,105
LID	6,76	2,71	1,04	167,85	148,5	9,67	7,96	0,89	2,96
MA	5,165	0,34	0,445	43,095	115,5	1,44	9,305	0,26	1,46
MAD	5.37	0.00	0.45	18.58	102.50	1.45	4.86	0.25	1.54

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S1.2: Diversity evaluation of the shotgun metagenome analysis from the rhizosphere of healthy and NCLB diseased maize.

SAMPLE SITES	LI	LID	MA	MAD
	UPLOADING INFORMATION			
bp Count	1,615,034,280	526054117	725518084,5	1105047462
Sequences Count	8417925,5	3523219,5	4822221	7607577,5
Mean Sequence Length	147+70	149+72	151+71	151.5±72
Mean GC percent %	64±12	65+12	64+12	65±13
	POST QC INFORMATION			
bp Count	1165623372	487655198,5	673172171	1025190816
Sequences Count	7416748,5	3057518	4198824,5	6608547
Mean Sequence Length	155±67bp	259±61bp	256±64	159±68
Mean GC percent %	65±9	68+9	67+9	67±8
	PROCESSED READS			
Identified Protein Features	6298432	2586953,5	3500670,5	5431363
Identified rRNA Features	11537	5030	8813	13431,5
	ALIGNED READS			
Identified Protein Features	2284008,5	1027412,5	1342054	2098079,5
Identified rRNA Features	3733	1814	25655	4084

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S1.3: Diversity evaluation of major microbiome phyla in the rhizosphere of healthy and NCLB diseased maize plant.

Phylum	**LI**	**LID**	**MA**	**MAD**	**p-value**
Acidobacteria	4,00 ± 0,13	2,29 ± 0,45	4,63 ± 0,15	4,32 ± 0,61	0,20
Actinobacteria	29,51 ± 0,19	45.38 ± 2,92	44.65 ± 1,09	47,28 ± 3,30	0,21
Ascomycota (Fungi)	0,36 ± 0,02	0,26 ± 0,03	0,76 ± 0,09	0.64 ± 0.12	0,10
Bacteroidetes	4,20 ± 0,02	3,01 ± 0,09	3,10 ± 0,36	2,59 ± 0,25	0,16
Basidiomycota (Fungi)	0,04 ± 0,00	0,03 ± 0,00	0,05 ± 0,01	0,04 ± 0,01	0,17
Chlorobi	0,41 ± 0,01	0,30 ± 0,02	0,25 ± 0,02	0,24 ± 0,01	0,11
Chloroflexi	2,71 ± 0,01	1,99 ± 0,28	3,00 ± 0,11	2,98 ± 0,25	0,11
Crenarchaeota (Archea)	0,14 ± 0,00	0,09 ± 0,02	0,12 ± 0,00	0,13 ± 0,01	0,09
Cyanobacteria	2,34 ± 0,05	1,55 ± 0,30	1,61 ± 0,03	1,54 ± 0,13	0,26
Deinococcus-Thermus	0,77 ± 0,01	0,66 ± 0,05	0,68 ± 0,01	0,68 ± 0,00	0,26
Euryarchaeota (Archea)	0,74 ± 0,02	0,56 ± 0,08	0,61 ± 0,00	0,60 ± 0,06	0,24
Firmicutes	4,82 ± 0,12	4,01 ± 0,78	4,05 ± 0,04	4,13 ± 0,24	0,42
Gemmatimonadetes	1,21 ± 0,15	1,10 ± 0,10	0,54 ± 0,01	0,64 ± 0,16	0,14
Korarchaeota (Archea)	0,02 ± 0.00	0,01 ± 0,00	0,02 ± 0,01	0,01 ± 0,00	0,39
Planctomycetes	2.68 ± 0,02	1,37 ± 0,34	1,87 ± 0,14	1,58 ± 0,02	0,14
Proteobacteria	41,36 ± 0,25	34,49 ± 0,38	30,10 ± 0,87	31,08 ± 0,17	0,10
Thaumarchaeota (Archea)	0,14 ± 0,01	0,07 ± 0,04	0,04 ± 0,01	0,05 ± 0,04	0,23
Verrucomicrobia	2,17 ± 0,09	1,17 ± 0,20	1,02 ± 0,15	0,83 ± 0,19	0,20

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

5.8 Supplementary Data 2

Table S2.1: Average percentage of sequences similar to notable metabolisms in soil microbiomes across rhizosphere of healthy and NCLB diseased maize plant

SUBSYSTEMS LEVEL 1	LI	LID	MA	MAD	P value
Amino Acid	10.57±0.002	10.70±0.008	10.8±0.06	10.76±0.05	0.10
Carbohydrates	13.73±0.02	14.21±0.002	14.99±0.08	14.8±0.07	0.08
Cell Division and Cell Cycle	0.93±0.00	0.93±0.002	0.92±0.00	0.92±0.003	0.42
Cell Wall and Capsule	3.65±0.029	3.47±0.03	3.30±0.015	3.36±0.038	0.08
Clustering-based subsystems	12.88±0.04	12.97±0.03	12.93±0.03	12.97±0.03	0.28
Cofactors, Vitamins, Prosthetic Groups, Pigments	5.63±0.07	5.77±0.005	6.05±0.01	6.10±0.08	0.11
DNA Metabolism	4.60±0.03	4.71±0.13	4.56±0.03	4.56±0.01	0.68
Dormancy and Sporulation	0.14±0.003	0.16±0.01	0.17±0.00	0.16±0.00	0.20
Fatty Acids, Lipids, and Isoprenoids	2.71±0.02	2.72±0.00	2.90±0.01	2.87±0.01	0.14
Iron acquisition and metabolism	0.71±0.02	0.66±0.02	0.51±0.01	0.56±0.01	0.08
Membrane Transport	3.86±0.05	3.62±0.04	3.40±0.03	3.42±0.01	0.10
Metabolism of Aromatic Compounds	1.87±0.01	1.87±0.01	2.08±0.00	2.02±0.03	0.10
Miscellaneous	6.39±0.00	6.31±0.09	6.31±0.08	6.38±0.00	0.57
Motility and Chemotaxis	0.98±0.05	0.90±0.04	0.998±0.00	1.01±0.01	0.08
Nitrogen Metabolism	1.41±0.02	1.44±0.05	1.25±0.00	1.29±0.03	0.11
Nucleosides and Nucleotides	3.31±0.01	3.38±0.02	3.30±0.01	3.31±0.01	0.21
Phages, Prophages, Transposable elements, Plasmids	0.82±0.01	0.86±0.02	0.88±0.01	0.87±0.01	0.21
Phosphorus Metabolism	1.30±0.00	1.29±0.01	1.21±0.00	1.21±0.01	0.15
Photosynthesis	0.10±0.00	0.11±0.00	0.10±0.00	0.10±1.19	0.24
Potassium metabolism	0.51±0.01	0.47±0.01	0.44±0.00	0.44±0.01	0.10
Protein Metabolism	8.36±0.00	8.35±0.05	8.17±0.08	8.09±0.02	0.14
RNA Metabolism	3.46±0.03	3.43±0.04	3.67±0.01	3.28±0.02	0.13
Regulation and Cell signaling	0.98±0.01	0.96±0.00	0.93±0.01	0.96±0.01	0.11
Respiration	4.24±0.04	4.16±0.06	4.36±0.01	4.28±0.00	0.10
Secondary Metabolism	0.30±0.00	0.30±0.01	0.29±0.00	0.31±0.01	0.39
Stress Response	2.58±0.03	2.63±0.01	2.43±0.04	2.46±0.02	0.10
Sulfur Metabolism	1.01±0.02	0.96±0.04	0.95±0.01	0.95±0.01	0.39
Virulence, Disease and Defense	2.95±0.01	2.66±0.01	2.50±0.04	2.56±0.06	0.10

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

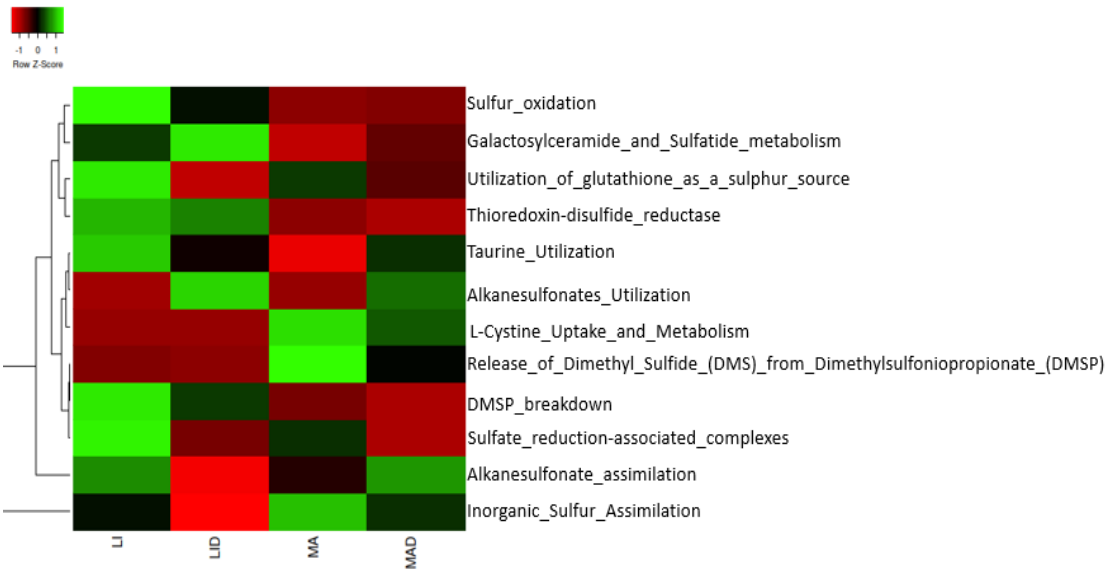

Figure S2.1: Heat map showing the relative abundance of pathways involved in sulfur metabolism in samples across the sites. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the University of North West farm.

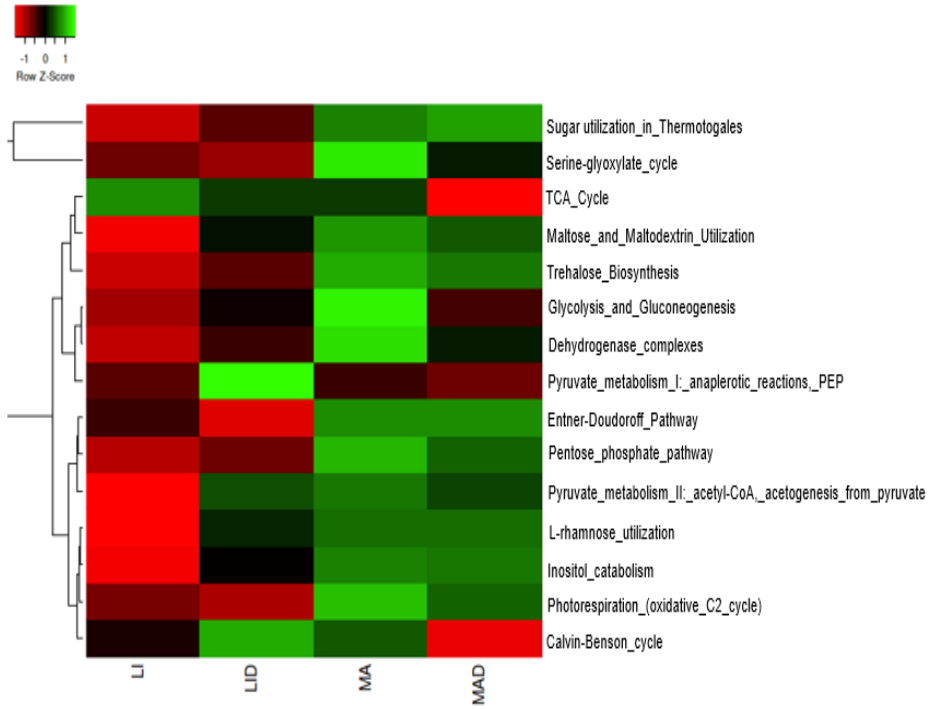

Figure S2.2: Heat map showing the relative abundance of pathways involved in carbohydrate metabolism in samples across the sites. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the University of North-West farm.

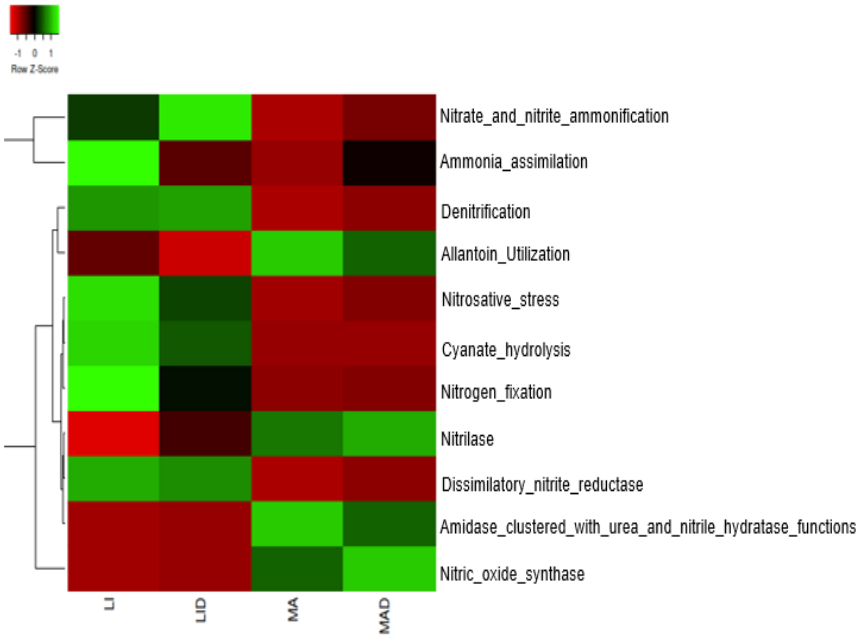

Figure S2.3: Heat map showing the relative abundance of pathways involved in nitrogen metabolism in samples across the sites. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the University of North West farm

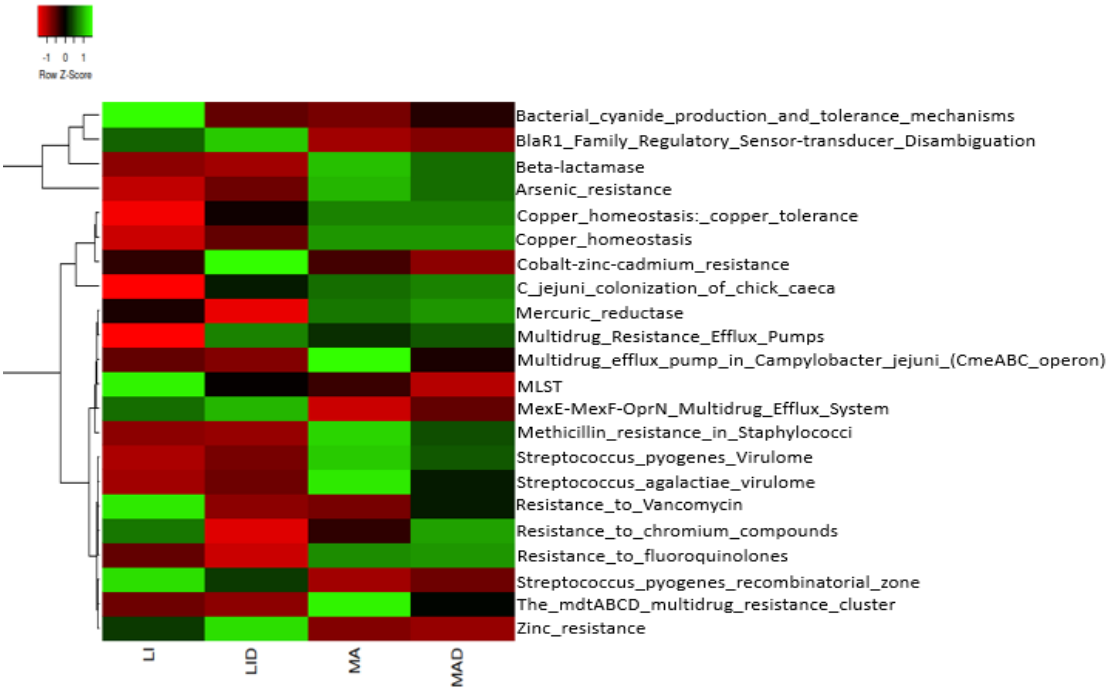

Figure S2.4: Heat map showing the relative abundance of pathways involved in pathways of virulence, disease, and defense of samples across the sites. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the University of North-West farm.

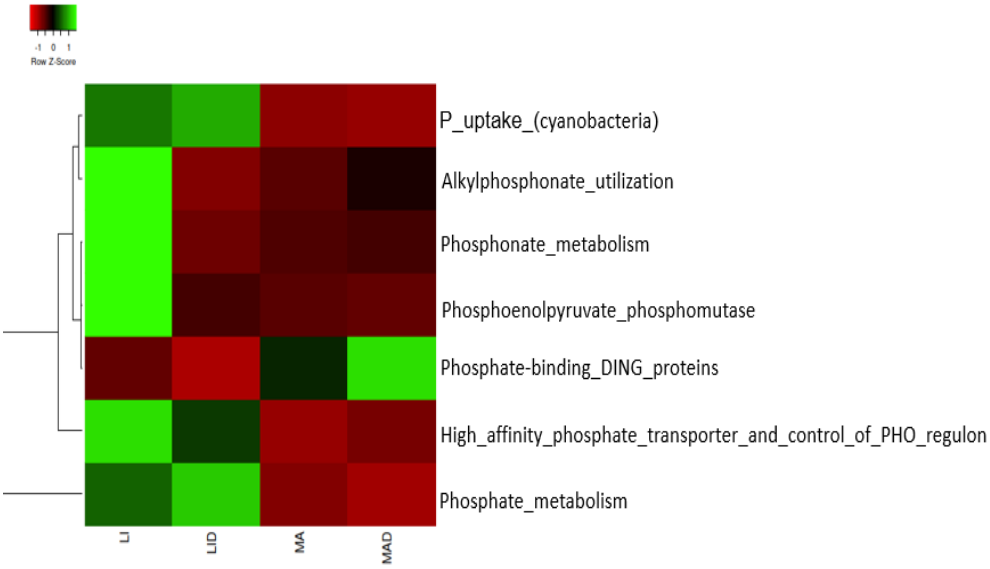

Figure S2.5: Heat map showing the relative abundance of pathways involved in phosphorous metabolism in samples across the sites. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the University of North West farm

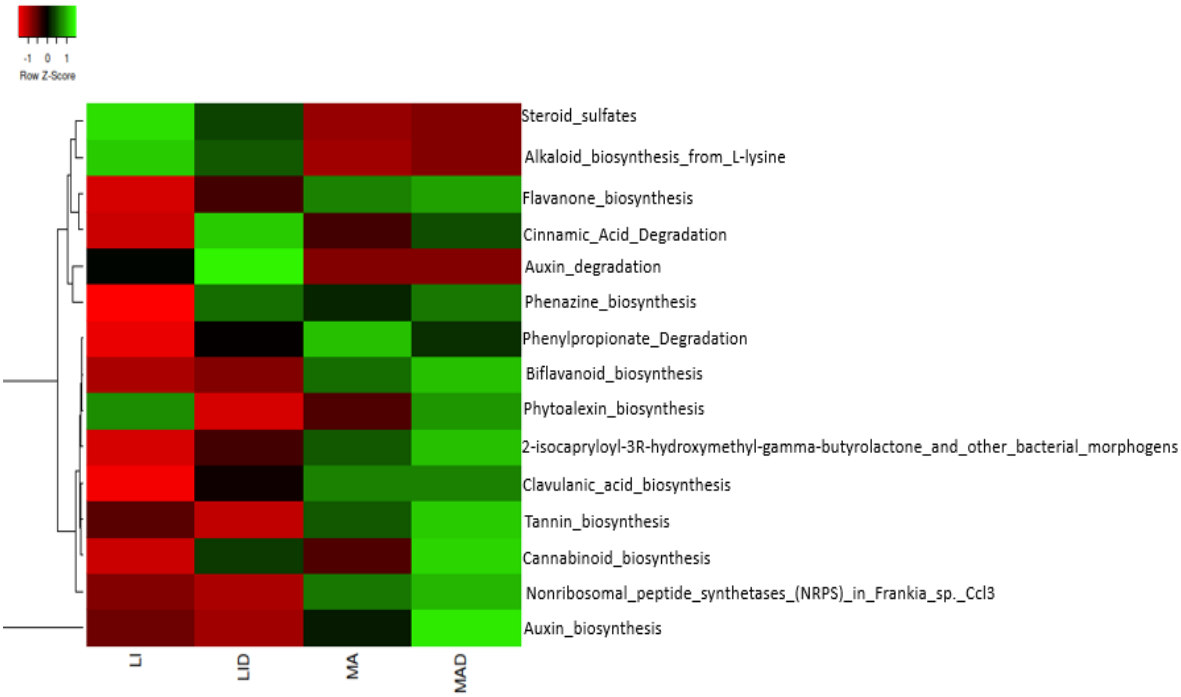

Figure S2.6: Heat map showing the relative abundance of pathways involved in secondary metabolism in samples across the sites. LI healthy rhizosphere soil, LID diseased rhizosphere soil from the Lichtenburg Farm. MA healthy rhizosphere soil, MAD diseased rhizosphere soil from the University of North-West farm

Table S2.2: Relative abundance of sulfur metabolism in the Lichtenburg (healthy and diseased maize plant) and Mafikeng (healthy and diseased maize plant) samples

Metabolic pathway	LI	LID	MA	MAD
Alkanesulfonate assimilation	0.17	0.16	0.16	0.17
lkanesulfonates utilization	0.03	0.03	0.03	0.03
DMSP breakdown	0.0	0.0	0.0	0.0
Galactosylceramide and sulfatide metabolism	0.11	0.11	0.10	0.10
Inorganic sulfur assimilation	0.39	0.38	0.40	0.40
L-Cysteine uptake and metabolism	0.02	0.02	0.02	0.02
Release of Dimethyl Sulfide (DMS) from Dimethylsulfoniopropionate (DMSP)	0.0	0.0	0.0	0.0
Sulfate reduction associated complexes	0.0	0.0	0.0	0.0
Sulfur oxidation	0.12	0.09	0.08	0.08
Taurine Utilization	0.04	0.04	0.03	0.04
Thioredoxin-disulfide reductase	0.06	0.06	0.05	0.05
Utilization of glutathione (sulfur source)	0.07	0.06	0.07	0.07

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S2.3: Relative abundance of Carbohydrates metabolism in the Lichtenburg (healthy and diseased maize plant) and Mafikeng (healthy and diseased maize plant) samples

Metabolic pathway	LI	LID	MA	MAD
Sugar utilization in Thermotogales	0.82	0.86	0.69	0.67
Serine glyoxylate cycle	1.27	1.26	1.33	1.30
TCA Cycle	0.57	0.57	0.57	0.56
Maltose and maltodextrin utilization	0.52	0.58	0.61	0.60
Trehalose biosynthesis	0.56	0.60	0.69	0.67
Glycolysis and Gluconeogenesis	0.45	0.46	0.48	0.46
Dehydrogenase complexes	0.45	0.46	0.48	0.47
Pyruvate metabolism I: PEP	0.50	0.52	0.50	0.49
Entner-Doudoroff Pathway	0.38	0.37	0.40	0.40
Pentose phosphate pathway	0.36	0.37	0.43	0.42
Pyruvate metabolism II: acetyl-CoA	0.40	0.42	0.43	0.42
L-rhamnose utilization	0.30	0.33	0.34	0.34
Inositol catabolism	0.28	0.33	0.35	0.35
Photorespiration (oxidative C2 cycle)	0.31	0.31	0.36	0.34
Calvin-Benson cycle	0.31	0.32	0.32	0.31

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S2.4: Relative abundance of nitrogen metabolism in the Lichtenburg (healthy and diseased maize plant) and Mafikeng (healthy and diseased maize plant) samples

Metabolic pathway	LI	LID	MA	MAD
Nitrate_and_nitrite_ammonification	0.43	0.49	0.35	0.37
Ammonia_assimilation	0.56	0.55	0.55	0.56
Denitrification	0.09	0.09	0.03	0.04
Allantoin_Utilization	0.07	0.07	0.08	0.08
Nitrosative_stress	0.02	0.02	0.01	0.01
Cyanate_hydrolysis	0.02	0.02	0.01	0.01
Nitrogen_fixation	0.04	0.02	0.02	0.02
Nitrilase	0.0	0.0	0.0	0.0
Dissimilatory_nitrite_reductase	0.01	0.01	0.0	0.0
Amidase clustered with urea and nitrile hydratase functions	0.01	0.01	0.01	0.01
Nitric_oxide_synthase	0.16	0.16	0.18	0.19

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S2.5: Relative abundance of virulence, disease, and defense metabolism in the Lichtenburg (healthy and diseased maize plant) and Mafikeng (healthy and diseased maize plant) samples

Metabolic pathway	LI	LID	MA	MAD
Bacterial cyanide production and tolerance mechanisms	0.24	0.23	0.23	0.23
BlaR1 family regulatory sensor-transducer disambiguation	0.20	0.20	0.19	0.19
Beta-lactamase	0.22	0.22	0.31	0.29
Arsenic resistance	0.33	0.34	0.38	0.37
Copper homeostasis: copper tolerance	0.08	0.09	0.09	0.09
Copper homeostasis	0.08	0.08	0.09	0.09
Cobalt-zinc-cadmium resistance	0.09	0.10	0.09	0.09
***Campylobacter jejuni* colonization of chick caeca**	0.12	0.14	0.15	0.15
Mercuric reductase	0.03	0.02	0.03	0.03
Multidrug resistance efflux pumps	0.02	0.03	0.03	0.03
Multidrug efflux pump in *Campylobacter jejuni* (CmeABC operon)	0.02	0.02	0.02	0.02
Multilocus sequence typing (MLST)	0.03	0.02	0.02	0.02
MexE-MexF-OprN multidrug efflux system	0.02	0.02	0.02	0.02
Methicillin resistance in Staphylococci	0.02	0.02	0.03	0.03
Streptococcus pyogenes virulome	0.0	0.0	0.01	0.0
***Streptococcus agalactiae* virulome**	0.0	0.0	0.0	0.0
Resistance to Vancomycin	0.0	0.0	0.0	0.0
Resistance to chromium compounds	0.0	0.0	0.0	0.0
Resistance to fluoroquinolones	0.0	0.0	0.0	0.0
***Streptococcus pyogenes* recombinatorial zone**	0.0	0.0	0.0	0.0
The mdtABCD multidrug resistance cluster	0.0	0.0	0.0	0.0
Zinc resistance	0.0	0.0	0.0	0.0

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S2.6: Relative abundance of phosphorus metabolism in the Lichtenburg (healthy and diseased maize plant) and Mafikeng (healthy and diseased maize plant) samples

Metabolic pathway	LI	LID	MA	MAD
P_uptake_(cyanobacteria)	0.10	0.10	0.09	0.09
Alkylphosphonate_utilization	0.08	0.06	0.07	0.07
Phosphonate_metabolism	0.01	0.01	0.01	0.01
Phosphoenolpyruvate_phosphomutase	0.01	0.01	0.01	0.01
Phosphate-binding_DING_proteins	0.0	0.0	0.0	0.0
High affinity phosphate transporter and control of PHO regulon	0.25	0.25	0.24	0.24
Phosphate_metabolism	0.01	0.01	0.01	0.01

Table legend: LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.

Table S2.7: Relative abundance of secondary metabolism in the Lichtenburg (healthy and diseased maize plant) and Mafikeng (healthy and diseased maize plant) samples

Metabolic pathway	LI	LID	MA	MAD
Steroid sulfates	0.04	0.03	0.01	0.01
Alkaloid biosynthesis from L-lysine	0.03	0.03	0.02	0.02
Flavanone biosynthesis	0.02	0.02	0.02	0.02
Cinnamic acid degradation	0.02	0.02	0.02	0.02
Auxin degradation	0.03	0.03	0.03	0.03
Phenazine biosynthesis	0.03	0.04	0.04	0.04
Phenylpropionate_Degradation	0.0	0.0	0.0	0.0
Biflavanoid_biosynthesis	0.0	0.0	0.0	0.0
Phytoalexin_biosynthesis	0.0	0.0	0.0	0.0
2-isocapryloyl 3R hydroxymethyl-gamma butyrolactone and other bacterial morphogens	0.0	0.0	0.01	0.01
Clavulanic acid biosynthesis	0.0	0.01	0.01	0.01
Tannin biosynthesis	0.01	0.0	0.01	0.01
Cannabinoid biosynthesis	0.0	0.01	0.01	0.01
Nonribosomal peptide synthetases (NRPS) in Frankia sp. Ccl3	0.01	0.01	0.01	0.01
Auxin biosynthesis	0.10	0.10	0.10	0.10

LI, rhizosphere soil from the healthy maize in Lichtenburg; LID, rhizosphere soil from the diseased maize in Lichtenburg. MA, rhizosphere soil from the healthy maize in Mafikeng, MAD, rhizosphere soil from the diseased maize in Mafikeng.