Metagenomic analysis of 16S sequences of nitrifying bacteria and archaea inhabiting maize rhizosphere

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DECLARATION

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

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DEDICATION

This work is dedicated to the Almighty God the ruler of the universe who has guided me throughout this journey.

ACKNOWLEDGMENTS

Achieving this goal would not have been possible without the people that surround me. Thank God for bringing them my way.

I wish to express my appreciation to my esteemed supervisor , Professor O.O. Babalola for her care, guidiance, encouragement, patience and motivation throughout the program. I also want to thank the entire staff and member of the Department of Microbiology and Faculty of Natural and Applied Science. I am grateful to North-West University for the Postgraduate and Faculty bursary which eased the financing of the degree.

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LIST OF ABBREVIATION

16S rRNA- 16S ribosomal ribonucleic acid

amoA- ammonia monooxygenase

AOA- ammonia oxidizing archaea

AOB- ammonia oxidizing bacteria

DMPP- 3,4-dimethylpyrazole phosphate

DNA- deoxyribonucleic acid

DNRA- dissimilatory nitrite reduction to ammonium

FAOSTAT- Food and Agriculture Organization Corporate Statistical Database

FGDG- flue gas desulphurization gypsum

FR- fruiting

HAO- hydroxylamine oxidoreductase

HAO- hydroxylamine oxidoreductase

HATS- high affinity transport system

IR- industrial revolution

LATS- low affinity transport system

MG-RAST- metagenomic rapid annotation using subsystems technology

NBPT- N-(n-butyl) thiophosphoric triamide

NOAA- National Oceanic and Atmospheric Administration

NP- nitrification process

NUE- nitrogen use efficiency

NXR- nitrite oxidoreductase

NXR- nitrite oxidoreductase

PCA- principal Component Analysis

PCoA- principal coordinate analysis

PGPR- plant growth promoting rhizobacteria

PR- pretasseling

TA- tasseling

USDA- United States Department of Agriculture

GENERAL ABSTRACT

The global nitrogen cycle has been disrupted, mainly as a result of nitrogen-based synthetic fertilizers and other agrochemicals used in plant cultivation. Microbial inoculation is fast becoming an environmentally friendly choice of biofertilizer. Nitrifying bacteria and archaea are the chief contributors of nitrogen available for plant use in the soil. The growing demand for maize has intensified its cultivation. Hence, there is a need to identify nitrifying microorganisms associated with maize plants, understand their relationship with the soil environmental factors, other microbes, and the soil community level physiological profile. This would enable efficient management of the studied group of organisms to maximize their potential for the growth of maize. Soil samples from the rhizosphere were obtained at various phases (pretassling, tassling and fruiting) of maize growth at North-West University Farm Molelwane, South Africa. Bulk soil was also collected. The nitrifying bacteria, archaea, and other microorganisms found in the maize rhizosphere were identified using 16S amplicon sequencing. The DNA was isolated from the soil samples using the nucleospin soil DNA extraction kit and sequenced on the Illumina Miseq. The acquired sequences were examined and processed using MG-RAST. The physical and chemical parameters of the rhizosphere were determined, and their impact on the nitrifying community was assessed. Also, the community level physiological profile was carried out using the Microresp Technique. The result revealed 9 genera of nitrifying bacteria; *Nitrospira, Nitrosospira, Nitrobacter, Nitrosovibrio, Nitrosomonas, Nitrosococcus, Nitrococcus,* unclassified (derived from Nitrosomonadales), unclassified (derived from Nitrosomonadaceae) and 1 archaeon, *Candidatus Nitrososphaera*. The Nitrospirae phyla group, which had most of the nitrifying bacteria, was more abundant at the tasselling stage (67.94%). Alpha diversity showed no significant difference. However, the Beta diversity showed a significant difference ($P = 0.01$, $R = 0.58$) across the growth stages. Although growth stages had no effect on nitrifying

bacteria and archaea diversity, there was variation in microbial structure as it related to maize growth stages. The bulk of the microorganisms were detected during the fruiting stage, whereas the nitrifying bacteria were most abundant during the tasselling stage. The pH values for soil chemical characteristics obtained varied from 5.35 to 6.22, with a mean of 5.93. The carbon-nitrogen ratio is around 9:1. The NH_4 to NO_3 ratio is about 1:1.4. There was a significant correlation between some of the parameters and the nitrifying microorganisms. The relationship between nitrifying bacteria, archaea, and other microbial groups revealed a significant negative and positive correlation. The Pearson correlation further showed a positive relationship between unclassified Nitrosomonadales and *Bacillus* (r = 0.59), unclassified Nitrosomonadales and *Azospirillum* (r = 0.52), *Nitrobacter* and *Azospirillum* (r = 0.54), *Nitrosomonas* and *Stenotrophomonas* (r = 0.54), *Candidatus Nitrosphaera* and *Rhizobium* (r = 0.68), *Nitrospira* and alanine (r = 0.52), and Lysine and *Nitrobacter* (r = 0.54). This study found previously known and undiscovered nitrifying bacteria and archaea linked with the maize rhizosphere. It has also demonstrated the relationship between the identified nitrifiers and soil chemical properties. The findings of this study will aid in the improvement of maize growth and development by altering the structure of the rhizosphere microbial community.

Keywords: Environmental challenge; nitrification inhibitor; nitrifying microorganism; synthetic fertilizer; food security; industrial revolution; sustainable agriculture

LIST OF PUBLICATIONS

Chapter Two: Factors influencing soil nitrification process and the effect on environment and health. *Published in Frontiers in Sustainable Food Systems*. (2021) 6: 821994 https://doi.org/10.3389/fsufs.2022.821994

Authors: Oluwatobi Esther Ayiti and Olubukola Oluranti Babalola

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

Chapter Three: Sustainable intensification of maize in the industrial revolution: potential of nitrifying bacteria and archaea. *Published in Frontiers in Sustainable Food Systems*. (2021) 6: 827477 https://doi.org/10.3389/fsufs.2022.827477

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Chapter Four: 16S metagenomics of nitrifying bacteria and archaea inhabiting maize rhizosphere and the influencing environmental factors. *This chapter is under review in Agriculture.*

Authors: Oluwatobi Esther Ayiti, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, carried out the experiment, performed the data analyses, interpreted the results, and wrote the first and final draft of the manuscript.

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Authors: Oluwatobi Esther Ayiti, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, carried out the experiment, performed the data analyses, interpreted the results, and wrote the first and final draft of the manuscript.

Chapter Six: Influence of nitrifying microorganism on plant growth promoting bacteria and community level physiological profile of the maize rhizosphere. *This chapter is under review in Journal of Microbiology, Biotechnology and Food Science.*

Authors: Oluwatobi Esther Ayiti, Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, carried out the experiment, performed the data analyses, interpreted the results, and wrote the first and final draft of the manuscript.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Nitrogen (N) is the most abundant element in the atmosphere. Although nitrogen is naturally found in the environment, it is provided for the use of plants and animals through the nitrogen cycle (Soliman and Eldyasti, 2018). It is one of the critical elements of life required for biochemical processes such as the formation of amino acids, proteins, DNA, and chlorophyll. They are mainly used up by organisms in their compound state. The compounds of nitrogen include; dinitrogen (N_2), ammonia (NH_3), ammonium (NH_4), nitric oxide (NO), nitrous oxide (N_2O), nitrogen dioxide (NO_2), nitrite (NO_2-), nitrate (NO_3-), hydroxylamine (H_3NO), ammonium sulfate $(NH_4)_2SO_4$, and urea (CH_4N_2O). However, in plants, only ammonium (NH_4^+) and nitrate (NO_3^-) can be assimilated (Kuypers et al., 2018).

Nitrogen compounds (NH_4 and NO_3) are needed for the formation of chlorophyll an important pigment that is crucial in plants for photosynthesis, a process through which plants produce their food for growth and other metabolic activity and plants usually reflect symptoms of nitrogen deficiency or abundance (Figure 1.1). The higher the amount of chlorophyll in a plant, the higher the rate of photosynthesis (Hidayati and Anas, 2016). This accounts for the high quantity usually required (Table 1.1). Through the complex activity of microorganisms, biotic and abiotic processes, nitrogen cycling provides plants with access to these nitrogen compounds.

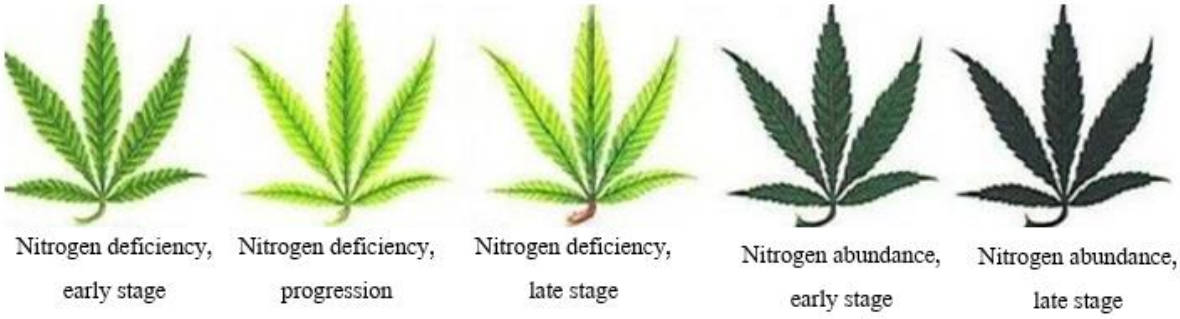

Figure 1.1: Plant showing different stages of nitrogen deficiency and abundance (Grows, 2016)

Table 1.1: Nutrient uptake and removal by bushel maize (Bender et al., 2013)

Nutrient	Requirement to produce (lb/acre)	Removed with grain (lb/acre)	Harvest Index (%)
N	256	148	58
P_2O_5	101	80	79
K_2O	180	56	32
S	23	13	57
Zn	7.1	4.4	62
B	1.2	0.3	23

The cycling of nitrogen is a complex biogeochemical process in which nitrogen is converted from one chemical form to another. Although the classical nitrogen cycle does not exist in the orderly fashion of distinct processes following each other, microorganisms in the cycle form complex networks that link nitrogen transforming reactions (Kuypers et al., 2018). However, it can be divided into five major stages: nitrogen fixation, ammonification, nitrification, assimilation and denitrification (Figure 1.2). Nitrogen fixation is the incorporation of atmospheric nitrogen into the soil by the metabolic action of nitrogen-fixing bacteria (Valentine et al., 2018). Nitrogen-fixing bacteria could be in symbiotic relationship with

leguminous plants or free living among the soil microbiota (Dynarski and Houlton, 2018). Ammonification is the production of ammonia and ammonium by microbial conversion of organic nitrogen gotten from decomposing organic debris into inorganic nitrogen (Jorgensen and Fath, 2014).

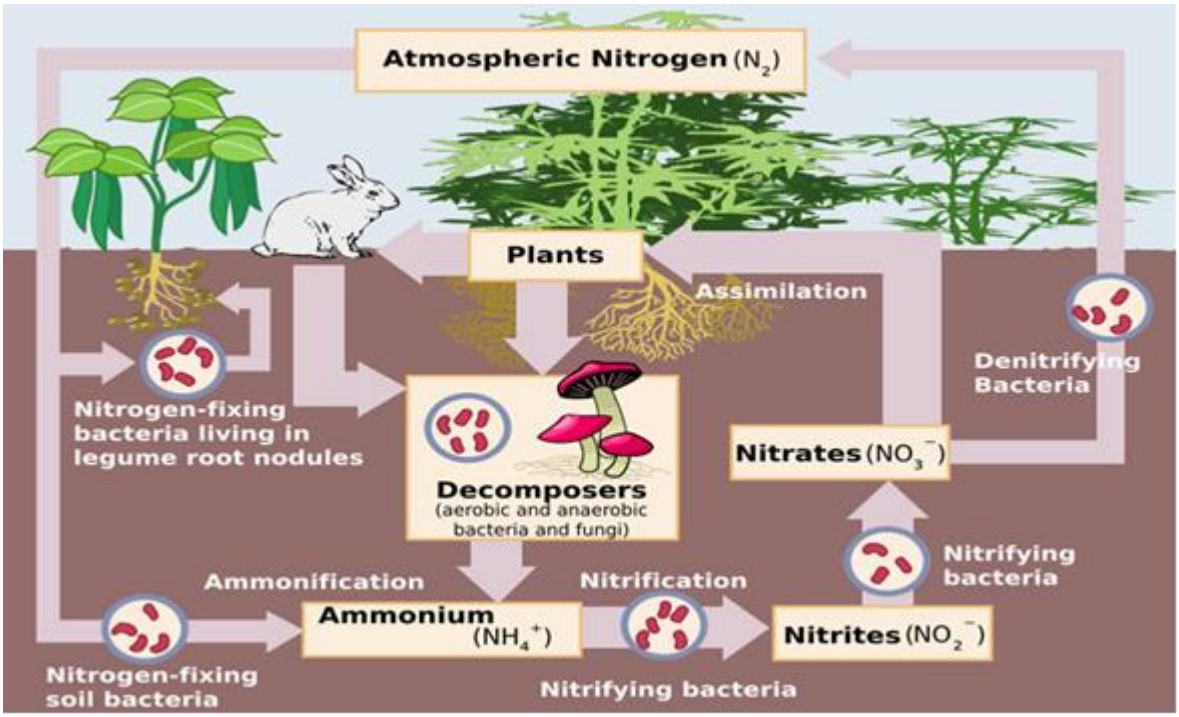

Figure 1.2: Nitrogen cycling in the soil ecosystem (Dreo, 2009).

At the nitrification stage, ammonia is converted to nitrite by ammonia oxidizing bacteria (AOB) and archaea (AOA), and then to nitrate by nitrite oxidizing bacteria and archaea. In acidic soils, AOA dominates the nitrification process, whereas AOB is more prevalent in neutral, alkaline, and nitrogen-rich soils (He et al., 2012). A change can occur in the community structure and abundance of AOA and AOB in agricultural soil depending on cultivation style. As observed by Wang et al. (2014), the achaeal ammonia monooxygenase enzyme (*amoA*) gene abundance increased while bacterial *amoA* gene abundance decreased after conversion from soybean cultivation to rice. Nitrification is a crucial process necessary for the healthy growth of plants (Burrell et al., 2001).

At the assimilation stage, absorption of nitrate or/and ammonium takes place through the active transport across the plasma (Krapp, 2015). Denitrification is the reduction of the unabsorbed nitrate to nitrogen. Nitrates that are not absorbed by the plant at the required rate are reduced to nitrogen molecules by the action of denitrifying bacteria and diffused into the atmosphere. Recently, anaerobic ammonia oxidation, a process called anammox, gives rise to nitrogen gas and water. Anammox reactions are involved in the transformation of ammonium, nitrite, and nitrate to dinitrogen without N_2O as an intermediate, acting as mitigating processes to nitrification (Klotz, 2016).

As seen in Figure 1.3, numbered circles represent the reactions that comprise the nitrogen cycle processes. Ammonification can be accomplished through the first process (indicated as '1' in the figure), which is dinitrogen reduction (also known as nitrogen fixation) or the second process (2) which involves, dissimilatory nitrite reduction to ammonium (DNRA). The third process (3) is the oxidation of ammonia to nitrite (also known as nitritation), while the fourth process (4) is the oxidation of nitrite to nitrate (also referred to as nitratation). The fifth process (5) involving reduction of nitrate to nitrite can be linked to the second, sixth (6) and seventh (7) processes in a population or community. Denitrification, also known as 'nitrogen-oxide gasification,' is depicted as the sixth process. Anammox is illustrated as the seventh process and is also known as coupled nitrification–denitrification.

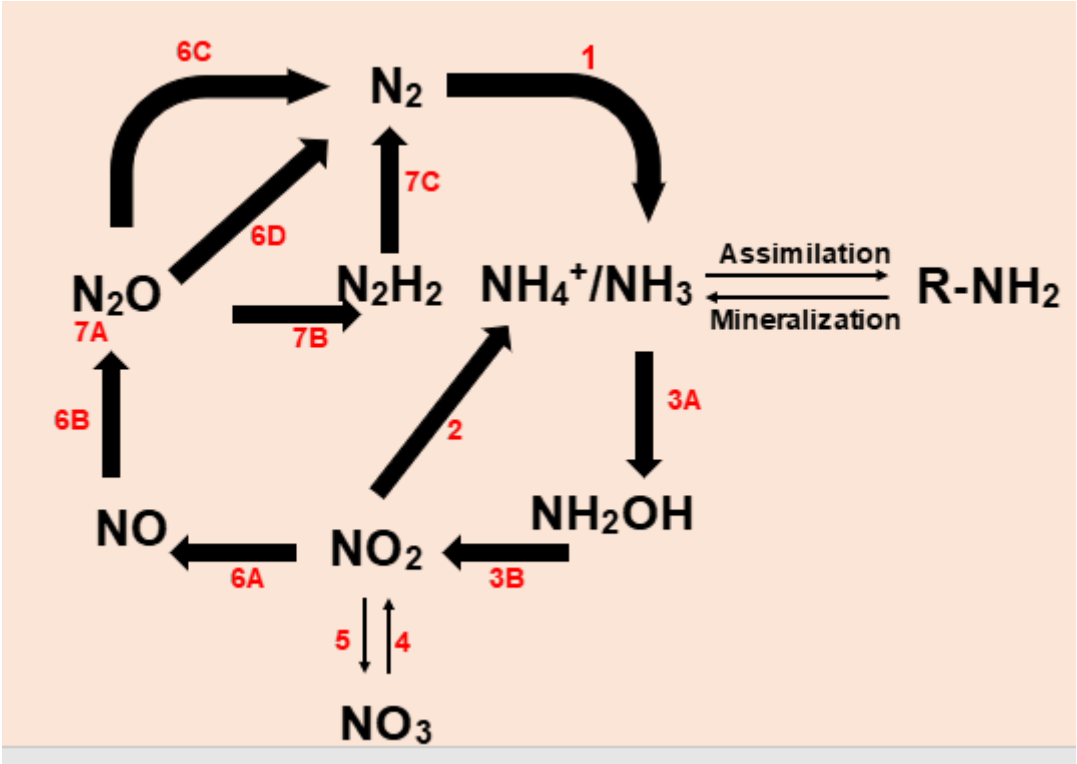

Figure 1.3: Chemical conversion in the nitrogen cycle

An ammonium cation (NH_4) is a positively charged polyatomic ion formed by the reaction of ammonia with hydrogen. The degree to which ammonia forms the ammonium ion depends on the pH of the soil solution (Hachiya and Sakakibara, 2016). Although ammonia is a source of nitrogen for plant species, especially those growing on hypoxic soils, it is toxic to most crop species. Prolonged application of ammonium as the sole nitrogen source may result in physiological and morphological disorders that lead to decreased plant growth and toxicity (Esteban et al., 2016). Nitrate anion (NO_3^-) is a negatively charged ion carrying a formal charge of -1 and it is the most used form of nitrogen in plants (Pinton et al., 2016).

Nitrates are preferred over ammonium because they are non-volatile, more stable, more mobile, directly taken up by plants with higher efficiency, less acidic, and synergistically promote the uptake of cations (K, Ca and Mg), while ammonium competes with the uptake of these cations (Lehtovirta-Morley, 2018). Nitrate salts occur naturally on Earth as large nitratite deposits; they are a naturally occurring form of sodium nitrate ($NaNO_3$) that appears

as a coating of white, grey, to yellowish brown mases. Due to scarcity, other types of synthetic fertilizer were sought. The use of synthetic fertilizer has increased the amount of anthropogenic nitrogen in the environment and this has resulted to pollution (Hundey et al., 2016). Biologically, nitrates are produced by nitrifying bacteria and archaea that obtain their substrate (ammonium and ammonia) from the process preceding nitrification in the nitrogen cycle.

Nitrifying bacteria and archaea are plant growth promoting rhizobacteria (PGPR) that engineer the nitrogen cycle's nitrification process. They influence plant growth by producing nitrate. Symptoms of nitrogen deficiency in plants include yellowing of leaves, which is as a result of a decrease in chlorophyll content (a condition known as chlorosis), and may lead to reduction in flowering, fruiting, proteins, and starch. Also, plant growth is stunted with dormant lateral buds and they become susceptible to disease, therefore without sufficient nitrogen, plants are as good as dead. PGPR has been shown to be an environmentally friendly method of increasing crop yields by promoting plant growth (Pagnani et al., 2018). This demonstrates a critical role in the sustainable agricultural industry.

Climate change will have major effects on food security, hence scientists call for research that directly informs the measures needed to solve food security concerns (Campbell et al., 2016). Globally, policymakers and scientists are advocating for various approaches to conventional agricultural intensification that improve the ecosystem services provided by biodiversity (Garibaldi et al., 2017). Innovation to improve maize production could lead to poverty alleviation (Ayinde et al., 2019). Feeding a growing world population amidst climate change requires optimizing the reliability, resource use, and environmental impacts of food production. One way to assist in achieving these goals is to integrate beneficial plant microbiomes that enhance plant growth, nutrient use efficiency, abiotic stress tolerance, and disease resistance into agricultural production (Busby et al., 2017).

Due to the exceptional phenotypic and molecular diversity possessed by maize plant, it is one of the most significant and economically important crops in developing countries, such as South Africa and the world at large (Dowswell et al., 2019). Substantially, they are influenced by environmental conditions and genetic variation. Since reducing fertilizer use in agriculture is a global necessity, particularly in maize production due to its widespread cultivation, there is a great interest in understanding their bacterial diversity in order to better explore their potential (Arruda et al., 2014). In addition, given its widespread planting in monoculture, maize may be viewed as an ecosystem engineer strongly responsible for shaping the agricultural environment because it cohabits with species, especially microbes, in its rhizosphere (Peiffer et al., 2013). Plant genotype differences affect microbes that colonize plant roots, but their agronomic significance is unknown (Walters et al., 2018). Thus, the need to understand the microbial processes taking place in the rhizosphere and their importance in plant growth.

Metagenomics is a modern molecular tool for analyzing DNA obtained from environmental samples in order to study the community of microorganisms present without the need for pure cultures (Ghosh et al., 2019). Metagenomics analysis of bacteria and archaea is applicable for identification, taxonomic classification, comparing sequencing that lead to recognition of novel organisms, providing species-specific signature sequences, rapid and cheap alternative to phenotypic methods of identification, reclassification of bacteria into new species or even genera, and identifying species that has never been cultured (Culligan and Sleator, 2016).

Understanding and managing plants and microbes interactions for the benefit of modern agricultural systems requires collaboration between researchers and agriculturists. Some of the identified priorities for research include; developing model host microbiome systems for plants with associated microbial culture, collections, and reference genomes, defining core

microbiomes and metagenomes in these model systems, elucidating synthetic and functionally programmable microbiome assembly rules, determining functional mechanisms of plant-microbiome interactions (Busby et al., 2017). Achieving these objectives would speed up the ability to design and implement effective agricultural microbiome manipulations and management strategies, which would benefit both consumers and producers of the world's food supply.

1.2 Problem Statement

There is need to secure food now more than ever considering the continual exponential increase in the world population (Ramankutty et al., 2018). In order to improve farm yields, farmers have resulted in several forms of practices, which have led to land degradation. Land degradation has affected the bioavailability of nitrate, an important macronutrient for plants, which is produced in the soil by the action of nitrifying bacteria and archaea. Synthetic fertilizer used to substitute nitrate deficiency is constantly lost from the soil, which is detrimental to health and environment (Wang and Li, 2019).

Historically, *Nitrosomonas europea* has generally been believed to be the bacterium responsible for nitrification because it was the major bacteria isolated from nitrifying systems by traditional culturing techniques. Presently, researchers have been able to identify nitrifying bacteria and archaea from soil collected from fields and farmlands. However, from literature studied, there are still many more yet to be discovered and their contributions to the process of nitrification understood since there is no full comprehension of how these groups of organisms carry out their activities (Beirn et al., 2017, Peiffer et al., 2013, He et al., 2012). Many undiscovered nitrogen-transforming reactions that are thermodynamically feasible remain undiscovered, as do the microorganisms catalyzing these reactions and the involved pathways (Kuypers et al., 2018). The 16S amplicon sequencing technique, which

has been successfully used to discover novel microorganisms can be used to identify and study nitrifying bacteria and archaea inhabiting it (Fujitani et al., 2015).

In respect to the aforementioned, the problem this research considers is that, till date, only a few nitrifying bacteria and archaea have been identified as being associated with the maize rhizosphere. It is also unknown how they interact with the soil environment and other microbes. Hence, the research would lead to an increased database of beneficial plant growth promoting nitrifying bacteria and archaea that can be useful as biofertilizer. Also, it would initiate a better understanding of how to manage the nitrification process of the soil.

1.3 Research Questions

I. What is the diversity of nitrifying bacteria and archaea in the maize rhizosphere and how do they relate to the soil chemical properties?
II. What is the relationship between nitrifying bacteria, archaea, and other microbial groups in the maize rhizosphere?
III. What is the influence of nitrifying microorganisms on plant growth promoting bacteria and the community level physiological profile of the maize rhizosphere?

1.4 Research Aims and Objectives

The aim of this study is to evaluate the diversity of nitrifying bacteria and archaea and determine the relationship between them and the soil properties, other microbial functional groups, plant growth promoting microorganisms, and community level physiological profile of the rhizosphere.

The objectives are to:

I. identify the diversity of nitrifying bacteria and archaea in the maize rhizosphere and understand how they relate to the rhizosphere’s physical and chemical properties,

II. determine the relationship between nitrifying bacteria, archaea, and other microbial groups in the maize rhizosphere,
III. evaluate the influence of nitrifying microorganisms on plant growth promoting bacteria and the community level physiological profile of the maize rhizosphere.

1.5 Relevance of Research

Agriculture contributes significantly to household food security and plays an important role in the process of economic development. This study would:

I. increase the database of nitrifying bacteria and archaea that can be useful as biofertilizers,
II. provide a better understanding of the relationship between nitrifying bacteria, archaea, and other microbial groups, and
III. understand the influence of nitrifying bacteria and archaea on plant growth promoting microorganism and rhizosphere's community level physiological profile.

CHAPTER TWO

FACTORS INFLUENCING SOIL NITRIFICATION PROCESS AND THE EFFECT ON ENVIRONMENT AND HEALTH

Abstract

To meet the global demand for food, several factors have been deployed by agriculturists to supply plants with nitrogen. These factors have been observed to influence the soil nitrification process. Understanding the aftermath effect on the environment and health would provoke efficient management. We review literature on these factors, their aftermath effect on the environment and suggest strategies for better management. Synthetic fertilizers and chemical nitrification inhibitors are the most emphasized factors that influence the nitrification process. The process ceases when pH is less than 5.0. The range of temperature suitable for the proliferation of ammonia oxidizing archaea is within 30^{o}C to 37^{o}C while that of ammonia oxidizing bacteria is within 16^{o}C to 23^{o}C. Some of the influencing factors excessively speed up the rate of the nitrification process. This leads to excess production of nitrate, accumulation of nitrite as a result of decoupling between nitritation process and nitratation process. The inhibition mechanism of chemical nitrification inhibitors either causes a reduction in the nitrifying micro-organisms or impedes the *amoA* genes function. The effects on the environment are soil acidification, global warming, and eutrophication. Some of the health effects attributed to the influence are methemoglobinemia, neurotoxicity, phytotoxicity and cancer. Biomagnification of the chemicals along the food chain is also a major concern. The use of well researched and scientifically formulated organic fertilizers consisting of microbial inoculum, well-treated organic manure and good soil conditioner are eco-friendly. They are encouraged to be used

to efficiently manage the process. Urban agriculture could promote food production, but environmental sustainability should be ensured.

Keywords: Agricultural intensification, agroecosystems, environmental challenge, nitrification inhibitor, nitrifying microorganism, synthetic fertilizer

2.1 Introduction

Nitrification process (NP) is an oxidation reaction that usually occurs under aerobic conditions. The process serves as an intermediate of oxidized and reduced forms of nitrogen in its cycling. The nitrate produced serves as a substrate for denitrification and a nutrient for plant growth. This has made it important to environmental sustainability and agricultural intensification. Compounds such as ammonium (NH_4), ammonia (NH_3), hydroxylamine (NH_2OH), nitrous oxide (NO), nitrite (NO_2^-), and nitrate (NO_3^-) are the major forms of nitrogen associated with the process. The soil nitrification process is divided into two major phases which are nitritation and nitratation, and the order of microbial oxidation of ammonia via nitrite to nitrate is sequential (Amoo and Babalola, 2017). Nitritation accomplishes the oxidation of ammonia to nitrite, while nitratation phase oxidizes nitrite to nitrate. This process is mainly engineered by some group of nitrifying bacteria and archaea in a complex chemical transformation, and they are affected by several factors. The factors include synthetic fertilizers, chemical nitrification inhibitors and other agrochemicals. The effects are evaluated with total soil nitrogen, mean annual temperature, pH and microbial biomass nitrogen (Li et al., 2020).

The universal cycling of nitrogen is being massively distressed due to the activities of man on the lithosphere. Manipulation of the soil nitrification process for agricultural benefit has been one of such activity. This has led to and would continue to lead to negative effects which many researchers do not foresee. Reviewing this will enlighten scientists on the

importance of the soil nitrification process, its influencing factors and the effect on environment and biotic health (Figure 2.1). This would provoke better management and cause amendments to be made. The influence is measured by the rate at which associating chemicals are produced or by the dynamics of the soil organism, especially those directly associated with the process 'the nitrifying bacteria and archaea'.

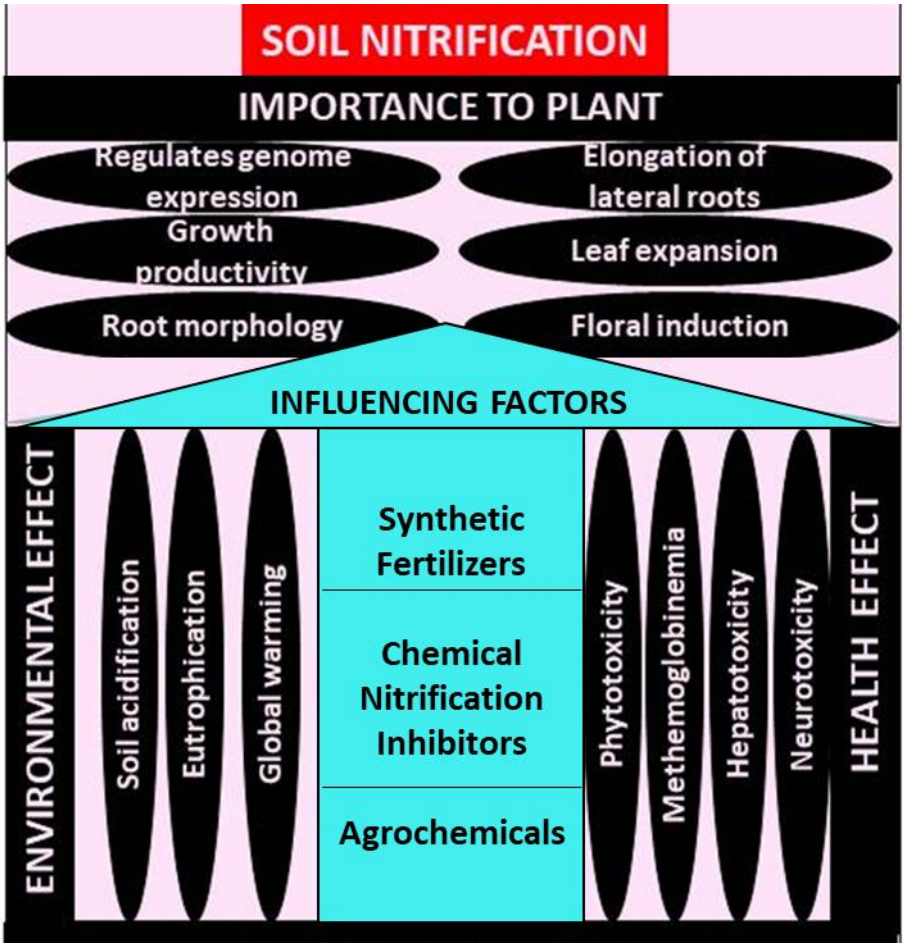

Figure 2.1: Effect of influencing soil nitrification process negatively

2.2 Importance of Nitrification

The modern nitrification process has led to a 50% loss of nitrogen and has reduced the availability of nitrogen for the use of plants (Beeckman et al., 2018). Despite the present situation, the importance of the nitrification process cannot be overemphasized. Its most important goal is to provide nitrate for plant use. Although there are other available nitrogen

forms in the soil, nitrate seems preferable to most plants and other soil organisms and leads to better functioning of the ecosystem if produced in the right proportion.

Crop nitrogen demand is unpredictable. The time of greatest demand is normally during the stem elongation phase, except for crops targeted for high protein grain whose highest demand is during the flowering phase (Angus, 2001). However, the presence of external NO_3^- induces the expression of the NO_3^- transporter gene, causing elongation of lateral roots (Mantelin and Touraine, 2004). Also, high-affinity transport system (HATS) becomes active if concentration of NO_3 in soil is low (< 250 micrometers) and low-affinity transport system (LATS) becomes activated if the concentration of NO_3 is high (>250 micrometers) (Plett et al., 2018). Subsequently, an excess supply of nitrate reduces the demand for nitrate (Mantelin and Touraine, 2004); therefore, it is needed in a gradual release and at the right time.

In addition to being a nutrient, nitrate is a local and systemic signal that regulates genome-wide gene expression, root morphology, leaf expansion, seed dormancy and floral induction (Hachiya and Sakakibara, 2016). It helps in the production of embryos during the early stages of reproduction and carries out anthesis (Yoneyama et al., 2016). Several responses to nitrate are mediated via calcium and phytohormone signaling pathways including auxin, cytokines and abscises acid (Hachiya and Sakakibara, 2016). A decrease in nitrate assimilation causes a decline in protein concentration in cereals. This leads to retardant growth, and the subsequent effect on animal and human nutrition can be detrimental (Dier et al., 2018).

An additional benefit of nitrification is the oxidation of ammonia. Ammonia has a negative effect on plant, biotic and abiotic components of the environment. Excess ammonia affects the uptake of nutrients, disturbs hormonal balance, decreases soluble carbohydrates of

plants, and distorts photosynthesis and metabolic pathways (Wang et al., 2016b). Directly or indirectly, ammonia plays a crucial role in environmental damage (Lehtovirta-Morley, 2018). This could be the result of its higher acid level when compared to the oxidized nitrogen forms. Ammonia in agricultural runoff negatively affects water bodies as it reduces dissolved oxygen resulting in aquatic biota toxicity (Wang et al., 2016b). Plant tolerance of ammonia varies within plant species (Byrnes et al., 2017), and few plants can conveniently use ammonia.

The availability of nitrates is one of the main factors that determine the productivity and growth of plants. Unfortunately, they are scarce in natural soil due to soil physical and chemical properties, microorganism activities and drainage (Kiba and Krapp, 2016). Of all the nitrogen forms, nitrate is the most susceptible to leaching, thus making it often unavailable for plant use at the moment needed. The anthropogenic input of nitrogen has done more harm than good to the agricultural system. Although done purposely to improve crop yield, the excessive and repeated input of anthropogenic nitrogen has increased nitrate leaching (Nevison et al., 2016) and reactive nitrous oxide gas production. This is alarming as agriculturists believing they have made available sufficient nitrates for plant growth have indirectly affected productivity.

2.3 Mechanism of Nitrification Process

The mechanism of nitrification is a complex one, being a mixture of biological and chemical processes (Figure 2.2). The biochemical reaction takes place on the membrane site of the associating microorganisms. Primarily, ammonia (NH_3) is used as the major substrate. It is transformed by ammonia monooxygenase enzyme (*amoA*) into hydroxylamine (NH_2OH), while hydroxylamine with the aid of the enzyme hydroxylamine oxidoreductase (HAO) reacts with water to produce nitrite (NO_2) (Amoo and Babalola, 2017). Nitrite oxidoreductase (NXR)

found in nitrite-oxidizing bacteria transforms nitrite into nitrate (NO_3) (Fu et al., 2020b). The reaction requires the use of oxygen and hydrogen; electrons are usually released from the membrane. In an unperturbed environment, the nitrification process is usually stable, however, when disturbed by anthropogenic activities it varies. The variation is dependent on factors that affect the availability of ammonia as well as the abundance and function of nitrifying bacteria. At suitable conditions such as such a sufficient amount of substrate and pH that is balanced, the rate of nitrification as reported by Tarre and Green (2004) is 0.55g of N.g of biomass $^{-1}$, day $^{-1}$.

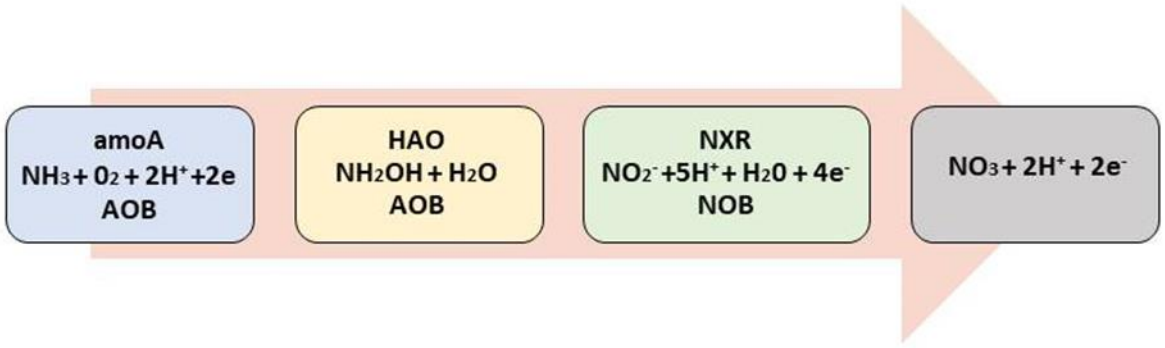

Figure 2.2: Schematic diagram showing biochemical reaction in nitrifying bacteria

2.4 Factors Influencing the Nitrification Process

Categorically, the factors that affect the nitrification process can be chemical or physical. These factors were adopted to intensify crop production and meet global food demand. The chemical factors include synthetic fertilizer, chemical nitrification inhibitors and pesticides. Some of the notable physical factors are temperature, pH, and oxygen (Schaefer and Hollibaugh, 2017). Li et al. (2020), evaluated the global soil nitrification rate across terrestrial ecosystems. It was observed that the total soil nitrogen contributed mostly to the nitrification with a coefficient of 0.29, next was the mean annual temperature (0.25), followed by the pH (0.24), and microbial biomass nitrogen (0.19).

2.4.1 Synthetic Fertilizer

Synthetic fertilizers come in various types, brands, and formulae (Table 2.1), and they could be in solid, liquid, or gaseous state. The different kinds of fertilizer majorly are made of phosphorus, potassium, nitrogen, and a combination of either two or the three elements (Cai et al., 2019). Koli et al. (2019) classified them as straight (supply only one nutrient), complex (containing two or three nutrients), and mixed fertilizer (has more than three nutrients). Majority are nitrogen-based as a result of high requirement of the element by the plants. Farmers rely on fertilizers made of nitrogen to have an exponential increase in crops produced. However, the efficiency of its use is low (30–50%) when comparing it to the amount of crop produced (Liang et al., 2019).

Table 2.1: Nitrogen content of nitrogen-based synthetic fertilizer.

Nitrogenous synthetic fertilizer	Chemical state	Chemical formula	Approximate % of nitrogen
Anhydrous ammonia	Gas	NH_3	82%
Urea	Solid	$CO(NH_2)_2$	46%
Urea ammonium nitrate	Liquid	$[CO(NH_2)_2]$ $[NH_4NO_3]$	32%
Ammonium nitrate	Solid	NH_4NO_3	34%
Ammonium phosphate	Solid	$(NH_4)_3PO_4$	11%

Sodium nitrate	Solid	$NaNO_3$	16%
Ammonium sulfate	Solid	$(NH_4)_2SO_4$	21%
Calcium nitrate	Solid	$Ca(NO_3)_2$	17%
Diammonium phosphate	Solid	$(NH_4)_2HPO_4$	18%
Monoammonium phosphate	Solid	$NH_4H_2PO_4$	12%
Potassium nitrate	Solid	KNO_3	13%
Calcium ammonium nitrate	Solid	$5Ca\ (NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$	27%
Ammonium thiosulfate	Solid	$(NH_4)_2S_2O_3$	12%
Magnesium nitrate	Solid	$Mg(NO_3)_2$	18%

In time past, the rotation of crops was carried out in farming to exploit endophyte nitrogen-fixing rhizobia inhabiting legumes and microorganisms in organic waste to produce beneficial nutrients, ammonia, and nitrate for plant use. The practice was safe but could not continually be relied on because it does not provide enough for the plant usage. This resulted in the use of synthetic fertilizer which provides an immediate replacement to naturally

produced nutrients. Unfortunately, it negatively affects the rate of nitrification in the long run (Verma et al., 2018). Those with ammonia speed up the rate of nitrification excessively since they provide an immediate substrate for ammonia oxidizers to act on. Also, synthetic fertilizers with phosphate elevate the process of nitrification twelve times by raising soil pH to favor the process (DeForest and Otuya, 2020). This often leads to an oversaturation of nutrients beyond what the biota in the environment can assimilate. Generally, where there is an increase in soil nitrifying microorganisms as a result of synthetic fertilizer application, it is only temporal (Quemada et al., 2019).

2.4.2 Chemical Nitrification Inhibitors

Nitrogen is lost from the soil through leaching, volatilization of NH_3 and other nitrogenous gases associated with the microbial reaction in the denitrification and nitrification processes (Coskun et al., 2017). Due to the detrimental effects of the gases on health and the environment, inhibitors have been recently used to restrict the rate of nitrification. This causes the transformation of ammonium (NH_4^+) to nitrate (NO_3^-) to be delayed in the soil. The actions of the inhibitors are noticed by restraining the action of the genes associated with process (Liu et al., 2020). Also, growth of the acting bacteria and archaea be inhibited (Elrys et al., 2020). However, their use and mechanism of inhibition are yet to be fully understood.

Nitrapyrin (NP), Dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) are well known synthetic nitrification inhibitors (Lu et al., 2019). They are usually used along with synthetic nitrogen fertilizers or organic waste. Infusing organic waste with DMPP can prolong the nitrification time (Kong et al., 2018). This is achieved by chelating chemicals like Cu which inhibit the first enzymatic step of nitrification (Wu et al., 2018a). Moreover, the mechanisms of inhibition vary within the different nitrification inhibition (Rodrigues et al.,

2018). Application of DCD with urea decreased the rate of NH_4^+ loss (1.8 mg N kg^{-1} soil day^{-1}) which could have been a result of an inhibitory effect on ammonia-oxidizing microbial communities (Duncan et al., 2016).

DMPP is considered less toxic than DCD because its recommended application rate is one-tenth of DCD (Rodrigues et al., 2018). However, according to Yang et al. (2016), an increase in yield by the application of DMPP was noticed only in alkaline soil. The shortcomings of chemical nitrification inhibition as reported by Lu et al. (2019) include; difficulties in application, high cost, environmental pollution and food safety risks (Table 2.2). Other than these few mentioned shortcomings, there are likely to be more. Knowing the specific species that are targeted by this organism would be of great advantage to agricultural and environmental management.

Table 2.2: Environmental risk of chemical nitrification inhibitors

Chemical Nitrification inhibitor	**Environmental risk**	**Reference**
3,4-dimethylpyrazole phosphate (DMPP)	Absorption and accumulation of chemicals in plant tissue	Rodrigues et al. (2019)
Dicyandiamide (DCD)	Increases ammonia released from the soil	Elrys et al. (2020)
Nytrapyrin (NP)	Transported off agricultural fields with possible effect on non-target organism.	Woodward et al. (2016)

3,4-dimethylpyrazole-succinic acid (DMPSA)	It affects nontarget organisms that are of agricultural benefit	Corrochano-Monsalve et al. (2020)
N-(*n*-butyl) thiophosphoric triamide (NBPT)	More leaching and denitrification loss	Meng et al. (2020)
Benzotriazole (BTA)	Contamination of Groundwater	Trcek et al. (2018)
Potassium thiosulfate	Accumulation of nitrite	Cai et al. (2018)

2.4.3 Other Agrochemicals and Substance

Aside from the use of fertilizer and nitrification inhibitors, there are some other agrochemicals and substances used in farms that influence the nitrification process. They are frequently used to promote plant productivity. Pesticides are one of them and they are of various categories, such as fungicides, insecticides, and rodenticides. Iprodione a fungicide has an antagonistic effect on *amoA* genes, it decreases their abundance and reduces the rate of nitrification (Zhang et al., 2018a). Another is herbicides which can be synthetic or organic. Atrazine and glyphosphate are synthetic herbicides observed to grossly reduce the rate of nitrification in the soil by inhibiting the microbial functional genes responsible for the process (Zhang et al., 2018b).

Clinoptilolites are synthetic substances with high cation exchange properties with the potential to retain ammonium ions (Jakkula and Wani, 2018). Hydrogel, polyvinyl alcohol, and anionic polyacrylamide are soil conditioners reported by Seddik et al. (2019) noticed to increase the total nitrogen content of the soil. Although, according to Youssef et al. (2019), polyvinyl alcohol had no significant effect on the nitrification process. Also, quartz sand used to control soil nutrient leaching in agricultural soil affects nitrogen transformation dynamics. It was observed to grossly inhibit the autotrophic nitrification process and stimulate the immobilization of NO_3^- and thus should be used cautiously (Wang et al., 2017b). This must have been a result of altering the agricultural soil's physical and chemical properties.

Flue gas desulphurization gypsum (FGDG) has also been used as a soil amendment and noticed to influence the nitrification process by inhibiting and delaying the occurrence of *amoA* genes (Li et al., 2016). Industrial waste from dairy factories escalates the availability of ammonium, this rapidly increases nitrification process. Other forms of human activities that have brought excess influx of nitrogen include, combustion of fossil fuel, biomass burning, and biological activities in the natural soil. The terrestrial anthropogenic activities have been increasing tremendously over the past years and would continue to increase. Researchers need to find a way to create a pseudo-balanced ecosystem continuously.

2.4.4 Climate Change

Agriculture practices such as bush burning, tree cutting have affected climate change. One of the observed effects of climate change is an unusual increase in atmospheric temperature. Increased temperature increases the volatilization and emission of nutrients. The nitrification process driven by AOA and AOB is strongly affected by elevation and fundamental differences in temperature. Taylor et al. (2017) evaluated AOA and AOB across a gradient of (4^oC - 42^oC), it was observed that the maximum nitrification potential rates of

AOA are within the range 30^{o}C to 37^{o}C while that of AOB is within the range 16^{o}C to 23^{o}C. Hu et al. (2016) reported an increase in AOA and a gradual decrease in AOB under elevated temperatures. Akram et al. (2018) observed a correlation between change in climate, nitrogen fertilizer application and emission of N_2O. According to Sahrawat (2008), plotting the response of temperature to climate change gives a bell-shape with an optimum temperature of 30-35^{o}C.

2.4.5 Physical Factors

Anthropogenic activities often affect physical factors of soil environment. These in turn affect the soil nitrification process. Notable physical factors that affect the process are temperature, pH, moisture, oxygen, and aeration. The two most important physical factors are temperature and pH. The response of the process to temperature is similar to that observed in climate change. Le et al. (2019) reported that ammonia oxidation is inhibited at pH 5 while nitrite oxidation is inhibited at pH 8.5, optimum activity of AOB and NOB are 7.5 and 7.0. The optimum pH varies but there is an agreement of the process ceasing at 5.0 since oxidation of ammonia is the first. Also, Soil moisture closes up pore spaces, this affects aeration and reduces the oxygen level. Nitrification is a biochemical oxidation process, low oxygen levels in the soil would negatively affect the process.

AOB diversity differs among soil types; the presence of clay in soil affects the nitrification process. Waterlogging which could arise as a result of frequent irrigation reduces the soil oxygen level decreasing the nitrification potential rate and the abundance of ammonia oxidizing microorganisms (Nguyen et al., 2018). Tillage is an age-long agricultural practice done to increase productivity by removing weeds and increasing soil aeration. However, it has a subsequent disadvantage of reducing soil biomass, which negatively affects soil structure and quality (Vazquez et al., 2019). The mechanism of the influencing physical

factors is not fully understood as a result of the complex interaction among the various factors.

2.5 Effect of the Influencing Factors on the Environment

In the past, scientists managing the nitrification process have focused on agricultural intensification, paying little or no attention to environmental degradation. The addition of fertilizer initially brought an enormous boom in agricultural productivity with little or no side effects. However, it is presently clear that the use of nitrogen fertilizer is causing serious environmental issues. Excessive levels of NO_3^- in the soil can be imputed to the increasing use of fertilizer made of synthetic nitrogen in agroecosystems (Zhai et al., 2017). Significant changes were observed in soil bacteria community structure, and soil organic matter mineralization tends to be negatively affected by the use of DMPP (Zhang et al., 2017).

The efficiency of nitrogen use in crops is low. Fifty percent of the synthetic nitrogen applied to agricultural systems is not mopped up, instead, it is distributed to the surroundings as oxides of nitrogen (NOx) and ammonia (Coskun et al., 2017). The excess nitrogenous compounds are lost to surface water, groundwater, and the atmosphere as a result of over saturation in the soil, propelling detrimental effects to the environment. The increased ammonia leads to soil acidification and eutrophication of surface water bodies (Ni et al., 2018).

2.5.1 Soil Acidification

Fertilizers with ammonia, especially urea, reduce the pH of the soil; this increases its acidity (Goulding, 2016). An acidic soil affects the normal functioning of the ecosystem, especially the biotic component. Also, high acid levels of soil negatively affect the biodiversity dwelling in it, and this is detrimental to soil quality (Li et al., 2017). Farmers resolve the challenge by

manipulating the soil with various chemicals and substances, thus the land eventually becomes degraded and undesirable for planting in the long run.

Nitrification inhibitors can also decrease the rate of nitrification by disrupting the activities of the bacteria leading to low soil pH (Alonso-Ayuso et al., 2016). Soil with low pH affects the uptake of nutrients such as calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P) and molybdenum in plants (Shi et al., 2019). Inhibiting nitrification is believed to reduce agricultural production costs, pollution, and climate change (Coskun et al., 2017). However, the detrimental effects of nitrification inhibition in increasing the volatilization of NH_3 outweigh its benefits.

2.5.2 Eutrophication

Eutrophication is a global challenge that impairs the quality of marine and inland waters (Le Moal et al., 2019). Ammonia, nitrite, and nitrate are widely spread in natural waters, and they increase the occurrence of eutrophication (Wu et al., 2018b). The leached nitrogenous substances result in eutrophication and they affect surface and groundwater, causing algal blooms and loss of biodiversity (Beeckman et al., 2018). The occurrence of eutrophication often results in the production of cyanobacteria in rivers and waterways (Le Moal et al., 2019). This has led to the threatening of aquatic resources (Paerl, 2018).

The management and mitigation of the global expansion of toxic cyanobacterial harmful algal blooms (CyanoHABs) is a major challenge facing researchers and water resource managers (Paerl et al., 2019). In June 2016, St Lucie River in Florida had high concentrations of cyanotoxins that greatly exceeded WHO guidelines for consumable and recreational water (Metcalf et al., 2018). The degradation of the environment and abuse of agrochemicals has prompted researchers into searching for environmentally friendly ways

of improving crop yields (Enagbonma and Babalola, 2019). Replacing synthetic fertilizers with a more environmentally friendly biofertilizer could limit the occurrence of algal blooms.

2.5.3 Global Warming

According to NOAA (2021), in 2020 the average temperature globally was 0.98^{o}C warmer than in previous years. Modernized agriculture areas would contribute to global warming as they depend on fertilizer and other agrochemicals to maximize plant growth. The inputting of synthetic and organic nitrogenous materials in the soil by agroecosystems has contributed largely to anthropogenic N_2O emissions (Charles et al., 2017). In a study carried out by Xiaomin Feng et al. (2019) in Northeastern China, chemical fertilizer was observed to increase nitrous oxide emission by increasing nitrifying and denitrifying microorganisms. Decoupling is usually observed in the two stages of nitrification (Heiss and Fulweiler, 2016). Accelerating soil nitrification with rapid microbial activity could cause decoupling (Figure 2.3) as a result of nitrite accumulation and a reduction in nitrogen use efficiency (NUE) of plants. This often leads to the escape of excess nitrite and other reactive nitrogen into the environment (nitrogen cascade). Nitrogen dioxides are greenhouse gases with 300 times greater global warming effect than carbon dioxide (Beeckman et al., 2018). NO is chemically reactive, the gas is involved in photochemical processes in the troposphere and acts as the major pioneer of ozone (O_3) formation at ground level (Recio et al., 2019).

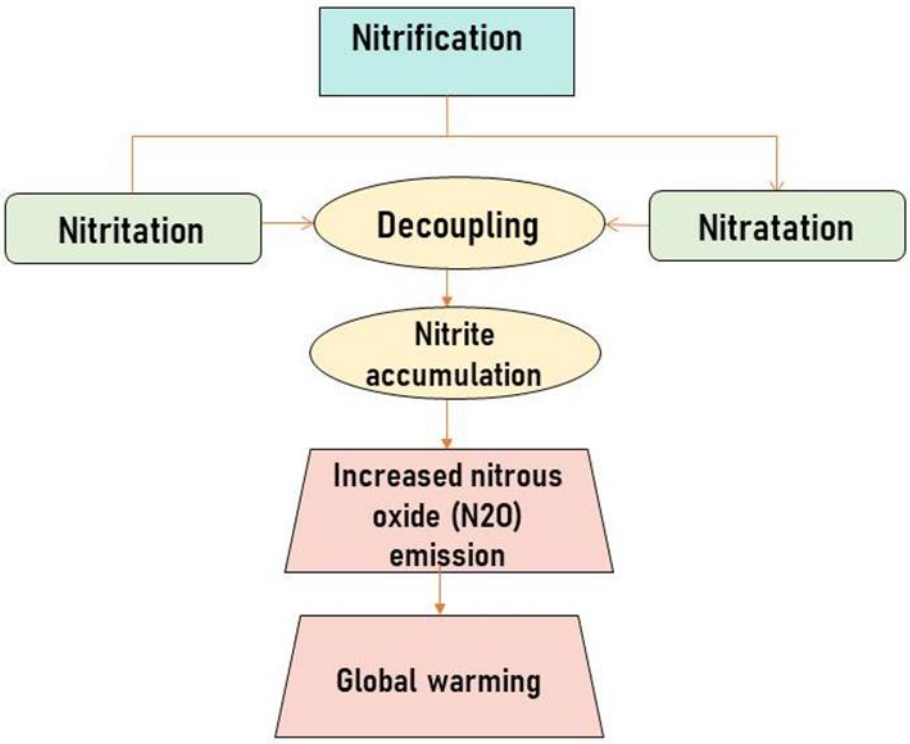

Figure 2.3: Decoupling in soil nitrification process resulting to global warming

Nitrification and denitrification are closely related, and the types of gas used up and produced by the different processes pose a challenge to scientific researchers. Denitrification produces higher amounts of N_2O when compared to nitrification, as nitrification simply produces the substrate on which denitrifying bacteria act (Siljanen et al., 2019). If this is so, then it would be more appropriate to inhibit denitrification process and not nitrification process for reducing global warming which is the goal of chemical nitrification inhibitors.

2.6 Effect of the Influencing Factors on Health

The influencing factors on the nitrification process have directly and indirectly affected biotic health. Biomass, crops and animals' health are affected at low pH (Zou et al., 2018). Acidic soil increases the bioavailability of heavy metals making the soil toxic for organisms (Ayangbenro et al., 2018). Low pH accumulates and increases the toxicity of aluminium (Al)

and manganese (Shi *et al.*, 2019). The metals accumulate in plants and biomagnify along the food chain, disrupting the physiology of animals. Furthermore, bacterial wilt disease develops more quickly and severely in acidic conditions, causing mechanical blockage of the water transport system in the plant (Li et al., 2017). Also, retarding the nitrification process by using nitrification inhibitors might effectively decrease the emission of N_2O. However, more NH_3 would be retained in the soil and its volatilization to the atmosphere would be increased (Fan et al., 2018, Ni et al., 2018). The emission of ammonia negatively affects the health of humans and vegetation (Ni et al., 2018).

2.6.1 Plants

The continual application of nitrification inhibitors in a farm can negatively affect the growth and development of plants. According to Rodrigues et al. (2018), plants can take up N-(n-butyl) thiophosphoric triamide (NBPT) urease inhibitor, which can affect their metabolism by influencing their endogenous urease. NBPT reduces the possibility of urea reaching the nickel atom. This causes transient yellowing of leaf tips as a result of urea toxicity soon after application (Cantarella et al., 2018). Nitrapyrin used with liquid fertilizers shows symptoms of phytotoxicity (Rodrigues et al., 2018). Bioaccumulation of DMPP in plant leaves showed signs of phytotoxicity and affects plant metabolism and hormone signaling (Rodrigues et al., 2019). Soil factors, management factors and crop types often determine the efficiency of nitrification inhibitors (Yang et al., 2016). Also, the hindrance in NO formation as a result of inhibiting the nitrification process could negatively affect the resistance of plants to disease (Yun et al., 2016). Plants produced are weak, disease-prone with less fruiting, accumulate salt and burn plant roots at high concentrations. Although NO can have a positive effect on plants; however, at high concentrations it poses potential damage to cellular structures under conditions of redox imbalance (Farnese et al., 2016). An excessive increase in the rate of nitrification which would produce high concentration of NO should also be checked.

2.6.2 Soil Organisms

High concentration of nitrite is caused by varying factors which include heavy use of synthetic fertilizer and treatment of soil with biocidal chemicals (Siontorou and Georgopoulos, 2016). It can also accumulate in soil when oxidation of ammonia proceeds faster than the consumption of nitrate and when nitrate consumption is slower than its reduction (Heil et al., 2016). Nitrite at high concentrations is toxic to soil organisms. Nitrification inhibitors might have an undesirable effect on non-target soil organisms (Rodrigues et al., 2018).

2.6.3 Animals and Humans

Contamination of water bodies has been on the increase in emerging urban cities of developing countries (Fashae and Obateru, 2021). Fashae and Obateru (2021) observed a river located at Ibadan, Nigeria was polluted. This was partly attributed to agricultural activities. Also, groundwater with nitrate, the by-product of the soil nitrification process, is a global challenge, particularly in agrarian countries. The influence on the nitrification process has made it readily available in the environment. Nitrate dissolves easily in water, diffusing quickly towards the groundwater especially in sandy soil. Consumption of groundwater contaminated with nitrate can cause adverse health challenges. The health hazard of nitrate contamination varies for individuals in a population, and often it is in decreasing order from infants, children, adult females, and adult males (Zhai et al., 2017). Infants and children are seen to be most susceptible to the contaminant.

Methemoglobinemia is a common physiological disorder in infants as a result of ingesting high levels of nitrate either through formula or water. The nitrate binds with methemoglobin and this affects the ability of the blood to react with oxygen, it often leads to death (Ward et al., 2018). Besides methemoglobinemia, other health effects associated with nitrate

consumption include cancer of the colon, disease of the thyroid, neural tube defects, and adverse pregnancy outcomes (Ward et al., 2018). Nitrate can transform into N-nitroso compounds which have the potential to cause cancer, especially cancer of the colon (Schullehner et al., 2018).

Ammonia volatilization would increase with increasing urea-based fertilizer application. Ammonia has been associated with irritation of the eyes and respiratory system, and it also intensifies the production of particulate matter which damages the respiratory tissue (Naseem and King, 2018). Excessive amount of both ammonia and nitrate in the soil increases the occurrence of eutrophication. The toxins produced by cyanobacteria associated with eutrophication are known to be hepatotoxic, neurotoxic, irritating to the gastro intestine and cause contact dermatitis (Metcalf et al., 2018).

Nitrification inhibitors have been detected in open water environments and their effects on aquatic ecosystems and human health are still unclear (Qin and Lin, 2019). DCD has been discovered in milk products obtained from animals fed on plants cultivated with DCD (Rodrigues et al., 2018), and consumption of contaminated products is a potential health risk in humans (Ning et al., 2018). The health of people living in the region where nitrification inhibitors are continuously applied can be negatively affected (Yang et al., 2016).

2.7 Managing Nitrification Process

Recent agroecosystem practice depends heavily on chemicals, machinery, and other forms of management that dilapidate soil structuring and quality (Rillig and Lehmann, 2019). Additional expenditure on fertilizer is still increasing and encouraged in many areas even when the nitrogen fertilizer efficiency is not profitable. Management of nitrous oxide is best done locally and regionally since no best solution is permanent. Continual feedback from the agricultural system is necessary and immediate mitigation should be proffered where

necessary (Coyne and Ren, 2017). An efficient nitrification program can be established by the stakeholders. They are to determine if, and when nitrification is a challenge, which parameters are associated with the challenge and proffer solutions.

The rate of nitrification is observed to be positively correlated to the abundance of AOB (Tao et al., 2017). Monitoring it and factors that tend to overtly influence their growth would initiate a good procedure for management. Afterward, some organisms known to counteract the adverse effect of the nitrification and denitrification process could be used. Inoculating microbes into soil has been considered an environmentally sustainable means to increase production (Alori et al., 2017). Enebe and Babalola (2018), suggested integration of microorganisms with other mediums as biofertilizers. Modern biotechnologies can be used to decrease the contamination of food associated with organic and microbial biofertilizers.

Verma et al. (2018) suggested that agrochemicals produced should be incorporated with organic manures or biofertilizer, a system referred to as integrated plant nutrient management. However, Pathak et al. (2016) recommend a management system that eradicates chemicals by using microbial bioinoculants and organic manure. Organic fertilizer could be made from living organism, dead organism, or their waste. They could directly or indirectly increase the supply of nitrogen in the soil naturally and in a stable way. According to Wang et al. (2018b), *Trichoderma viride* inoculated into the topsoil increases the abundance of AOA and AOB. *Phanerochaete chrysosporium* and *Bacillus thuringiensis* can promote nitrate and ammonia supply in soil (Shang et al., 2017). Organic manure has been produced using the combination of microbial bioinoculants and vermicomposting (Arumugam et al., 2017).

Biochar made from the burning of organic waste is a carbon-rich product used also as soil amendment. According to He et al. (2016), rice straw biochar causes an increase in nitrifiers

activities and enhances the nitrification process. Zeolites are naturally occurring mineral compounds used in agriculture as soil conditioners. It is known to have a nutrient holding capacity, retain nitrogenous substances and gradually release them in a controlled manner (Jakkula and Wani, 2018). Zeolites have the potential to efficiently stabilize the nitrification process. Scientifically formulated organic fertilizers have been produced by researchers (Table 2.3). The acceptance of organic fertilizer for agricultural intensification should promote crop yields by improving nutrient storage, physical and chemical parameters of the soil (Cai et al., 2019). Applying the right amount of manure to plants when needed is also very crucial. This would require the agriculturist to know the growth stage when individual species of plants need nitrate the most and the quantity needed.

Table 2.3: Scientifically formulated organic fertilizers.

Organic fertilizer	**References**
Pelleted feather meal + soybean meal	Evans (2019)
Mixture of various animal excreta	Bhalla et al. (2017)
Poultry Excreta + wood shavings	Bhalla et al. (2017)
Lime	Zhang et al. (2019)
Cattle Manure	Tao et al. (2017)
Livestock excreta + *Musca domestica* larvae	Kitazumi et al. (2016)

Ipomoea vermicompost	Hussain et al. (2017)
Arbuscular mycorrhizal fungi organic fertilizer pellets encapsulated with alginate film	Pitaktamrong et al. (2018)
Microalgae	Coppens et al. (2016)
Grounded Fish waste	Bond (2017)
Seaweeds	Verma et al. (2014)
Sugarcane bagasse	Shaarani et al. (2019)
Vegetable waste + *Nitrosomonas sp* + *Nitrobacter sp*	Naghdi et al. (2018)
Azotobacter candida	Alami (2017)
Bacillus candida	Alami (2017)

Biological nitrification inhibitors are produced by certain plants which include *Brachiaria humidicola cv.* (Byrnes et al., 2017), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), wheat relative (*Leymus racemosus*), Neem (*Azadirachta indica*) (Cantarella et al., 2018) and peanut (O'Sullivan et al., 2016). *Brachiaria humidicola* is known to produce brachialactone (a powerful nitrification inhibitor) in its rooting systems and has the highest biological nitrifying inhibiting capacity established so far (Subbarao et al., 2017). 1,9-decanediol, a biological nitrification inhibitor in rice root exudates, was

recently identified and proved to inhibit nitrification in bioassays using *Nitrosomonas (Lu et al., 2019)*. The use of these biological nitrification inhibitors is better options if nitrification inhibition must be used. Also, since inhibition aims to retain nitrate and reduce nitrous oxide emission, then denitrification should be focused on to reduce the emission of greenhouse gas. Biological denitrification inhibition would be a better strategy to make nitrate more available in the soil for the use of plants (William et al., 2019).

2.8 Urban Agriculture

Cultivation of food in the cities, termed "urban agriculture", is becoming popular and of paramount importance globally. If well managed in a sustainable way, it would be a good strategy for combating food security. McDougall et al. (2019), evaluated the stress of urban agriculture on the ecosystem in Sydney, Australia, it was observed that the environmental loading ratio was on the increase (5.82) with 14.66% renewable input. They concluded that the system was inefficient. However, with a better management strategy, there was a drastic improvement, with an environmental loading ratio of 1.32. The use of synthetic fertilizers and other agrochemicals should be discouraged. Alternatively, organic waste and self-composting that promote plant growth should be encouraged, and bioinoculants proven to be safe could be incorporated for efficiency. Considering proximity to human settlement, urban agriculturists should be trained, certified, and continuously monitored before and during agricultural practice. Failure to do this could result in the indiscriminate use of synthetic fertilizers and agrochemicals, thereby increasing the exposure of many to their environmental and health risks.

2.9 Limitations and Prospect

Intensification of agriculture has proved to be a threat to the security of food at the present and in the future (El Mujtar et al., 2019). Techniques in the agronomic management of soil

should be improved. Research on soil nitrification process still has gaps to be covered and should be continuous. Considering the urgent need to manage the process as a result of its environmental and health effect, some of the prospects and suggestions for further research include:

1. Extensive environmental toxicological studies of the influencing factors should be carried out on agrochemicals and weighed with their intended benefit before approval for usage. Also, bioaccumulation and biomagnification along the food chain should be evaluated. Already, the use of agrochemicals (herbicide, fungicide, insecticide and synthetic fertilizer) is discouraged because of their negative effects in the long run. However, nitrification inhibitors are being encouraged and the usage is gradually increasing. Many of them are still under long term toxicological studies, they appear alright at first usage, but with time the negative effect is noticed. There should be a thorough investigation of its effects on the environment and health.
2. Production of scientifically formulated and modified organic fertilizer that can serve as an alternative to nitrogen-based fertilizer. Plants express inert proteins that could promote or suppress growth in plants when they are in contact with factors externally (Olanrewaju et al., 2019). Also, the fertility of soil needs to be considered when increasing crop production (Omomowo and Babalola, 2019). Fertilizers made from neem oil or cake can stabilize the nitrification process and increase nitrogen use efficiency (Sarwar et al., 2019). Using biotechnologically improved organic substances to immobilize nitrate for later gradual release into the soil environment would be beneficial.
3. Identifying and classifying nitrifying bacteria and archaea associated with specific crop plants species using new generation sequencing (NGS). The divergent thoughts of researchers on nitrification processes result from incomplete knowledge of the full

range of its microbial network. During the 4th International Conference on nitrification, early career investigators were encouraged to manage nitrogen concentrations for the benefit of soil biodiversity (Klotz, 2016). Nitrifying bacteria and archaea can be biotechnologically worked on and their proliferation in soil environment can be optimized for the management of nitrification process.

4. Influenced nitrification and denitrification processes' contribution to global warming, and the use of microbial inoculants as a management strategy. Without the influencing factors, the nitrification process's contribution to global warming would likely be minimal. However, yield may be low except with the use of bioinoculants, which would provide a gradual release of nutrient.

2.10 Conclusions

The process of nitrification affects global cycling of nitrogen and its derivatives, nitrogen use efficiency, ecosystem health and services. In unperturbed natural agroecosystems, only small amounts of nitrogen and its derivatives are lost. However, the present agroecosystem has highly increased the rate of nitrification beyond what the biotic system can absorb. They depend on synthetic fertilizers, nitrification inhibition and other agrochemical substance which influences the soil nitrification process. The effects of their influence are observed negatively on the environment and biotic health in general. Proper management and biotechnology need to be put in place to reduce and remediate their effect. Managing nitrification can be achieved by having an in-depth understanding of the process, initiating a well-planned monitoring strategy, using eco-friendly and sustainable materials to improve the availability of nitrogen in soils, deploying several strategies wholly and specifically for the various chemicals and organisms distributed within its system. Urban agriculture can be used to boost food production, but it must be managed properly t o ensure environmental sustainability.

CHAPTER THREE

SUSTAINABLE INTENSIFICATION OF MAIZE IN THE INDUSTRIAL REVOLUTION: POTENTIAL OF NITRIFYING BACTERIA AND ARCHAEA

Abstract

Sustainable intensification is a means that proffer a solution to the increasing demand for food without degrading agricultural land. Maize is one of the most important crops in the industrial revolution era, there is a need for its sustainable intensification. This review discusses the role of maize in the industrial revolution, progress toward sustainable production, and the potential of nitrifying bacteria and archaea to achieve sustainable intensification. The era of the industrial revolution (IR) uses biotechnology which has proven to be the most environmentally friendly choice to improve crop yield and nutrients. Scientific research and the global economy have benefited from maize and maize products which are vast. Research on plant growth-promoting microorganisms is on the increase. One of the ways they carry out their function is by assisting in the cycling of geochemical, thus making nutrients available for plant growth. Nitrifying bacteria and archaea are the engineers of the nitrification process that produce nitrogen in forms accessible to plants. They have been identified in the rhizosphere of many crops, including maize, and have been used as biofertilizers. This study's findings could help in the development of microbial inoculum, which could be used to supplement reduced amounts of synthetic fertilizer in the short term and subsequently optimized in the medium to long-term to replace synthetic fertilizers.

Keywords: archaea; bacteria; food security; industrial revolution; sustainable agriculture

3.1 Introduction

An agroecosystem where yields are increased without an adverse effect on the environment and a need for additional non-agricultural land is referred to as sustainable intensification (SI) (Pretty and Bharucha, 2014). The focus on agricultural intensification to increase yield for the growing population has escalated environmental degradation (Armstrong McKay et al., 2019). Furthermore, many farmers have yet to adopt environmental sustainability because the problem of low yields has not been addressed. (Figure 3.1). Sustainable intensification can concurrently address environmental security and food security. This is because as agricultural production would be increased, environmental degradation would be reduced simultaneously without acquiring more land for farm use (Hunt et al., 2019). The components of SI (Figure 3.1) protect the process of an ecosystem and biological diversity while achieving an increase in food production. However, to achieve this aim, the development of suitable techniques for estimating both the sustainability and intensification of agriculture is needed (Hunt et al., 2019). Therefore, studying the interaction of microorganisms and the ecosystem would help maximize their services to ensure a better ecosystem.

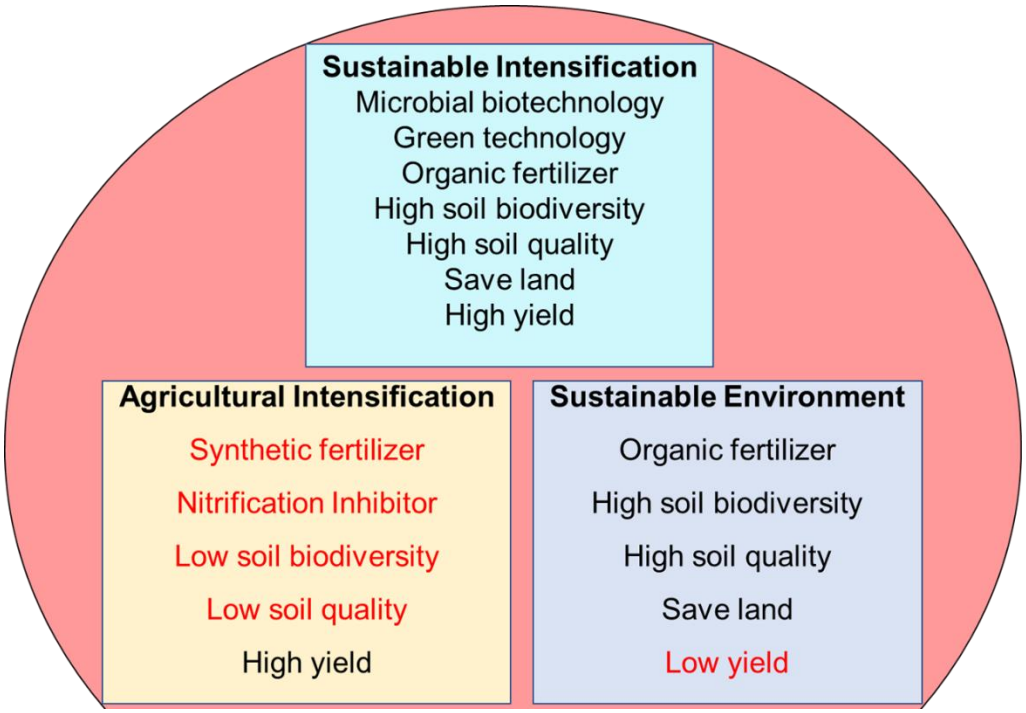

Figure 3.1: Components of sustainable intensification, agricultural intensification, and sustainable environment

Industrial revolution (IR) connotes industrialization that began way back in the 17th century (More and More, 2002). The industrial revolution brought about the expansion of farm crop yield and goods produced from them. Over the years IR has improved drastically as a result of mechanical production, electricity, electronics, telecommunication, computers, cyber-physical systems, genetic engineering, green revolution and the internet (Prisecaru, 2016, Vu and Le, 2019). This has affected the agricultural sector also because the innovation of technology is crucial to the renovation and cultivation of food. Food security is part of the challenges the industrial revolution intends to resolve (Prisecaru, 2016). Expectations have been raised regarding using new technologies to conserve resources and improve food nutrients. People are malnourished because nutrient requirements are not being met. Therefore, there is a need for further global green revolution if the world needs to be fed. The industrial revolution could contribute to the security of food by improving crops by artificially adjusting important microbes associated with crops.

Maize is an important staple crop in the industrial revolution and is still in high demand worldwide, considering its importance as food, additives in industrial products, scientific research, and economy. The necessity to intensify its production sustainably is of paramount importance. Modifications in the nitrogen cycle have acutely disturbed the structuring and functioning of the natural ecosystem. The suitable range of nitrogen levels has been altered within the ecospheres and has posed a challenge to the issue of nitrogen maintenance (Xu et al., 2016). The increasing nitrogen level is partly caused by the input of nitrogen-based synthetic fertilizers. Consequently, to avert the challenge with the use of synthetic fertilizers, the inoculation of plant growth-promoting microbes wholly or together with manures would be critical in improving maize productivity for industrial revolution. Nitrifying bacteria with traits that promote plant growth have the potential of achieving sustainable intensification. This review discusses the role of maize in the industrial revolution, progress toward sustainable production, and the potential of nitrifying bacteria and archaea to achieve sustainable intensification.

3.2 Significance of Maize in the Industrial Revolution

Maize accounts for a significant amount of daily food in most developing regions. It is referred to as yellow gold because of its usefulness as food, animal feeds, and manufacturing processed food and non-food materials. Several studies have been carried out on maize because of its economic importance. Maize is one of the few crops that have attracted the attention of researchers in the area of genetic enhancement (Badu-Apraku and Fakorede, 2017). Aside from its importance, researchers choose to work with maize because it is suitable to cultivate and easy to collect data from (Chen et al., 2015). Considering the foresight of industrial revolution, it is necessary to elaborate its role and point out how it can be cultivated in an environmentally friendly way.

3.2.1 Maize Products

The IR has caused an increase in agricultural products, both raw materials and industrialized. The processing of food rapidly has created sufficient time for human liberation, market participation and children care (Reardon et al., 2019). This has resulted in a reduced death rate and increased birth rate, causing a sharp increase in population, and placing high demand on resources. According to Dowswell et al. (2019), 20 million tons of maize is used for starch, 10 million tons are used for ethanol fuel production, 3 million for cereal and baked products, 0.7 million for cereal and hybrid seed sales. As a result of its reduced price as compared to other crops, maize has been used as feed formulae in animal rearing.

Maize is of high nutritional value and has been considered raw material for many industrial productions (Adiaha et al., 2016). This includes biomethane production, bioplastic, paper making, packaging and many additives. The agricultural sector substantially contributes to job creation and international marketing (Rekha and Singh, 2018). The effect of any technology in agriculture should be weighed against product output, profits, health, and environmental effect (Reardon et al., 2019). Over processed food has led to obesity, diabetes and several health problems, hence the need to ensure the fortification of foods with sufficient nutrients (Reardon et al., 2019).

3.2.2 Economic Importance of Maize

Since the transcend of IR, global economic growth has been increasing. The production of maize ranks first in Latin America and Africa, while in Asia it is ranked third after rice and wheat (Dowswell et al., 2019). The demand and supply for maize globally for food and non-food products are usually on the increase. Yearly, 15 million metric tons (MMT) are used for animal feed, 4.25 MMT for industrial use, 1.36 MMT is used as food (Yadav et al., 2016). Considering its value for domestic, industrial, and economic use (Adiaha et al., 2016),

investing in the increase in maize production is an opportunity for any country. Maize is grown in 170 countries using 184 M ha of land with a production of about 1016 MMT (FAOSTAT, 2017). Various countries have benefited from the exportation and importation of maize.

In India maize has an annual production of 24.26 million metric tons (MMT) (Yadav et al., 2016). There was a rapid increase in the production of maize from 1950 to 1980, while 1983 marked a sharp decrease in maize production (Dowswell et al., 2019). It generates income for the government as it is used by countries as a commercialized product (Adiaha et al., 2016). Companies and individual entrepreneurs are collaborating with large-scale farmers to produce high-quality maize seeds. This helps mitigate their high demand and insufficient supply (Jonga et al., 2018). Seed quality determines crop yield and productivity. Jonga et al. (2018) advised that a quality management system should be put in place by the companies and entrepreneurs to ensure better products continuously.

3.2.3 Scientific Research on Maize

The role of maize in scientific research for the industrial revolution cannot be overemphasized (Table 3.1). Some upcoming scientists wonder why there is intensive research on maize when compared to other cereals. Aside from its importance as food and uses in industrial products, maize is easy to cultivate and manage, thus the results are observed easily and juxtaposed occasionally to other plants. Notable of its use in genetic studies, Jiao et al. (2017) referred to it as a model species for agricultural and genetic research. Maize plant has been used to check the quality of soil (Adiaha et al., 2016). The cob is useful in the treatment of waste. Okoya et al. (2015) reported the efficiency of maize cob in the removal of lead and chromium from waste.

Table 3.1: Significant research findings related to the study of maize

Scientific research on maize	Result	Reference
Agronomic assessment of a Controlled-Release Polymer-Coated Urea-Based Fertilizer in Maize	20% significant increase in maize yield compared to traditional fertilizer. Soil property was improved, and nitrogen loss was reduced	Gil-Ortiz et al. (2021)
Evidence for phloem loading via the abaxial bundle sheath cells in maize leaves	The transfer of sucrose towards phloem was carried out by abaxial bundle sheath cell and it is subject to dorsoventral pattern	Bezrutczyk et al. (2021)
How to increase maize production without extra nitrogen input	Increasing the density of plant increase the yield of maize by 5.59% Greenhouse gas reduced	Hou et al. (2020)
Early isotopic evidence for maize as a staple grain in the Americas	Maize consumption started 4000 calendar years before present	Kennett et al. (2020)
Comparison between organic and inorganic fertilizer	The cost of production using organic fertilizer is one fourth cheaper than inorganic	Deba et al. (2019)

	Significant increase in broadness and number of leaves in the plants with organic fertilizer	
The function of ZmUBP15, ZmUBP16 and ZmUBP17.	Help plant tolerate cadmium and salt stress They are mostly found in the plasma membrane	Kong et al. (2019)
The role of cytoplasmic diversification on plant agronomic productivity and trait	A significant influence on the yield component of plants as a result of interaction between cytoplasm, nucleus, and testers	Calugar et al. (2018)
Determination on how cells and tissues rely on autophagy	The evident alteration was seen in plants missing the core autophagy component ATG12 Autophagy influences eukaryotic membrane under nutrient stress	McLoughlin et al. (2018)
Effect of climate change on maize cultivation	Yield loss majorly as a result of drought stress Elevated CO2 and heat had no effect on the crop	Webber et al. (2018)

Assemble and annotation of maize genome using single molecule real-time sequencing and high-resolution optical mapping	Contig length was significantly increased and there was a deletion in the low gene density region	Jiao et al. (2017)
Effect of heat and drought on rubisco activity which is associated with photosynthetic limitation	Rubisco activities was most affected at high temperature, but it was unrelated to the amount of rubisco activities The reduced rubisco affected CO2 assimilation rate Rubisco can be used to improve plant photosynthetic performance in warm climate	Perdomo et al. (2017)
Molecular basis of carpel fusion in ovary development	Certain miRNAs influence incomplete carpel fusion which code for auxin response factor and growth regulating factor	Li et al. (2017)
Cadmium stress tolerance of plant using dark septate endophyte	Cadmium phytotoxicity reduced significantly while maize growth increased	Wang et al. (2016a)

	This was done by triggering the antioxidant system, altering cadmium and partitioning the subcellular cadmium into the cell wall.

3.2.4 Food Security

The quantity and quality of food have been threatened by unfavourable environmental conditions. To meet the needs of the high population, the quantity of food must be increased without jeopardizing the quality. In search of a solution, maize has been a choice crop by researchers (Otsuka and Muraoka, 2017, Adiaha et al., 2016). According to Abate et al. (2015), after considering factors that can be used to combat food security, maize was chosen as the best cereal to be cultivated in Ethiopia. He further explained that in terms of calorie intake, maize is the most important staple food. Otsuka and Muraoka (2017) acknowledged maize to be the most important cereal, considering its production and consumption. The development of the agricultural sector is necessary to reduce poverty and secure food. The need to secure food should be reinforced with green revolution that would drastically increase the yield of crops in a sustainable way. Therefore, maize which is easily cultivated and possess lots of nutrient has the potential to combat food insecurity globally. Maize cultivation has dropped the rate of poverty and improved the lives of local farmers, especially in developing countries Adiaha et al. (2016).

3.3 Industrial Revolution of Maize

The agricultural sector, in general, has benefitted from industrial revolution using green and microbial biotechnology. Presently, green revolution has been anchored on genetically modified food and agrochemicals alongside several other inventions and technology (Llewellyn, 2018). Otsuka and Muraoka (2017) stated that the green revolution has helped

resolve food crisis however, some countries are yet to meet the global standard of maize yield and attributed this to low soil quality. Also, food insecurity is rising, crop yields are lower than expected when compared to farmers' input, many crop plants are susceptible to disease and the environment is being depleted. An improvement in the present green revolution is necessary, this could be achieved by scientific and biotechnological research towards agricultural production.

The focus is now on sustainably feeding the growing population. Increasing land productivity is a crucial requirement in meeting the growing demand for food in every region. Implementing technology in agriculture can cause a global transformation. Brill (1981) suggested the possibility of getting a hybrid plant with foreign genetic material that would make it possible for the plant to efficiently use atmospheric nitrogen. The possibility of using recombinant DNA techniques in microbial breeding for agriculture is still at a primitive stage, while engineering of beneficial soil microorganisms associated with the specific crop is ongoing. The inoculation of bacteria into soil has been seen to have a positive effect on plant growth (Ndeddy Aka and Babalola, 2016). The beneficial microorganisms can be cultured, grown in fermentation tanks and isolated for use. This can be taken practically to revolutionize industrial maize production.

3.4. Achieving Sustainable Intensification

In-depth knowledge of the dynamism of nitrogen would require research on the distribution, function, structure, and contribution of Bacteria and Archaea associated with its cycling process (He et al., 2012). Inoculation of microorganisms is a biotechnological environmentally safe alternative to increase crop production (Alori et al., 2017, Olanrewaju et al., 2017). The microorganism with the highest benefit could be useful for biotechnology breeding (Walters et al., 2018). Integration of microbes with organic material can also be considered Enebe and Babalola (2018). This would reduce the need for synthetic fertilizer

and achieve SI. A new system incorporating different components that can boost maize production can be put in place (Figure 3.2).

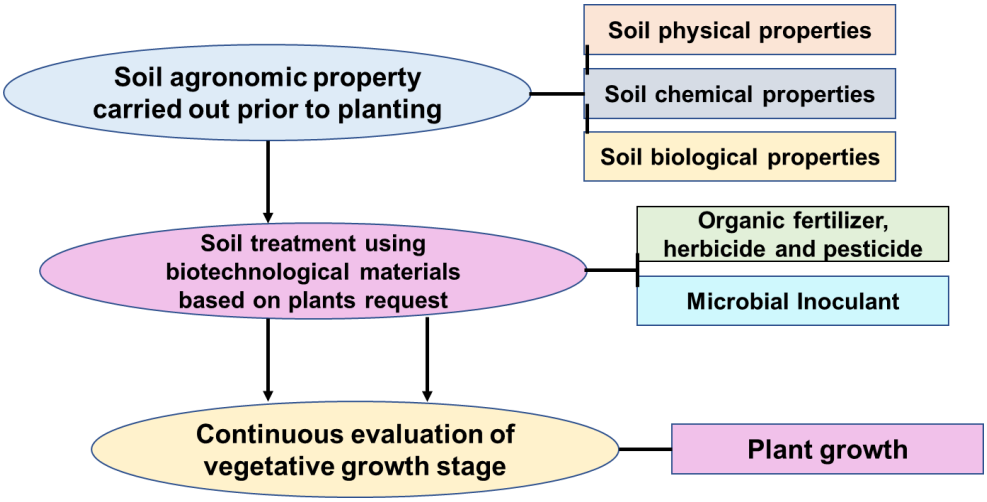

Figure 3.2: Components that can help achieve sustainable intensification

3.5 Plant Growth Promoting Microorganisms

Unavailability of nutrients, pest infestation, and drought are some of the challenges to plant growth. Some microorganisms referred to as plant growth promoters have been observed to have traits that could help combat these challenges. One of the ways to address these challenges is to assist in the cycling of geochemical making nutrients available for plant growth (Etesami and Adl, 2020). Inoculation of microorganisms is a biotechnological alternative to increase crop productivity, increase the availability of nutrients, reduce the use of synthetic fertilizer, and achieve SI (Table 3.2). *Bacillus subtilis* was reported by Zheng et al. (2018) to be able to influence the physical, chemical and hydrological characteristics of the rhizosphere, thus improving drought tolerance of plants in the long run. They ascribed this attribute of *Bacillus subtilis* to their production of extracellular polymeric substances. Using genomic information, Wang et al. (2018a) ascertained the usefulness of *Streptomyces albireticuli* and *Streptomyces alboflavus* as a biocontrol agent.

Table 3.2: Microorganisms with plant growth-promoting traits that have been used on maize

Microorganism Inoculate used in maize	Type of experiment	Plant Growth Promoting Trait	Result	Reference
Aspergillus niger	Field	Zinc and phosphate solubilization at a wider temperature and pH range	Inhibit production of aflatoxin Increase harvest index and yield Improves maize nutrient content	Naeem et al. (2021)
Rhizophagus Irregularis, Glomus mosseae, Paraglomus occultum	Greenhouse	Increase soil fertility and enhance plant growth	Significant increase in root colonization and maize growth	Fasusi et al. (2021)
Anabaena-Nostoc consortium, Anaebaena-Trichoderma biofilm	Field	Carbon Nitrogen mobilization	Higher efficiency was recorded in terms of economic, energy and	Sharma et al. (2021)

			environmental use Increased cob yield	
Azospirillum brasilense	Field	Increase chlorophyll content of plant	Increased yield and productivity	Cardozo et al. (2021)
Metarhizium sp	Greenhouse	Possess entomopathogenic properties	Antagonistic effect on maize pathogen *Spodoptera frugiperdia*	Silva (2021)
Azospirilum brasilense* and *Bacillus subtilis	Greenhouse	Zinc solubilization	Modified root system which efficiently improves water and nutrient use	Moreno et al. (2021)
Trichoderma harzianum	Field	Induce resistance of plant against herbivorous attack	Alter and reduce the community and abundance of pests	Contreras-Cornejo et al. (2021)

Bacillus sp* and *Paenibacillus	Field	Auxin production	Improve maize yield	De Carvalho Nascimento et al. (2021)
Bacillus subtilis* and *Pseudomonas koreensis	Greenhouse	Siderophore production	Reduces infectious disease caused by *cephalosporium maydis*	Ghazy and El-Nahrawy (2021)
Burkholderia cepacia* and *Acinetobacter baumannii	Net house	Zinc solubilization	Improve the level of protein and sugar accumulation	Upadhyay et al. (2021)
Claroideoglomus etunication	Greenhouse	Facilitate revegetation of contaminated soil	Enhance plant growth in lanthanum contaminated soil	Hao et al. (2021)
Azotobacter chroococcum	Field	Promotes absorption of plant nutrients	Increase total nitrogen and phosphorus content in plant	Song et al. (2021)

Anabaena cylindrical* and *Azospirillum brasilense	Field	Nitrogen-fixing bacteria	Higher nitrogen content of maize	Gavilanes et al. (2020)
Arthrobacter arilaitensis* and *Streptomyces Pseudovenezuelae	Greenhouse	Ammonia, Indole-3-acetic acid, and Siderophore activity	Plants tolerated drought better Physiological parameters show significant increase	Chukwuneme et al. (2020)
Trichoderma harzianum, Bacillus amyloliquefaciens	Greenhouse	Phosphate solubilization	Stimulate root growth which promotes the absorption of nutrients in the soil	Mpanga et al. (2019)

3.6 Nitrifying Bacteria and Archaea

Surprisingly, nitrifying bacteria and archaea (Table 3.3) have not been focused on as plant growth-promoting bacteria. Considering their importance in nitrate production and oxidizing ammonia in soils and substrates, this calls for attention in scientific research. Aside from their major function of nitrification, they could have other plant growth-promoting traits. They can be classified into three distinct groups depending on the key enzymes possessed. The first group is the ammonia-oxidizing bacteria and archaea, second is the nitrite-oxidizing

bacteria (Table 3.3) and the third is comammox bacteria (oxidation of ammonia to nitrate) (Stein and Klotz, 2016). Key enzymes used by these organisms are

ammonia monooxygenase (*amoA*), hydroxylamine oxidoreductase (HAO) and nitrite oxidoreductase (NXR) (Kuypers et al., 2018).

Table 3.3: Well-identified nitrifying bacteria and archaea genera and their physiological group

Domain	Phylum	Class	Order	Family	Genera	Physiological group	Reference
	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	*Nitrosospira*	Ammonia oxidation	Schaechter (2009)
	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	*Nirosococcus*	Ammonia oxidation	Gerardi (2003)
Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	*Nitrosomonas*	Ammonia oxidation	Koops and Stehr (1991)

Archaea								
Thaumarchaeota	Nitrospirae	Proteobacteria	Proteobacteria	Deltaproteobacteria	Nitrospinales	Nitrospinaceae	*Nitrospina*	Nitrite oxidation	Gerardi (2003)
Nitrososphaeria	Nitrospira	Gammaproteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Nitrobacter*	Nitrite oxidation	Brenner et al. (2005)
Nitrososphaerales	Nitrospirales	Chromatiales	Ectothiorhodospiraceae	*Nitrococcus*	Nitrite oxidation	Schaechter (2009)		
Nitrososphaeraceae	Nitrospiraceaa	*Nitrospira*	Nitrite oxidation	Schaechter (2009)				
Candidatus Nitrososphaera	Ammonia oxidation	Tourna et al. (2011)						

Thaumarchaeota	Nitrososphaeria	Nitrosopumilus	Nitrosopumilaceae	*Candidatus*	*Nitrosopumilus*	Ammonia oxidation	Qin et al. (2017)

Based on nutrition, the nitrifying bacteria and archaea could be divided into heterotrophs and autotrophs (Liu et al., 2015). The heterotrophs depend on other organisms or dead organic matter for food while the autotrophs can synthesize their food. The autotrophs could further be divided into photoautotrophs (possess bacteriochlorophyll and use solar energy to produce food) and chemoautotrophs (using the oxidation of certain chemicals to produce food). Cellular respiration of nitrifying bacteria and archaea could either be aerobic (with oxygen) or anaerobic (without oxygen) (Muck et al., 2019). The group of organisms involved in anaerobic ammonium nitrification is known as anammox, they carry out nitrification in oxygen-depleted zones (Rich et al., 2018).

Nitrifying microbes include chemolithotrophic members, members of Betaproteobacteria, Gammaproteobacteria, and members of the Thaumarchaeota (Stein, 2019). The reactions occur under varying soil characteristics with some abiotic components contributing to it (Heil et al., 2016). Also, there are heterotrophic and methanotrophic bacteria that oxidize ammonium to nitrite efficiently (Stein and Klotz, 2016). High temperature changes soil nitrifying communities as a result of an increase in the rate of chemical production (Nguyen et al., 2019). pH between 7 and 9 is best for the activity of ammonia oxidizing bacteria and nitrite-oxidizing bacteria, as higher than that disrupts their activity (Heil et al., 2016). Environmental factors determine the group of nitrifying microorganisms that would be prevalent in a habitat or substrate.

Nitrifying bacteria are widely used in aquaculture management (Ajijah et al., 2021, Ruiz et al., 2020) and waste management (Zhao et al., 2020, Sepehri et al., 2020). It is rarely used in cropping. *Nitrobacter*, on the other hand, has been used as a biofertilizer both alone (Doost et al., 2019) and in groups of micro-consortiums (Vatandoost et al., 2019). Doost et al. (2019) discovered that the protein content of Canola improved when compared to the control. Beyond aquaculture and waste management, there is still a need to expand the use of nitrifying bacteria in cropping systems. Nitrifire 5x, MicrobeLift Nite-out II, Scape bac up, Nitrobacter multi-probiotic, Nbc1 and Nbc2 are some of the commercially available application-based nitrifying bacteria. Although these products were intended for use in aquaculture, their novel application in crop management can be investigated.

Excess ammonia in the soil as a result of synthetic ammonium-based fertilizer affects the environment negatively (Lehtovirta-Morley, 2018). The presence of nitrifying bacteria in the soil reduces ammonia. This makes the soil less acidic and, as such, other beneficial microorganisms can proliferate, thus promoting soil quality. Also, nitrate, which is eventually produced from the nitrification process, elongates lateral roots (Mantelin and Touraine, 2004), mediates signalling pathways of phytohormones, expands leaves, and induces flowers in plants (Hachiya and Sakakibara, 2016). Furthermore, plants' yields and growth are increased and there is little or no dependence on synthetic fertilizer and other agrochemicals that degrade the soil.

3.7 Electron Transport Chain

The enzymatic process of nitrification can be divided into three pathways: NH_3 oxidation pathway, NH_2OH oxidation pathway, and NO_2 oxidation pathway. The enzymatic process is carried out by an electron transport chain and the reaction is exergonic (a biochemical reaction that releases energy) (Wendeborn, 2019). Ammonia monooxygenase (AMO) turns ammonia into NH_2OH with the gain of two electrons (Daims et al., 2015). The electron is

obtained from subsequent oxidation of hydroxylamine, and the energy liberated is obtained from the linked reaction of oxygen reduced to water (Wendeborn, 2019). AMO exists in an integral membrane protein and is a member of the copper membrane monooxygenase (CuMMO) family. The mechanism by which CuMMO carries out its oxidation could help in the development of monitored synthetic oxidation (Lancaster et al., 2018).

$NH_3 + 2H^+ + O_2 + 2e^- \rightarrow NH_2OH + H_2O$

$NH_3 + ½O_2 \rightarrow NH_2OH$

$½O_2 + 2H^+ + 2e^- \rightarrow H_2O$

Four electrons are used by hydroxylamine oxidoreductase (HAO), a multiheme enzyme used to oxidize NH_2OH to NO_2^-. Two of the electrons used in oxidizing hydroxylamine return to AMO while the remaining two enter the respiratory electron transport chain, terminating the electron acceptor using O_2 (Daims et al., 2015). This reaction is also exergonic and the energy produced is higher if coupled with a reduction of water (Wendeborn, 2019). According to Lancaster et al. (2018), oxygen is not required for HAO activity, and NO is the product of NH_2OH oxidation and not NO_2. The author further explained that NO is a reactive molecule, as its transformation to other forms of nitrous oxide could be a non-enzymatic reaction. This might be true, however, Wendeborn (2019), reported some organisms that can oxidize NO_2 to NO_3.

$NH_2OH + ½ O_2 \rightarrow NO_2^- + 2e^-$

Nitrite oxidoreductase possessed by some NOB oxidizes nitrite to nitrate with the use of electrons donated from oxygen. However, it can also be produced when nitrite donates electrons to reduce CO_2 to glucose by some photosynthetic bacteria (Wendeborn, 2019).

$NO_2^- + ½ O_2 \rightarrow NO_3$

$2NO_2^- + CO_2 + H_2O \rightarrow CH_2O + 2NO_3^-$

Comammox (complete ammonia oxidizer) was predicted by Costa et al. (2006) and discovered in *Nitrospira* by Daims et al. (2015) and Van Kessel et al. (2015). They can utilize eight electrons to oxidize NH_3 to NO_3^- (Lancaster et al., 2018). Broda (1977) predicted two chemolithotrophic organisms that can carry out anammox (Anaerobic ammonia oxidation). One of the bacteria responsible for anammox was identified as Planctomycetales in 1999 (Strous et al., 1999). Anammox microorganisms in an environment where oxygen is depleted can make use of nitrite instead of oxygen as the electron acceptor producing dinitrogen (Wendeborn, 2019). Considering the complex metabolic pathway in the nitrification process, there might be more discoveries to be made to manage the process efficiently.

3.8 Availability of Ammonia in the Soil and Organic waste

Ammonia-based substance is the substrate used by AOA and AOB. They can obtain it from ammonia-based organic waste or soil organic matter. Organic waste improves the quality of soil because it positively affects the growth of soil microorganisms. The natural process of nitrification does not provide sufficient nitrate. Therefore, to strike a balance between the modern process and the natural process, it would be good to provide a technology that would mimic the natural process. Organic fertilizers have been made from composting of organic waste and vermicomposting (Caceres et al., 2018). Plant growth-promoting microorganisms can be used along with these organic materials (Domenico, 2020). One of the biological approaches suggested for SI is to increase biological diversity in the agricultural systems (Petersen and Snapp, 2015). Nitrate has been successfully produced from ammonium contained in vegetable waste using *Nitrosomonas* sp and *Nitrobacter* sp by Naghdi et al. (2018). Synthetic fertilizer is the cause of excessive amounts of nitrate

because it speeds up the rate of nitrification. The gradual and systematic production of nitrate is considered safe for the ecosystem and a better alternative to synthetic fertilizer.

3.9 Identification and Isolation of Nitrifying Bacteria and Archaea

Microorganisms are ubiquitous; however, their composition varies in different habitats as a result of varying environmental factors. In time past isolation and identification of microorganisms are usually carried out after culturing. Recently, metagenomics survey has enabled the easy identification of microorganisms. Known and unknown nitrifying microorganism strain has been identified from different habitats via metagenomics analysis (Clark et al., 2021). The establishment of the presence of nitrifying bacteria and archaea provides a guide on what type is to be isolated and cultured. Although nitrifying bacteria and archaea have been difficult to culture, however, some researchers have been successful in that regard (Könneke et al., 2005, Mellbye et al., 2017). Könneke et al. (2005), isolated nitrifying archaea using serial dilution and incubated them with a medium enriched with ammonia at 21^{o}C to 23^{o}C. The use of mineral salt media with varying formulations has been used by Mellbye et al. (2017). Furthermore, Fujitani et al. (2015), explained the possibility of isolating them from nitrifying granules in a wastewater plant and cultivating them in a liquid culture rich in ammonia. Molecular characterization of the nitrifying microorganisms can also be carried out using 16S rRNA gene sequencing after serial dilution, DNA extraction and PCR amplification (Hastuti et al., 2019). Cultivating nitrifier community unique to maize plant can be carried out and used to increase their population in maize rhizosphere. This would increase the bioavailability of nitrogen in the soil, thereby replacing nitrogen-based fertilizers.

3.10 Conclusions and Perspective

Sustainable intensification proffers the solution to the conflicts of meeting the increasing demand for food and ensuring a sustainable environment. Industrial revolution merges

trends in intelligent automation with artificial intelligence, and this results in remarkable improvement in technology, growth in economy and unimaginable progress. Maize accounts for a significant amount of daily food in most developing regions and it is important to scientific and industrial use. Considering the need to increase maize production, microorganisms with growth-promoting properties can help achieve proper management, sustainable agriculture, and sustainable environments. Agriculture has used large amounts of land globally, with major implications for reactive nitrogen from synthetic fertilizers and the use of nitrifying inhibitors to inefficiently manage the system. Nitrifying bacteria and archaea can transform ammonia locked up in soil organic matter and organic waste matter. They can be inoculated wholly or together with ammonium-based organic waste into the rhizosphere of maize. Although the biotechnological formulation and use are still in their primitive stage. Identifying and isolating nitrifying microorganism communities and structures associated with maize is a step towards achieving sustainable intensification.

CHAPTER FOUR

16S METAGENOMICS OF NITRIFYING BACTERIA AND ARCHAEA INHABITING MAIZE RHIZOSPHERE AND THE INFLUENCING ENVIRONMENTAL FACTORS

Abstract

The maize rhizosphere soil is unique with diverse microorganisms. Nitrifying bacteria and archaea are ubiquitous and can transform ammonia locked up in soil or manure into nitrate, a more soluble form of nitrogen. However, nitrifying bacteria and archaea inhabiting maize rhizosphere are yet to be identified. We elucidate the diversity and abundance of nitrifying bacteria and archaea associated with maize rhizosphere across different growth stages using 16S metagenomics sequencing. Also, the influence of environmental factors (soil physical and chemical properties) on the nitrifying communities was evaluated. DNA was extracted from maize rhizosphere soil using Nucleospin Soil DNA extraction kit and sequenced on Illumina Miseq platform. MG-RAST was used to analyze the raw sequences. Some physical and chemical properties of the soil were measured using standard procedure. The result revealed 9 genera of nitrifying bacteria; *Nitrospira, Nitrosospira, Nitrobacter, Nitrosovibrio, Nitrosomonas, Nitrosococcus, Nitrococcus,* unclassified (derived from Nitrosomonadales), unclassified (derived from Nitrosomonadaceae) and 1 archaeon *Candidatus Nitrososphaera*. The Nitrospirae phyla group which had the most nitrifying bacteria was more abundant at the tasselling stage (67.94%). Alpha diversity showed no significant difference. However, the Beta diversity showed significant difference (P=0.01, R=0.58) across the growth stages. The growth stages had no significant effect on the diversity of nitrifying bacteria and archaea, but the tasselling stage had the most abundant. A correlation was observed among some of the chemical properties. The research outcome

can be put into consideration while carrying out a biotechnological process that involves nitrifying bacteria and archaea.

Keywords: Nitrospirae, biotechnology; nitrate; maize growth stages; rhizospheric soil; ammonia

4.1 Introduction

Metagenomics has exposed an extraordinary degree of diversity and novelty among microbial communities. Its analysis can involve sequence-based or functional approaches or a combination of both (Akinola et al., 2021a, Chukwuneme et al., 2021). It could help the development of management practices that maximize the beneficial use of microbial communities in and around the crops. Also, it could lead to the discovery of novel natural products, new antibiotics, new bioactive molecules, and new functions. Pyrosequencing of bacterial 16S genes has led to observed substantial variation in bacterial richness, diversity, and relative abundances of taxa between bulk soil and the maize rhizosphere, as well as between fields (Peiffer et al., 2013). Also, comparing 16S sequence profiles across samples clarifies how microbial diversity associates with environmental conditions (Sharpton, 2014).

Nitrifying bacteria and archaea are the microorganisms that carry out the biochemical reaction of transforming ammonia to nitrate. Their importance cannot be overemphasized because nitrates help in the regulation of gene expression, mediate hormone signals (Hachiya and Sakakibara, 2016) and are less acidic than ammonia. An acidic environment increases the bioavailability of heavy metals (Ayangbenro et al., 2018) and affects nutrient uptake (Shi et al., 2019). Synthetic fertilizer (Verma et al., 2018) has been used to replace the function of these organisms. Unfortunately, this has caused an adverse environmental effect which include increase in nitrous oxide emission and eutrophication (Verma et al., 2018, Zhai et al., 2017). A management process that would mimic the natural process would

be better to achieve both agricultural intensification and environmental sustainability (Alori and Babalola, 2018).

The rhizosphere serves as an interface that supports exchange of resources between plant and their associated soil environment. Its microbial diversity is influenced by the soil physical, biological, and chemical properties usually determined by the host plant. Microbes in the maize rhizosphere can be endophytic, epiphytic, or closely associated (Peiffer et al., 2013). They can be diverse with various organisms, such as fungi, bacteria, archaea, nematodes, and other invertebrates, due to the exudates (metabolite) secreted by the plant. The organisms either play a positive role by enhancing the growth of the plant or a negative role by causing diseases. Characterizing the ones associated with enhanced crop yield is an important first step towards understanding the role of the microbiota in soil fertility (Qiao et al., 2019). The structure and diversity of bacterial community in the rhizosphere vary significantly according to plant species (López-Carmona et al., 2019). This diversity is a result of differences in the type of exudates and signalling compounds they produce.

Substantial variation is being observed in the microbial diversity of maize rhizosphere. Their root exudate enables them to attract high diversity of microorganisms. It contains sugars, organic acids, aromatics and enzymes, which attract a wide range of microbial diversity (Peiffer et al., 2013). Evidence shows that the economic gains of farmers through maize production have led to an increased level of household food security primarily in relation to nutritional balance (Chowdhury, 2016). Maize is an important source of nutraceuticals, such as phenolics, carotenoids, anthocyanins, phlobaphenes, insoluble and soluble dietary fibre and polar and nonpolar lipids, which are known to prevent diseases and enhance health (Ekpa et al., 2018).

There exists a direct and indirect interlink within and between soil physical, chemical and biological parameters. The richness, diversity and structure of microbial communities can be affected by environmental parameters and edaphic properties, mainly pH and nutrients. Researchers have reported the relationship of pH with other soil parameters (Xiao et al., 2018, Tu et al., 2018, Li et al., 2019c). Organic carbon had a significant correlation with pH (Xiao et al., 2018, Tu et al., 2018). A high level of sulfur in the soil increases its pH (Li et al., 2019c). According to Kopáček et al. (2013), nitrogen cycling is intimately linked to sulfur and carbon cycling. Plant yield, quality and growth are optimized when the ratio of ammonia to nitrate is low; Liu et al. (2017) suggested a ratio of 1:3. The ratio of carbon to nitrogen and soil total nitrogen influence both microbial activity and soil quality (Xiao et al., 2018); these are pivotal to crop production.

One of the methods of biofertilization is increasing the abundance of microbes in the rhizosphere of plants (Igiehon and Babalola, 2017). Elucidating nitrifying bacteria and archaea associated with specific crop types and growth stages could provide information for its biotechnological application. To date, many of the maize rhizosphere resident nitrifying bacteria and archaea associated with varying growth stages are unknown. Identifying them and the influence of the rhizosphere physical and chemical properties would enhance microbiome-based management strategy for nitrogen utilization. We hypothesize that maize rhizosphere inhabits unique composition of nitrifying bacteria and archaea across different growth stages, and they are influenced by environmental factors. This study elucidates the diversity and abundance of nitrifying bacteria and archaea across different growth stages of maize rhizosphere using 16S metagenomics. Similarly, the study evaluates the relationship among the soil physical and chemical properties and their influence on the nitrifying bacteria and archaea.

4.2 Materials and Methods

4.2.1 Sampling

The samples were collected from the 32 years old maize plantation of the North-West University Farm, Molelwane, Mahikeng, South Africa (25° 47’ 23.9604” S, 25° 37’ 8.43348” E; altitude 1012 m Figure 4.1). The region has annual temperature ranging from 22^{o}C - 35^{o}C in summer, 2^{o}C - 20^{o}C in winter and an annual rainfall of 450 mm. The farm was irrigated and treated with NPK (20% Nitrogen, 7% Phosphorus and 3% Potassium) fertilizer before planting. The maize cultivar planted was QN.633. Three different growth stages of maize were identified: Pretasseling growth stage (PR), tasselling growth stage (TA), and fruiting growth stage (FR). The rhizosphere soil was collected between 0-15 cm depth and 0-5 cm breath of each maize root and bulk (BU) soil was also collected. The soil was collected in triplicate for each developmental stage and bulk soil, then transported to the laboratory and stored at -20^{o}C.

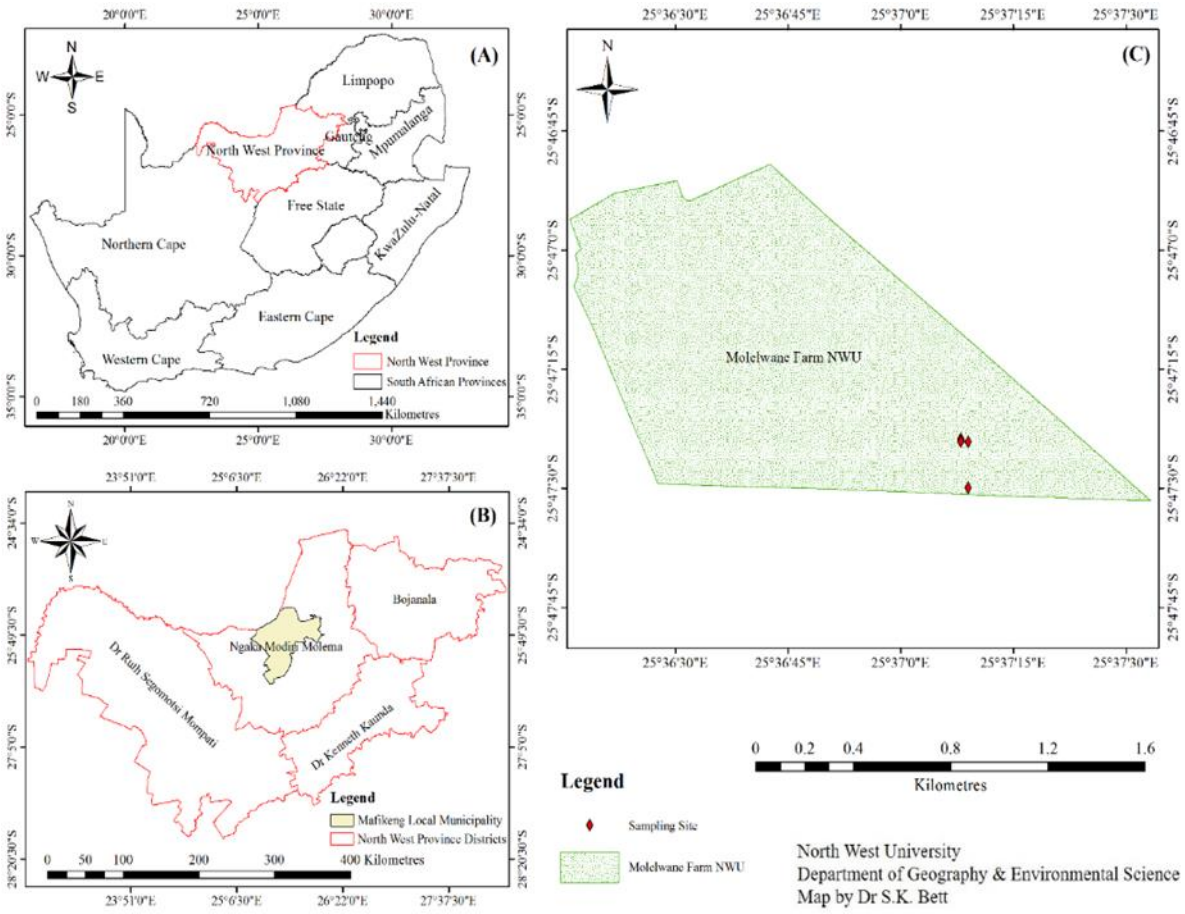

Figure 4.1 Sketch map of the study area, Molelwane farm, North West Province, South Africa

4.2.2 Physico-Chemical Analysis of the Rhizosphere and Bulk Soil

Physical and chemical properties of the soils were measured using standard chemical analysis. The particle size (sand, silt and clay) distribution was evaluated using the method of Kroetsch (2008). Nitrate and ammonium were measured using KCL extraction method as described by Keeny and Nelson (1982). Organic matter was measured using loss of ignition method (Nelson and Sommers, 1996). Total carbon was analyzed using dry combustion method (Santi et al., 2006). Organic carbon was measured using the method described by Walkley and Black (1934). Total nitrogen was analyzed using digestion method (Bremme and Mulvaney, 1982). HCl extraction method was used to determine the sulfur content of the rhizosphere as described by Smittenberg (1951). The pH was measured with Jenway

3520 pH meter (Cole – Palmer instruments, Staffordshire UK) after mixing the soil (2g) and deionized water (10ml).

4.2.3 DNA Extraction and 16S Metagenomics Sequencing

DNA was extracted using a Nucleospin soil DNA extraction kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. The V3 - V4 hypervariable portions of the 16S rRNA gene were targeted with universal primer pairs 341F (5'-CCT ACG GGN GGC WGC AG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Thijs et al., 2017). The amplicons were then gel purified, end-repaired, and Illumina-specific adaptor sequences ligated to each of them. The samples were individually indexed after quantification, and another purification step was conducted. The amplicons were sequenced using a MiSeq v3 (600 cycles) kit on Illumina's MiSeq platform. For each experiment, 20 Mb of data (2x300 bp long paired-end reads) was generated.

4.2.4 Metagenome Assembly and Gene Annotation

The MG-RAST server (http://www.mg-rast.org) was used to process and analyze the raw sequences, which were uploaded as a FastQ file (Meyer et al., 2008). Following that, the sequence reads were annotated using the BLAST technique (Kent, 2002) and the M5NR database (Wilke et al., 2012). The data normalization tool was applied to reduce experimental error. Default parameters were used for the bioinformatics tools. The abundance of bacterial and archaeal community at different growth stages were evaluated. Reads of eukaryotes and unclassified sequences were removed.

4.2.5 Data and Statistical Analysis

Microsoft Excel software was used in evaluating the mean of the triplicate samples and the relative abundance of the bacterial and archaea diversity. The richness of the species sequence was evaluated through rarefactions analyses on MG-RAST. Heat map of the relative abundance of bacteria and archaea was carried out using Heatmapper online

software (www.heatmapper.ca/expression). Alpha and beta diversity analysis was carried out using Past version 2.17 (Hammer et al., 2001). CANOCO 5 was used to carry out principal component analysis and principal coordinate analysis using default settings (Cajo et al., 1997). XLSTAT was used to determine the relationship between the soil physical and chemical properties and their influence on nitrifying bacteria and archaea.

4.3 Results

4.3.1 Rhizosphere Environmental Factors

The statistical analysis of the rhizosphere physical and chemical parameters is summarized in Table 4.1. The pH which is the focal point of the physical and chemical parameter ranges from 5.35 to 6.22 with a mean of 5.93. The soil sample contained a mean of 85% sand, 13% clay, 0.73% organic carbon, 0.73% total carbon, 2.4% organic matter, 0.08% total nitrogen, 336.5 mg/kg sulfur, 4.348 mg/kg ammonium, 6.123 mg/kg nitrate. The carbon to nitrogen ratio is approximately 9:1. The NH_4 to NO_3 ratio is approximately 1:1.4.

Table 4.1: Physico-chemical parameters of the maize rhizosphere.

Variable	Minimum	Maximum	Mean	Std. deviation
SA	84.00	86.00	85.00	1.16
CL	12.00	14.00	13.00	1.16
pH	5.35	6.22	5.93	0.41
S	246.00	576.00	336.50	159.85
OC	0.52	0.84	0.73	0.15
TC	0.52	0.89	0.73	0.15
OM	2.04	2.70	2.43	0.30
TN	0.06	0.09	0.08	0.01

NH_4	3.84	4.67	4.35	0.40
NO_3	4.02	9.76	6.12	2.72

SA-Sand (%), CL-Clay (%), pH (H_2O), S-Sulphur (mg/kg), OC-Organic carbon (%), TC-Total carbon (%), OM-Organic matter (%), TN-Total nitrogen (%), NH4-Ammonium (mg/kg), NO3-Nitrate (mg/kg), NB-Nitrifying bacteria.

4.3.2 16S Metagenomics Sequencing of Maize Rhizosphere Across Different Growth Stages

The information of the sequence read is listed in Table 4.2. Rarefaction curve shows the richness of species sequences with the fruiting stage having the highest among the different vegetative growth stages (Figure 4.2). Table 4.3 and Figure 4.3 show the bacteria and archaea phylum relative abundance represented in all growth stages. Over 99% of the reads were predominantly bacteria, while the archaea were less than 1%. Phylum Actinobacteria was the most dominant in all the growth stages and was highest (47%) at PR. The bulk soil sample showed the highest percentage of Proteobacteria (10.4%) and Bacteroides (5.2%). Gemmatimonadates (5.6%) and Chloroflex (2.6%) were highest at PR. At TA, Planctomycete and Acidobacteria were highest at 6.5% and 7.8% respectively. Phylum Firmicutes was highest (27%) at FR. Thaumarchaeota was the only phylum observed in the archaea domain. Although it was less than 1% in all the stages, it was highest at the FR. There was no significant difference (P=0.99) in the bacteria and archaea phylum groups across the different growth stages (Table 4.4). At P=0.01, R=0.58 the beta diversity showed a significant difference across the growth stages.

Table 4.2: 16S Metagenomic sequence information for maize rhizosphere across different growth stages

SAMPLE	DATA BEFORE QC					DATA AFTER QC			
	Size (bp)	**No. of raw sequence reads**	**Mean sequence length(bp)**	**Mean GC content(%)**	**No of artificial duplicate read**	**Size (bp)**	**No. of sequence reads**	**Mean sequence length (bp)**	**Mean GC content (%)**
B U1	3999 9874	93132	429 ± 60	59 ± 5	78725	554 444 7	13981	397 ± 92	59 ± 4
B U2	3395 9250	79190	429 ± 62	58 ± 5	66328	492 221 6	12368	398 ± 91	59 ± 4
B U3	3943 9263	91569	431 ± 59	58 ± 4	78070	520 903 2	13189	395 ± 94	58 ± 4

P	3476					507			
R1	5082	81200	428 ± 61	59 ± 4	68091	315 2	12759	398 ± 91	59 ± 4
P	3389					484 043			
R2	0551	78866	430 ± 57	59 ± 4	66470	5	12182	397 ± 91	59 ± 4
P	4508					596 270			
R3	8395	104815	430 ± 60	58 ± 5	89330	7	14913	400 ± 88	59 ± 4
TA	3908					538 705			
1	8384	91384	428 ± 60	58 ± 5	77224	8	13644	395 ± 91	59 ± 4
TA	3705					502 542			
2	2055	87143	425 ± 69	58 ± 7	73348	6	12809	392 ± 94	59 ± 4

						455			
TA	3145					776			
3	8263	74393	423 ± 67	58 ± 6	61962	8	11824	385 ± 95	59 ± 4
						548			
F	3572					640			
R1	8655	84510	423 ± 66	58 ± 5	69749	5	14237	385 ± 95	58 ± 4
						538			
F	3449					135			
R2	9959	81697	422 ± 68	58 ± 6	66905	8	14139	381 ± 100	58 ± 5
						508			
F	3335					882			
R3	7878	78587	424 ± 64	58 ± 5	65154	9	13101	388 ± 95	59 ± 4

BU- Bulk soil, PR- Pretasseling stage rhizosphere, TA- Tasseling stage rhizosphere, FR- Fruiting stage rhizosphere

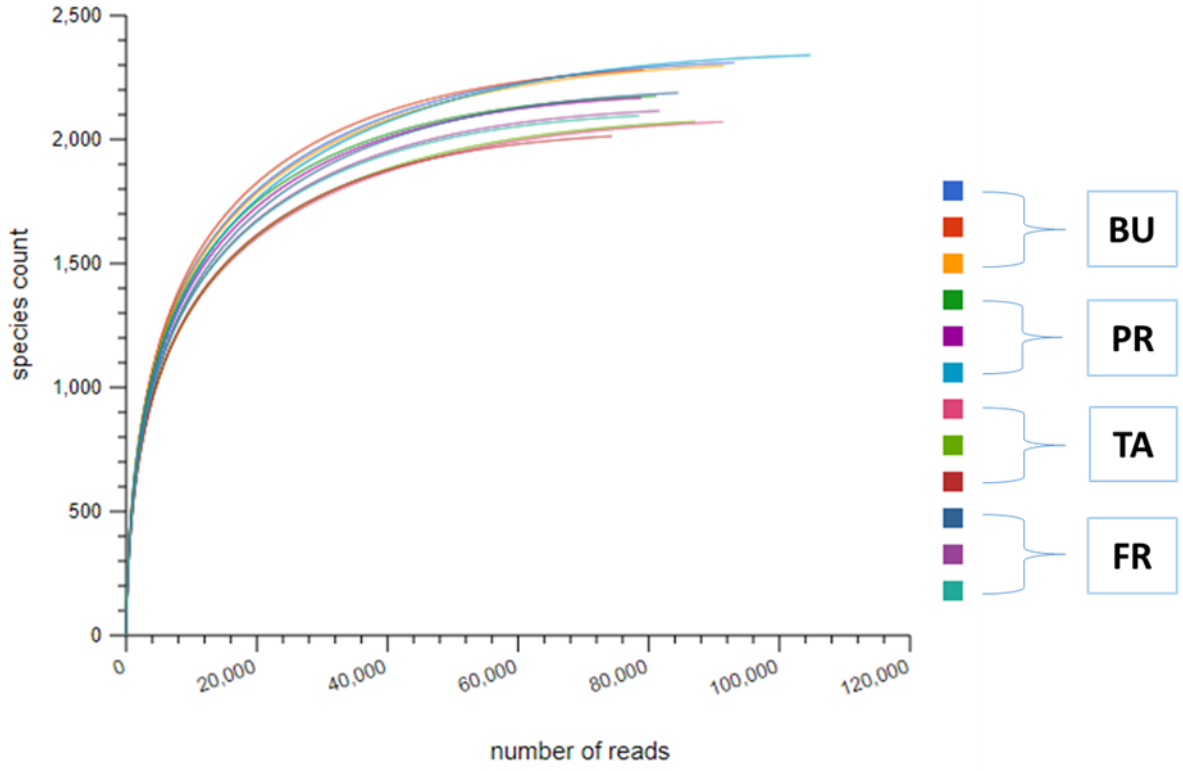

Figure 4.2: Rarefaction curve showing the richness of species sequences across the different vegetative growth. BU= samples from bulk soil, PR= samples from pretasseling growth stage, TA= samples from tassel growth stage, FR= samples from fruiting growth stage.

Table 4.3: Relative abundance (%) of the different phylum across the different growth stages

Phylum	BU	PR	TA	FR
Actinobacteria	46.053 ± 3.137	46.988 ± 3.377	41.997 ± 2.371	42.016 ± 2.183
Firmicutes	23.800 ± 4.266	24.171 ± 2.097	24.902 ± 4.510	26.752 ± 0.835
Proteobacteria	10.350 ± 0.857	9.210 ± 0.468	9.861 ± 0.797	10.066 ± 1.395
Gemmatimonadetes	4.920 ± 0.152	5.589 ± 0.651	3.664 ± 1.025	5.164 ± 0.444
Planctomycetes	3.600 ± 0.609	4.334 ± 0.795	6.452 ± 1.192	4.551 ± 0.484
Chloroflexi	2.256 ± 0.450	2.592 ± 0.418	1.527 ± 0.034	2.026 ± 0.363
Acidobacteria	2.099 ± 0.309	2.912 ± 0.726	7.837 ± 2.114	4.226 ± 1.271
Bacteroidetes	5.232 ± 6.370	2.215 ± 0.352	1.405 ± 0.094	2.322 ± 0.289
Verrucomicrobia	0.606 ± 0.128	0.853 ± 0.141	0.842 ± 0.111	1.476 ± 0.068
Nitrospirae	0.401 ± 0.036	0.414 ± 0.086	0.698 ± 0.305	0.500 ± 0.021
Cyanobacteria	0.250 ± 0.090	0.268 ± 0.070	0.107 ± 0.035	0.220 ± 0.068
Spirochaetes	0.176 ± 0.067	0.194 ± 0.041	0.366 ± 0.309	0.400 ± 0.096
Deinococcus-Thermus	0.123 ± 0.067	0.104 ± 0.020	0.035 ± 0.012	0.079 ± 0.011
Chlamydiae	0.040 ± 0.018	0.041 ± 0.014	0.055 ± 0.015	0.075 ± 0.043

Thermotogae	0.038 ± 0.003	0.050 ± 0.014	0.178 ± 0.018	0.073 ± 0.005
Thermodesulfobacteria	0.010 ± 0.009	0.008 ± 0.004	0.004 ± 0.004	0.008 ± 0.003
Aquificae	0.015 ± 0.003	0.016 ± 0.004	0.019 ± 0.004	0.011 ± 0.002
Tenericutes	0.009 ± 0.005	0.0188 ± 0.005	0.001 ± 0.001	0.006 ± 0.003
Synergistetes	0.013 ± 0.004	0.010 ± 0.003	0.032 ± 0.019	0.018 ± 0.003
Dictyoglomi	0.004 ± 0.002	0.008 ± 0.008	0.009 ± 0.005	0.004 ± 0.002
Deferribacteres	0.002 ± 0.002	0.001 ± 0.001	0.003 ± 0.001	0.002 ± 0.000
Chlorobi	0.001 ± 0.001	0.002 ± 0.001	0.004 ± 0.002	0.002 ± 0.002
Elusimicrobia	0.001 ± 0.001	0.002 ± 0.002	0.002 ± 0.002	0.001 ± 0.002
Fibrobacteres	0.001 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.002
Thaumarchaeota	0.001 ± 0.001	0.003 ± 0.002	0.002 ± 0.002	0.004 ± 0.003

BU- Bulk soil, PR- Pretasseling stage rhizosphere, TA- Tasseling stage rhizosphere, FR- Fruiting stage rhizosphere. Mean ± standard deviation (n=3).

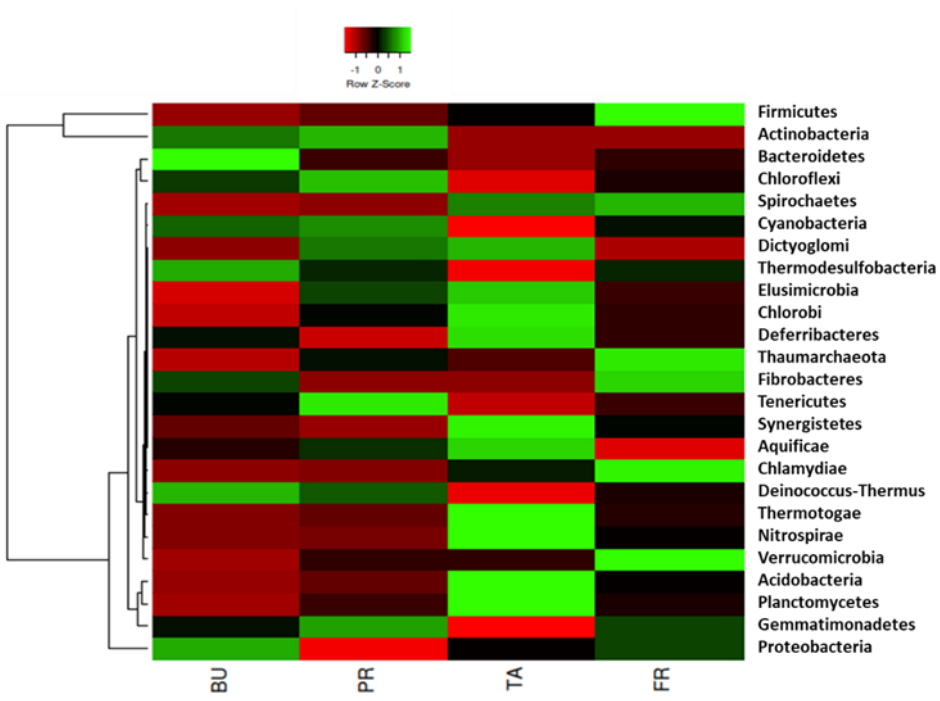

Figure 4.3: Heatmap showing the relative abundance of bacteria and archaea at each growth stage. Z- score with the scale bar shows the gradient of color saturation representing the relative abundance of the organisms. BU= samples from bulk soil, PR= samples from pretasseling growth stage, TA= samples from tasseling growth stage, and FR= samples from fruiting growth stage.

Table 4.4: Evaluation of evenness and diversity of bacteria and archaea across different growth stages.

	BU	PR	TA	FR	P-value
Phylum					
Simpson_1-D	0.71 ± 0.06	0.71 ± 0.07	0.74 ± 0.07	0.73 ± 0.06	0.99
Shannon_H	1.62 ± 0.19	1.61 ± 0.18	1.69 ± 0.19	1.69 ± 0.19	
Evenness_e^H/S	0.20 ± 0.10	0.21 ± 0.11	0.23 ± 0.10	0.22 ± 0.11	

The p-value is based on Kruskal-wallis test. mean ± standard error (n=3).

4.3.3 Taxonomic Profiling of Nitrifying Bacteria and Archaea Inhabiting Maize Rhizosphere Across Different Vegetative Growth Stages

At the genus level, 9 groups of nitrifying bacteria and 1 group of archaea were identified (Table 4.5 and Figure 4.4). *Nitrospira* groups are the most abundant with their relative abundance highest at the TA stage 67.94%. *Nitrosospira* and unclassified (derived from *Nitrosomonadales and Nitrosomonadaceae*) were also notably abundant. Figure 4.5. shows the principal component analysis (PCA) conducted to reveal how the nitrifying bacteria and archaea were distributed at the various growth stages**.** Nitrospirae is in close association with Thermotogae and Synergistetes (Figure 4.5A). The identified genus was widely distributed and dominated different vegetative growth stages (Figure 4.5B).

Table 4.5: Relative abundance (%) of nitrifying bacteria and archaea at genus level at the different growth stages

Genus	BU	PR	TA	FR
Nitrospira	66.82 ± 5.34	65.20 ± 2.27	67.94 ± 7.85	63.6 ± 2.22
Nitrosospira	12.56 ± 1.14	13.30 ± 0.38	9.67 ± 4.03	11.62 ± 1.47
Unclassified^	12.10 ± 3.18	11.75 ± 1.64	14.50 ± 1.73	15.54 ± 2.95
Unclassified*	2.67 ± 1.43	2.63 ± 0.32	0.89 ± 0.58	1.77 ± 0.14
Nitrobacter	2.10 ± 0.90	2.49 ± 1.22	3.81 ± 2.22	3.28 ± 0.40
Nitrosovibrio	2.01 ± 0.26	2.20 ± 0.81	1.65 ± 0.18	1.52 ± 0.30
Nitrosomonas	1.12 ± 0.59	0.87 ± 0.49	0.43 ± 0.13	1.10 ± 0.50
Candidatus Nitrososphaera	0.18 ± 0.16	0.42 ± 0.37	0.17 ± 0.15	0.56 ± 0.35
Nitrosococcus	0.17 ± 0.29	0.00 ± 0.00	0.37 ± 0.37	0.63 ± 0.47
Nitrococcus	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.18	0.08 ±0.13

BU- Bulk soil, PR- Pretasseling stage rhizosphere, TA- Tasseling stage rhizosphere, FR- Fruiting stage rhizosphere. Mean ± standard deviation (n=3). ^D*erived from Nitrosomonadales.* **D*erived from *Nitrosomonadaceae.*

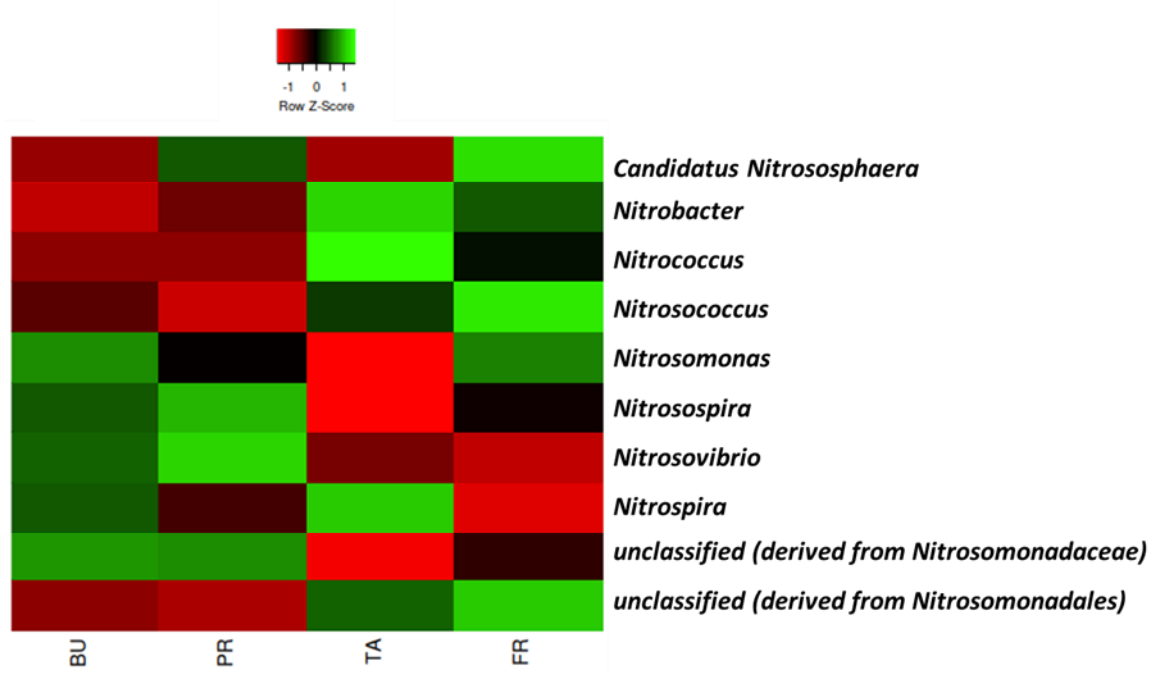

Figure 4.4: Heatmap showing list and relative abundance of nitrifying bacteria and archaea genera. Z- score with the scale bar shows the gradient of color saturation representing the relative abundance of the organisms.

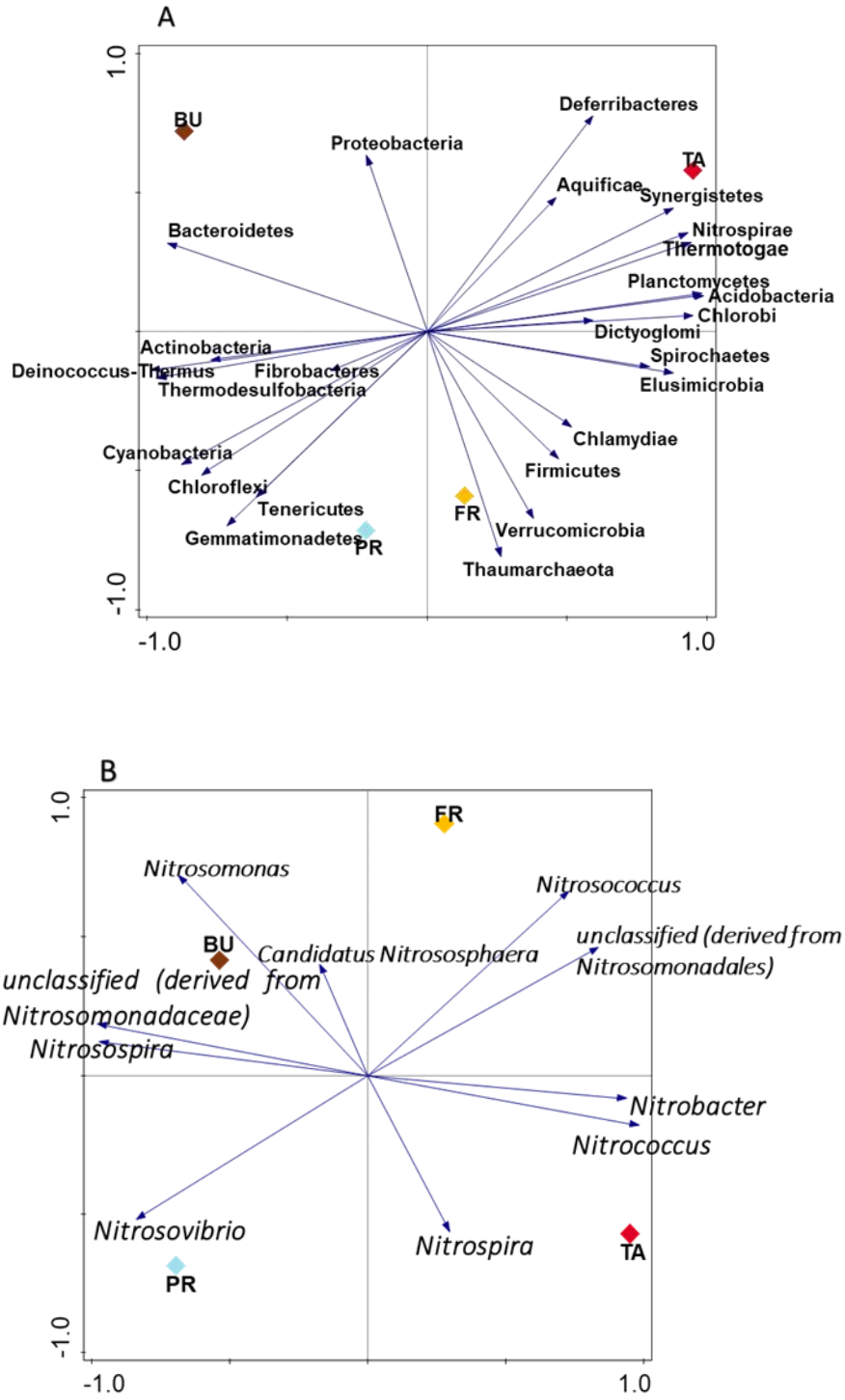

Figure 4.5: Principal Component Analysis (PCA) of nitrifying bacteria and archaea group 16S metagenomics sequence. The resultant vector showed the structural shift and the influence of nitrifying bacteria and archaea. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. [A; Phylum level axis 1 (83%), axis 2 (11%). B; Genus level axis 1 (69%), axis 2(21%)]

4.3.4 Assessment of Nitrifying Bacteria and Archaea Diversity Across Different Growth Stages

The diversity indices, Simpson, Shannon, and Evenness were used to evaluate alpha diversity of nitrifying bacteria and archaea across different groups. At P=0.99 the different genera groups showed no significant difference (Table 4.6). Beta diversity showed a significant difference (P=0.01; R=0.58) among the genera across the different growth stages. Principal coordinate analysis (PCoA) showed a distinct diversity exists across the different growth stages (Figure 4.6).

Table 4.6: Alpha diversity evaluation of nitrifying bacteria and archaea across different growth stages.

Diversity indices	BU	PR	TA	FR	P-value
Genus					
Simpson_1-D	0.52 ± 0.07	0.54 ± 0.11	0.51 ± 0.10	0.56 ± 0.10	0.99
Shannon_H	1.13 ± 0.19	1.19 ± 0.22	1.09 ± 0.20	1.21 ± 0.21	
Evenness_e^H/S	0.31 ± 0.10	0.33 ± 0.13	0.27 ± 0.12	0.28 ± 0.12	

The p-values are based on Kruskal-wallis test. Mean ± standard error (n=3).

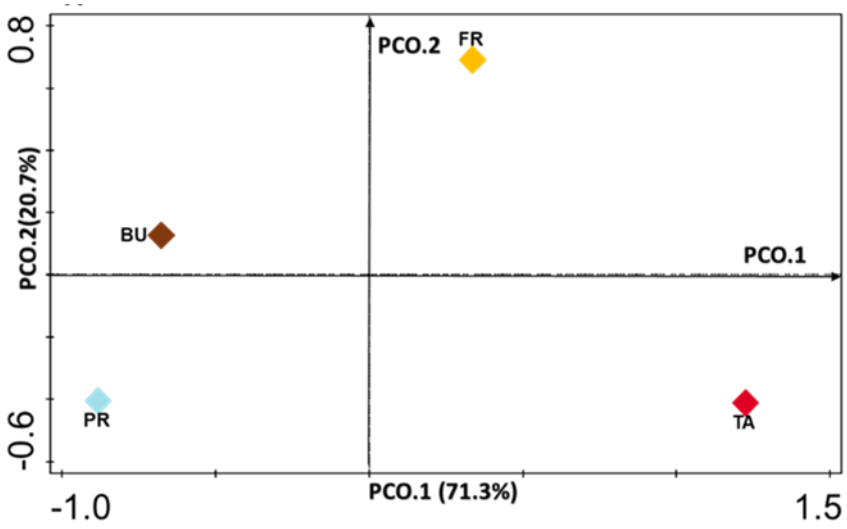

Figure 4.6: Principal coordinate analysis (PCoA) of nitrifying bacteria and archaea genera across different growth stages.

4.3.5 Relationship Among Maize Rhizosphere Environmental Factors and their Influence on Nitrifying Microorganism

The Pearson's correlation coefficient showed both positive and negative correlation among the physical and chemical parameters (Table 4.7). A significant positive and negative relationship was observed among some of the environmental factors. Notable is the relationship between sulfur and pH, organic carbon, total carbon. Also, between total carbon and pH, organic carbon, organic matter. Furthermore, between organic matter and total carbon, total nitrogen, pH, sulfur, organic carbon, total carbon, organic matter. Ammonium and sulfur, nitrate and pH, sulfur, organic carbon, total carbon, organic matter, total nitrogen also showed significant positive relationship.

Table 4.7: Pearson's correlation coefficient (r) matrix analysis shows the relationship among maize rhizosphere environmental factors.

Variables	SA	CL	pH	S	OC	TC	OM	TN	NH4	NO3	NB
SA	**1**										
CL	-1.00	**1**									

pH	**-0.81**	**0.81**	**1**								
S	**-0.60**	**0.60**	**0.51**	**1**							
OC	**-0.84**	**0.84**	0.10	**0.54**	**1**						
TC	**-0.81**	**0.81**	**0.98**	0.34	**0.97**	**1**					
OM	**-0.91**	**0.91**	**0.97**	**0.65**	**0.98**	**0.94**	**1**				
TN	**-0.74**	**0.74**	**0.98**	**0.62**	**0.98**	**0.91**	**0.95**	**1**			
NH4	0.27	-0.27	0.06	**0.53**	0.06	-0.13	0.04	0.27	**1**		
NO3	**-0.88**	**0.88**	**0.72**	**0.90**	**0.76**	**0.62**	**0.86**	**0.75**	0.16	**1**	
NB	0.22	-0.22	**-0.59**	0.38	**-0.55**	**-0.71**	-0.41	-0.49	0.31	0.12	**1**

Abbreviation of parameters are detailed in Table 4.1. r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$. Significant values in bold.

4.3.6 Influence of Maize Rhizosphere Environmental Factors on Nitrifying Bacteria and Archaea

Table 4.8 showed that a substantial number of the environmental factors had both positive and negative correlations on the nitrifying community. A significant positive and negative relationship was observed between some of the nitrifying microorganism and some environmental factors. The relationship varies among the different group of nitrifiers. pH, organic carbon, total nitrogen, and nitrate was observed to have a close relationship with *Nitrosospira*, unclassified Nitrosomonadaceae, *Nitrosovibrio*, and *Nitrosomonas.* Total carbon showed a close relationship with unclassified Nitrosomonadacea, *Nitrosovibrio*, and *Nitrosomonas*. Also, Organic matter showed a close relationship with *Nitrosospira*, *Nitrosovibrio*, and *Nitrosomonas. Nitrosomonas* and *Nitrosococcus* showed a close relationship with Ammonium.

Table 4.8: Pearson's correlation coefficient (r) matrix analysis shows the influence of environmental factors and nitrifying bacteria.

Variables	SA	CL	Ph	S	OC	TC	OM	TN	NH4	NO3
Nitrospira	-0.07	0.07	-0.49	0.29	-0.44	**-0.53**	-0.28	-0.49	-0.16	0.22
Nitrosospira	**-0.84**	**0.84**	**0.98**	0.37	**0.97**	1.00	**0.95**	**0.91**	-0.15	**0.66**
Unclassified^	**0.97**	**-0.97**	**-0.65**	**-0.51**	**-0.68**	**-0.67**	**-0.78**	**-0.55**	0.43	**-0.82**
Unclassified*	**-0.90**	**0.90**	**0.98**	**0.57**	**0.99**	**0.96**	1.00	**0.95**	-0.02	**0.81**
Nitrobacter	**0.94**	**-0.94**	**-0.92**	**-0.74**	**-0.94**	**-0.87**	**-0.99**	**-0.91**	-0.06	**-0.93**
Nitrosovibrio	**-0.95**	**0.95**	**0.67**	**0.37**	**0.70**	**0.73**	**0.77**	**0.54**	**-0.54**	**0.73**
Nitrosomonas	-0.41	0.41	**0.84**	**0.54**	**0.82**	**0.74**	**0.76**	**0.92**	**0.56**	**0.52**
Candidatus Nitrososphaera	0.20	-0.20	0.33	**-0.50**	0.28	0.41	0.11	0.30	0.01	-0.41
Nitrosococcus	**0.88**	**-0.88**	-0.49	-0.31	**-0.52**	**-0.55**	**-0.61**	-0.35	**0.63**	**-0.66**
Nitrococcus	**0.86**	**-0.86**	-1.00	**-0.53**	-1.00	**-0.98**	**-0.99**	**-0.97**	-0.01	**-0.76**

Abbreviation of parameters are detailed in Table 4.1. r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$. Significant figures in bold. ^D*erived from Nitrosomonadales.* **D*erived from Nitrosomonadaceae.*

4.4 Discussions

This study profiled the nitrifying bacteria and archaea associated with maize rhizosphere and evaluated their diversity across different growth stages. Also, the environmental factors were analysed and correlated with the nitrifying community. The pH is seen to be moderately acidic (5.93) according to USDA (2014) characterization. This could be as a result of the high level of sulfur noted (336 mg/kg). Agrochemicals have been culprit to high level of sulfur in farmlands (Burkitbayev et al., 2021). Sulfur is said to increase the acidity of soil when at a high level (Wang et al., 2008). The ratio of carbon to nitrogen (9:1) is slightly higher than USDA (2014) recommendation (8:1). Also, the NH_4 to NO_3 ratio (1:1.4) falls short of

expectation; Liu et al. (2017) suggested a ratio of 1:3 for the soil microorganism. The holistic physical and chemical parameter sustained the proliferation of nitrifying community with an average of 0.5% relative abundance (Table 4.3). Kong et al. (2018) report a favorable pH of 7.0 to 7.5 for nitrifying bacteria.

Nitrifying bacteria and archaea are ubiquitous and are found in varying environmental conditions. Some have been successfully used as biofertilizer in the form of single strains (Doost et al., 2019) and in a consortium (Vatandoost et al., 2019). The 9 genera of nitrifying bacteria identified in this study are *Nitrospira, Nitrosospira, unclassified (derived from Nitrosomonadales), unclassified (derived from Nitrosomonadaceae), Nitrobacter, Nitrosovibrio, Nitrosomonas, Nitrosococcus, Nitrococcus.* The order Nitrosomonadaceae and Nitrosomonadales still have unclassified and yet to be cultured bacterium species that are likely to be nitrifying bacteria. The only archaea genus discovered was *Candidatus Nitrososphaera*, which carry out ammonia oxidation (Zhalnina et al., 2014), it had also been reported by Melnichuk et al. (2020) and Enebe and Babalola (2021a) to be associated with crops including maize. There were fewer nitrite oxidizing bacteria genera than ammonia genera, however, the relative abundance of nitrite oxidizing genera were more (Table 4.5). The genera specification and proliferation could have accounted for the high level of nitrite than ammonia (Table 4.1).

Ammonia oxidizing bacteria noted in this study were *Nitrosospira, Nitrosomonas, Nitrosococcus* (Schaechter, 2009) and *Nitrosovibrio* (Fu et al., 2020c). *Nitrosomonas* was recently discovered in maize rhizosphere soil in low abundance by Wang et al. (2021). The nitrite oxidizing bacteria carrying out the second stage of nitrification were the genus *Nitrospira*, *Nitrobacter* and *Nitrococcus* (Schaechter, 2009). *Nitrospira* is known to be well distributed globally and was found to be most abundant. It was recently observed by Sun et al. (2021) in a maize rhizosphere. Also, *Nitrobacter* was noted in a maize-soybean rotation

system by Meier et al. (2021). Unclassified nitrifying microorganisms were seen in the order *Nitrosomonadaceae* and order *Nitrosomonadales.* This affirms the possible presence of novel nitrifying bacteria in the studied maize rhizosphere. Stein (2019), mentioned there has been an increasing number of novel nitrifying microorganisms discovered lately. This could be as a result of advanced technologies used in sequencing and sampling different soils.

Schlemper et al. (2017) affirm the existence of variation in bacteria population across different growth stages. The rarefaction curve shows that each of the growth stages had high and unequal number of species diversity (Figure 4.2.). The PCoA plot showed a distinct diversity and gap across the growth stages (Figure 4.6). The phylum Nitrospirae, which had the most abundant nitrifying bacteria showed an increase from the BU to the TA and a decrease at the FR (Table 4.3). Also, *Nitrospira* genus was most abundant at the TA stage. This could be because of increasing demand of nutrient as the plant increase in growth. According to Rocha et al. (2020), the abundance of microorganisms associated with nitrification increases with increasing developmental stages. Furthermore, Lu et al. (2018) explained that the increased and prolonged availability of nitrogen in the rhizosphere by nitrifying microorganisms delayed flowering.

The heatmap showed that all the nitrifying bacteria genus were unequally distributed across the different growth stages (Figure 4.5). It was also observed, in the overall microbial community of a study carried out by Fu et al. (2020a) at varying maize growth stages. This would probably be due to the varying composition of nutrients at the different growth stages. Although, the alpha diversity showed no significant difference. However, there was a significant difference ($P=0.01$) in the beta diversity of the different growth stages. Also, Peiffer et al. (2013) reported a significant difference between the beta diversity between maize bulk soil and rhizosphere soil. They attributed it to the maize genotype. The result obtained from the correlation affirms there is indeed a direct and indirect interlink within the

environmental factors. Also, between them and the nitrifying community, the soil physical and chemical properties showed both positive and negative correlations with a substantial number of the nitrifying community. This was also observed by Fu et al. (2020a) between microbial community and soil nutrients.

4.5 Conclusions

Profiling and diversity of nitrifying bacteria and archaea of maize rhizosphere across different growth stages were carried out. At the genus level, 9 genera of nitrifying bacteria and 1 archaeon were identified. Two out of the 9 genera were yet to be identified nitrifying bacteria from the order Nitrosomonadaceae and order Nitrosomonadales. The tasselling growth stage had the most abundant of the nitrifying bacteria. The correlation within the environmental factors shows the existence of a relationship between some parameter in the rhizosphere and it reveals possible impact or non-impact on nitrifying community. Prominent nitrifying bacteria and archaea associated with maize rhizosphere identified in this study and the understanding of soil physical and chemical properties on them can be used as a microbiome-based strategy to improve the productivity and yield of maize plants. More so, growth stages of maize should be considered in its management.

CHAPTER FIVE

RELATIONSHIP BETWEEN NITRIFYING MICROORGANISMS AND OTHER MICROORGANISMS RESIDING IN THE MAIZE RHIZOSPHERE

Abstract

The microbial network of rhizosphere is unique as a result of root exudates. Insights into the relationship that exists with the energy metabolic functional groups will help in biofertilizer production. We hypothesize that there exists a relationship between nitrifying microorganisms and other energy metabolic functional microbial groups in the maize rhizosphere across different growth stages. Nucleospin soil DNA extraction kit was used to extract DNA from soil samples collected from maize rhizosphere. The 16S metagenomics sequencing was carried out on Illumina Miseq. The sequence obtained was analyzed on MG-RAST. *Nitrospira* genera were the most abundant in the nitrifying community. Nitrifying microorganisms were more than each of the studied functional groups except for nitrogen-fixing bacteria. Also, majority of the microorganisms were noticed at the fruiting stage and there was variation in the microbial structure across different growth stages. The result showed that there exists a substantial amount of both negative and positive correlation within the nitrifying microorganisms, and between them and other energy metabolic functional groups. The knowledge obtained from this study will help improve the growth and development of maize through modification of the rhizosphere microbial community structure.

Keywords: Predictive functional analysis; root exudate; nitrogen-fixing bacteria; methane oxidizing bacteria; carbon fixation.

5.1 Introduction

The complexity of the microbial network in rhizosphere has become unique over time when compared to the surrounding bulk soil as plants grow. Aside from exudates produced by plants, which is one of the causes (Peiffer et al., 2013), the secretion and detection of signaling compounds are usually produced between microbes, from plants to microbes, and from microbes to plants (Venturi and Keel, 2016). These account for the different functional gene diversity between bulk soil and the rhizosphere, with the latter harboring many gene copies and different functional genes than bulk soils (Pascual et al., 2018). The signal from plants to microorganisms via small plant-secreted molecules allows microbial communities to form and synchronize (Venturi and Keel, 2016). This has been implicated in several specialized relationships and most probably occurs frequently in other interactions.

There are several functional groups in the soil microorganisms associated with energy metabolism. These groups of microorganisms obtain energy through the absorption of nutrients. One of such group is the nitrifying microorganisms, which make use of ammonia to produce nitrate. Another is the nitrogen fixers, which converts atmospheric nitrogen into ammonia (Wagner, 2011). The sulfur reducing bacteria group makes use of sulfate to produce hydrogen sulfide (Myhr and Torsvik, 2000) while carbon fixing bacteria groups carry out oxygenic photosynthesis using carbon as an electron source (Stanier and Cohen-Bazire., 1977). Also among the functional groups are the methane oxidizing bacteria, which make use of methane as their energy source (Anthony, 1983).

Previous studies have proven that there is a form of relationship between these functional groups and nitrifying bacteria (Peng et al., 2020, Zhang et al., 2020, Cao et al., 2021). A significant correlation was observed between ammonia oxidizing bacteria that carry out the first stage of nitrification and nitrogen fixation in a litter composition (Torres et al., 2005). Rocha et al. (2020) also noted that nitrogen fixers associated with grass belonging to the

genus *Urochloa* had a positive correlation with ammonium (a substrate for nitrification). The activities of nitrifiers correlate positively with carbon oxidizers. Also, relationship was observed between methane oxidation and nitrifiers (Qin and Lin, 2019). According to Costa et al. (2019), both organisms have similar enzymes. Sulfur reducing bacteria increase significantly with the presence of nitrifiers because they suppress the activity of denitrifiers that affect their growth (Peng et al., 2020).

The action of suppressing the activity of growth of another organism is known as antagonism. Saravanakumar et al. (2017) reported an antagonistic activity between some soil isolates and disease-causing microorganisms associated with maize plants. Microorganisms acquire antagonistic character as a result of nutrient competition, metabolic and antibiotic production (Singh and Faull, 2020). Nanjundappa et al. (2019) reported the synergistic effect of Arbuscular mycorrhizal fungi and *Bacillus sp.* They observed that together they produce resistance to soil microbial pathogens and higher tolerance to environmental stress. The combination of microorganisms can improve plant growth, replace synthetic fertilizer, and is useful for sustainable agriculture (Alori et al., 2017, Rafi et al., 2019).

Exploring the relationships among these groups could be insightful and offer a better understanding of their activities. DNA sequencing technologies have been widely used by researchers to communicate trends in microbial communities (Fadiji and Babalola, 2020, Enebe and Babalola, 2021b). Wang et al. (2019a) evaluated the antagonist effect of *Paenibacillus jamilae* on some plant pathogens using 16S metagenomic sequence data, and a significant reduction in the pathogens was observed. Using OTU sequences, *Pantoca agglomerans* was observed to have an antagonistic effect on *Alternaria sp.* (Links et al., 2014). Although the sequence obtained from the 16S metagenome is known to have the shortcoming of not being able to evaluate functional genes. However, to overcome the

limitations, software such as Picrust (Langille et al., 2013), Tax4Fun (Aßhauer et al., 2015) and Vikodak (Nagpal et al., 2016) have been developed. They are used to predict functions based on evolution in taxonomic profiles. This approach cannot be efficiently replaced by profiling using whole metagenome sequences, but it is a useful substitute where cost needs to be considered. The actions are carried out by predicting functional capabilities through marker genes.

Furthermore, correlation among microbial organisms can show an antagonistic or synergistic effect. It is one of the statistics used to evaluate the possibility of a relationship between two variables. Also, it can be used to evaluate the relationship between microorganisms (Zhang et al., 2020) and their genes (Ma et al., 2018). A genetic correlation network carried out by Ma et al. (2018) using genes derived from metagenomics analysis showed both positive and negative correlations. They further explained that a positive mapped correlation indicates functional associations, whereas a negative correlation indicates a regulatory process. The functional association can be viewed as a synergistic effect while the negative regulatory process could be observed as an antagonistic relationship.

The soil environment is very complex and the networking within the system is highly diverse with varying and similar functions (Enebe and Babalola, 2021b). The distinct functional groups have a relationship within and between their communities. There are uncertainties in the relationship between nitrifying microorganisms and other functional groups.

Understanding the association between microorganisms associated with carbon, nitrogen, and sulfur could facilitate the sustainable growth of maize and alleviate environmental pollution. Microbes are an environmentally healthier alternative to synthetic fertilizer in sustainable crop production (Chukwuneme et al., 2020, Fasusi et al., 2021). The 16S metagenomics correlation between these groups of organisms has not previously been

explored. This study aims to affirm the existence of a relationship between nitrifying microorganisms and other energy metabolic functional groups. The specific objectives are to evaluate the relationship between nitrifying microorganisms and with nitrogen-fixing bacteria, carbon fixing bacteria, methane oxidizing bacteria and sulfur reducing bacteria.

5.2 Materials and Methods

5.2.1 Sampling and Site Description

Rhizosphere soil samples were collected from maize plantation of the North-West University, Molelwane, Mahikeng, South Africa (25° 47’ 24.17604” S, 25° 37’ 9.08328” E; 25° 47’ 29.97048” S, 25° 37’ 8.62428” E; 25° 47’ 23.9604” S, 25° 37’ 8.43348” E; 25° 47’ 23.82252’ S, 25° 37’ 8.30064” E; 25° 47’ 24.11844” S, 25° 37’ 8.18148” E; altitude 1012 m). Active cultivation with irrigation and fertilizer NPK (20% Nitrogen, 7% Phosphorus and 3% Potassium) application has been ongoing for 32 years. The region has a mean annual temperature ranging from 22^{o}C - 35^{o}C in summer, 2^{o}C - 20^{o}C in winter and an annual rainfall of 450 mm. Maize cultivar used is the QN.633. Bulk soil, rhizosphere from pretasseling growth stage (PR), rhizosphere from tasseling growth stage (TA) and rhizosphere from fruiting growth stage (FR) were collected between 0-15 cm depth and 0-5 cm breath of each maize root. The pretasseling growth stage was characterized by emerging maize plants without silk, while tasseling was characterized by the presence of silk without cob and the fruiting stage was identified by the presence of maize cob with grains. Soil samples were collected in triplicate, transported to the laboratory in sterile plastic bags and stored at -20^{o}C.

5.2.2 Extraction and Sequencing of DNA

Nucleospin soil DNA extraction kit was used to extract the DNA by adhering to the manufacturer's instructions. Universal primer pairs 341F (5'-CCT ACG GGN GGC WGC AG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA TCC-3') were used to target the V3 - V4 hypervariable portions of the 16S rRNA gene (Thijs et al., 2017). The amplicons were then

purified, end-repaired, and Illumina-specific adaptor sequences ligated to each of them. The samples were individually indexed after quantification, and another purification step was conducted. The amplicons were sequenced using a MiSeq v3 (600 cycles) kit on Illumina's MiSeq platform. For each experiment, 20 Mb of data (2x300 bp long paired end reads) was generated.

5.2.3 Sequence Analysis and Statistics

The MG-RAST server (http://www.mg-rast.org) was used to process and analyze the raw sequence which was uploaded as a FastQ file (Meyer et al., 2008). The quality control included filtering of ambiguous bases, removal of chimeras, minimum read specification and length filtering (Bolger et al., 2014). Following that, the sequence reads were annotated using the BLAT technique (Kent, 2002) and the M5NR database (Wilke et al., 2012). The data normalization tool was applied to reduce experimental error. Default parameters were used for the bioinformatics tools. The predictive functional analysis was performed on Nephele (https://nephele.niaid.nih.gov) using default setting (Battré et al., 2010). The abundance of bacteria and archaea community at different growth stages was evaluated. Reads of eukaryotes and unclassified sequences were removed. The generated data from the 16S metagenomics sequence before and after quality control are in the supplementary material.

The mean of the triplicate samples and the relative abundance of the microorganisms were evaluated using Microsoft excel software. The principal component analysis (PCA) was carried out using CANOCO 5 at default settings (http://www.canoco5.com). The heat map of the functional genes was carried out using online software at www.heatmapper.ca/expression. Pearson’s correlation test was analyzed on XLSTAT v 2021.

5.3 Results

5.3.1 Energy Metabolism Function Predicted Within 16S Metagenomics Sequence of Maize Rhizosphere

The relative abundance of the energy metabolic function genes is presented in Figure 5.1 and 5.2. The figures showed that nitrogen metabolism genes were present and most abundant in the bulk soil (BU). However, the overall distribution of the energy metabolic functional genes across the different growth stages showed that tasseling stage (TA) had the most energy metabolic functional genes (Figure 5.1 & 5.2). Nitrogen metabolism has a highly negative correlation with photosynthesis, a moderate negative correlation with sulfur metabolism, a highly positive correlation with methane metabolism, and a moderate positive correlation with carbon fixation (Table 5.1).

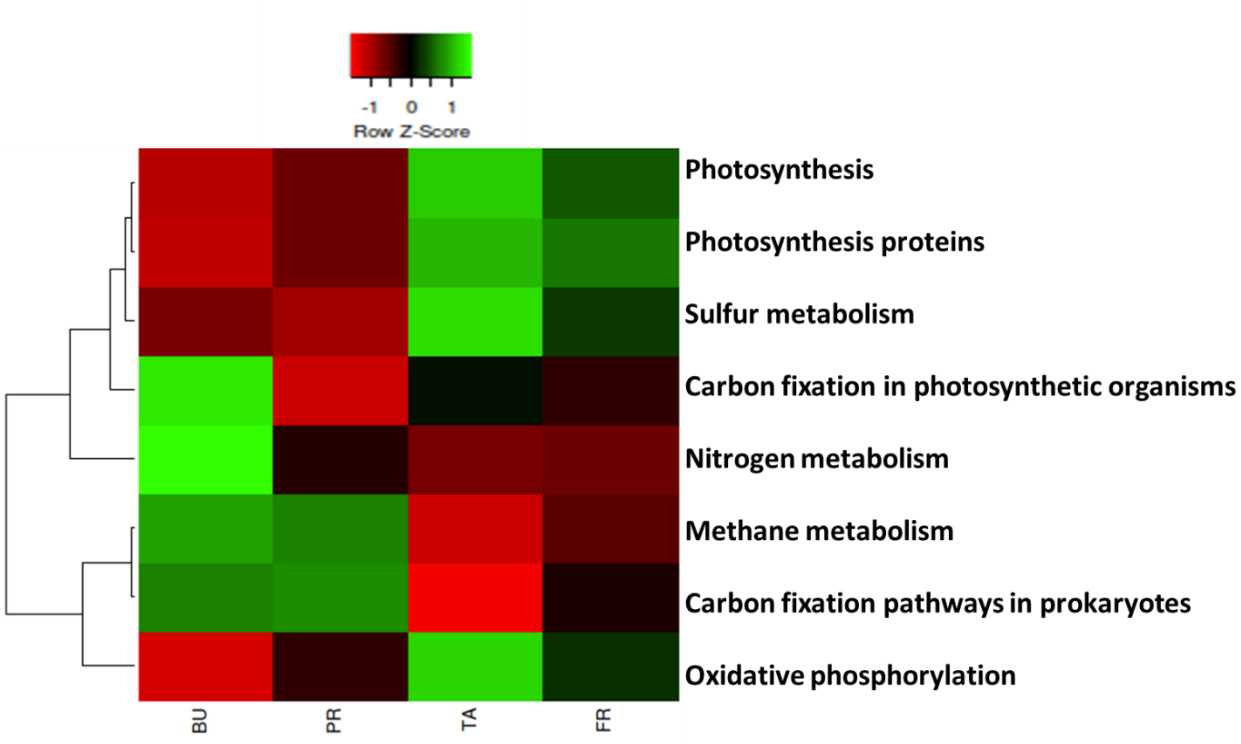

Figure 5.1: Heatmap showing list and relative abundance of energy metabolic function genes. Z- score with the scale bar shows the gradient of colour saturation representing the relative abundance of the organisms. BU- samples from bulk soil, PR- samples from

pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

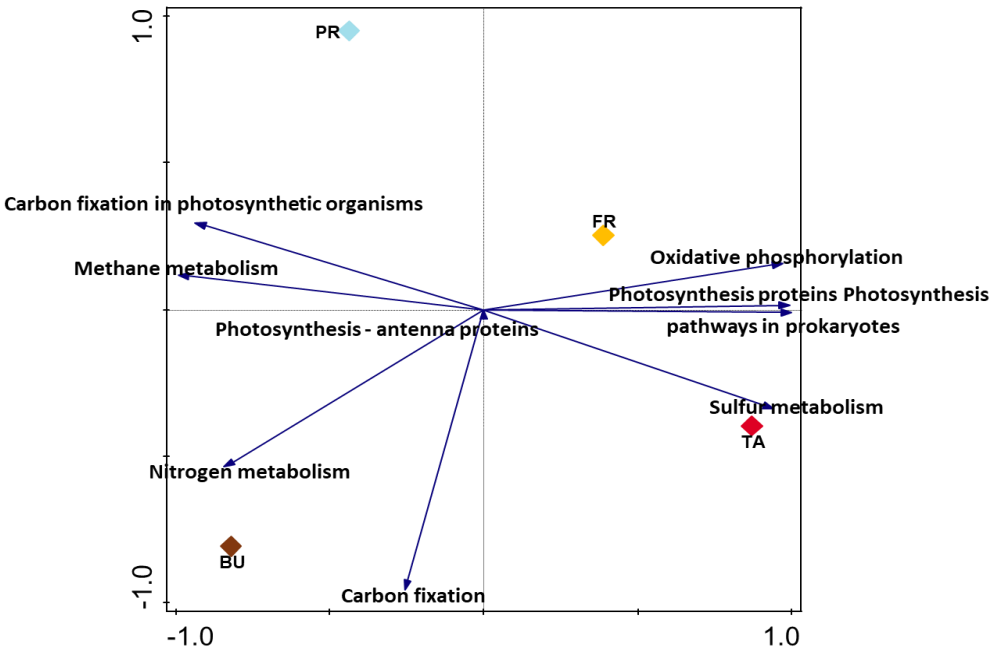

Figure 5.2: Principal Component Analysis (PCA) of energy metabolism function of 16S metagenomics sequence. The resultant vector showed the structural shift and the influence of energy metabolism functions. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (92%), Axis 2 (6%). BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

Table 5.1: Pearson's correlation coefficient (r) matrix between the energy metabolic functions.

Energy metabolic function	Carbon fixation	Methane metabolism	Nitrogen metabolism	Sulfur metabolism	Photosynthesis
Carbon fixation	**1.00**				
Methane metabolism	0.96	**1.00**			
Nitrogen metabolism	0.63	0.77	**1.00**		
Sulfur metabolism	-0.99	-0.97	-0.61	**1.00**	
Photosynthesis	-0.94	-0.99	-0.83	0.94	**1.00**

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$

5.3.2 Relationship Between Nitrifying Microorganisms and Nitrogen-fixing Bacteria

The abundance of the diversity of nitrifying microorganisms showed that *Nitrospira* were the most abundant (above 60%), *Nitrosospira*, and unclassified Nitrosomonadales were above 10% while unclassified Nitrosomonadaceae, *Nitrobacter, Nitrosovibrio, Nitrosomonas, Candidatus Nitrosophaera* were below 10% (Figure 5.3). The results of the principal component analysis showed that the fruiting stage had most of the microorganisms (Figure 5.4). In Figure 5.5 the relative abundance of nitrogen-fixing bacteria (> 80 %) was higher than nitrifying microorganisms (< 20%).

Table 5.2 shows the correlation between nitrifying microorganisms. There was both a positive and a negative correlation between the microorganisms. The very high positive correlation observed between *Nitrospira* and *Nitrobacter*, an unclassified derived from Nitrosomonadales and *Nitrobacter*, is noteworthy. A highly negative correlation was observed between *Nitrobacter* and *Nitrosospira,* unclassified Nitrosomonadaceae and unclassified Nitrosomonadales, *Nitrosovibrio* and unclassified Nitrosomonadales. The correlation between nitrifying microorganisms and nitrogen-fixing bacteria, presented in Table 5.3, showed a high positive correlation, moderately positive correlation, highly negative correlation, and moderately negative correlation between them.

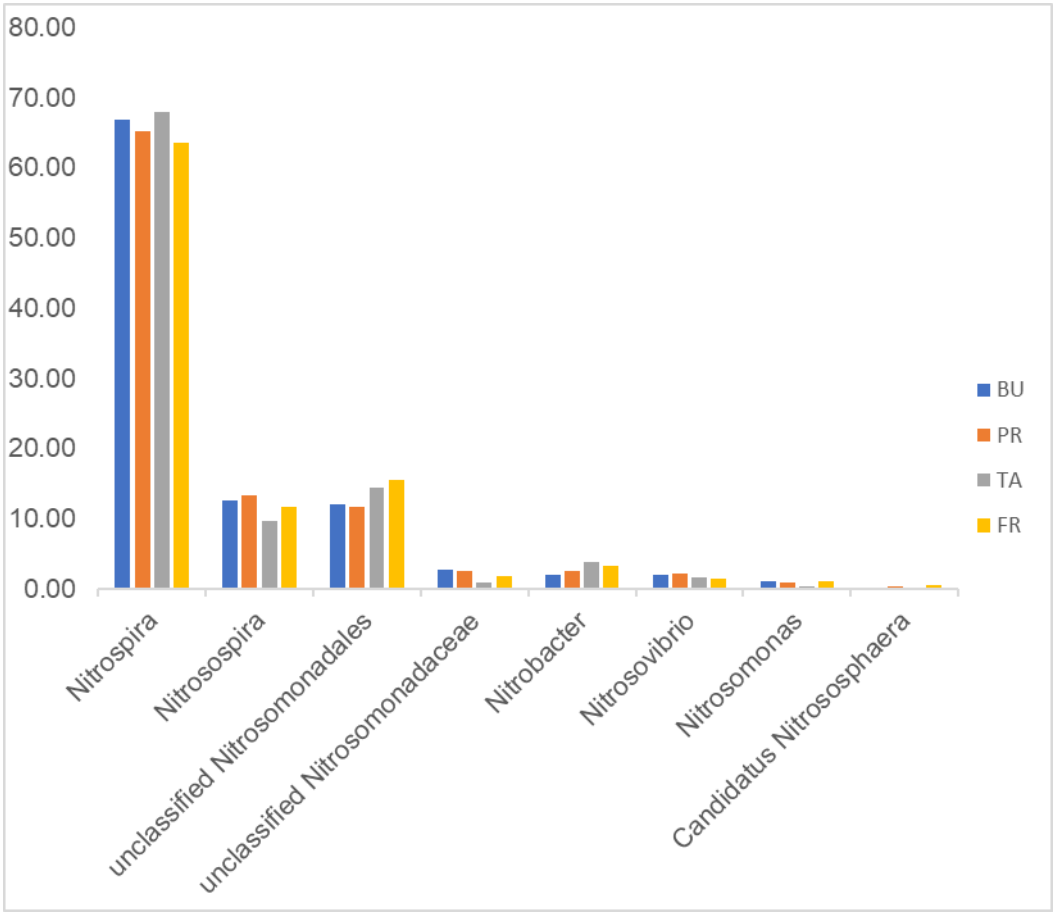

Figure 5.3: Abundance (%) of nitrifying microorganisms across the different growth stages. BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

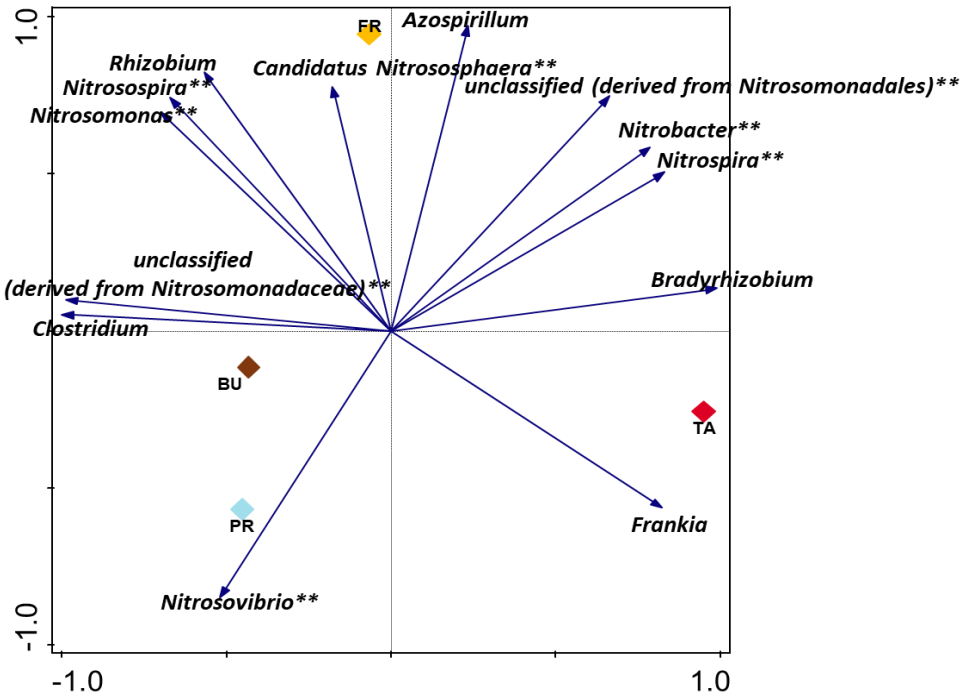

Figure 5.4: Principal Component Analysis (PCA) of nitrifying microorganisms and nitrogen-fixing bacteria. The resultant vector showed the structural shift and the influence of nitrifying microorganisms and nitrogen-fixing bacteria. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (82%), Axis 2 (17%). BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage. ** nitrifying microorganisms.

Table 5.2: Pearson's correlation coefficient (r) matrix between nitrifying microorganisms.

Nitriyng microorganisms	*Candidatus Nitrososphaera*	*Nitrobacter*	*Nitrosomonas*	*Nitrosospira*	*Nitrosovibrio*	*Nitrospira*	Unclassified Nitrosomonadaceae	Unclassified Nitrosomonadales
Candidatus Nitrososphaera	**1.00**							
Nitrobacter	0.13	**1.00**						
Nitrosomonas	0.50	-0.53	**1.00**					
Nitrosospira	0.54	-0.76	0.82	**1.00**				
Nitrosovibrio	-0.61	-0.50	-0.46	-0.02	**1.00**			
Nitrospira	-0.30	0.90	-0.64	-0.95	-0.31	**1.00**		
Unclassified Nitrosomonadaceae	0.07	-0.98	0.68	0.88	0.34	-0.96	**1.00**	
Unclassified Nitrosomonadales	0.31	0.94	-0.22	-0.57	-0.77	0.80	-0.86	**1.00**

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$

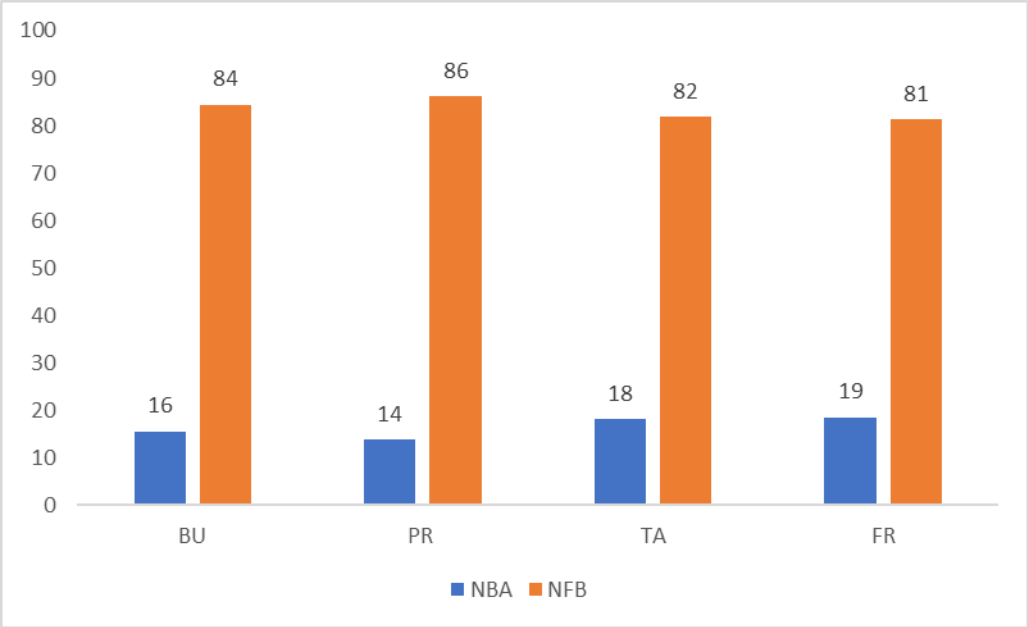

Figure 5.5: Relative abundance (%) of nitrifying microorganisms and nitrogen-fixing bacteria across the different growth stages. NBA- Nitrifying Microorganisms, NFB- Nitrogen-fixing Bacteria, BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

Table 5.3: Pearson's correlation coefficient (r) matrix between nitrifying microorganisms and nitrogen-fixing bacteria.

Nitrogen-fixing bacteria / Nitrifying microorganisms	*Azospirillum*	*Frankia*	*Rhizobium*	*Bradyrhizobium*	*Clostridium*
Candidatus Nitrososphaera	0.75	-0.55	0.77	-0.12	0.14
Nitrobacter	0.74	0.35	0.11	0.82	-0.82
Nitrosomonas	0.54	-0.97	0.95	-0.65	0.65
Nitrosospira	0.61	-0.96	0.98	-0.60	0.61
Nitrosovibrio	-0.94	0.02	-0.48	-0.57	0.56
Nitrospira	0.64	0.44	-0.01	0.88	-0.87
Unclassified Nitrosomonadaceae	-0.13	-0.88	0.56	-0.98	0.98
Unclassified Nitrosomonadales	0.88	0.13	0.34	0.69	-0.69

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$

5.3.3 Relationship Between Nitrifying Microorganisms and Carbon Fixing Bacteria

Principal component analysis in Figure 5.6 shows the distribution of energy metabolic functional genes across the different growth stages. The result showed that the fruiting stage had most of the microorganisms. The relative abundance of nitrifying microorganisms (> 70%) in Figure 5.7 was greater than that of carbon-fixing bacteria (30%). The correlation between nitrifying microorganisms and carbon fixing bacteria showed that there was both a positive and a negative correlation between them (Table 5.4).

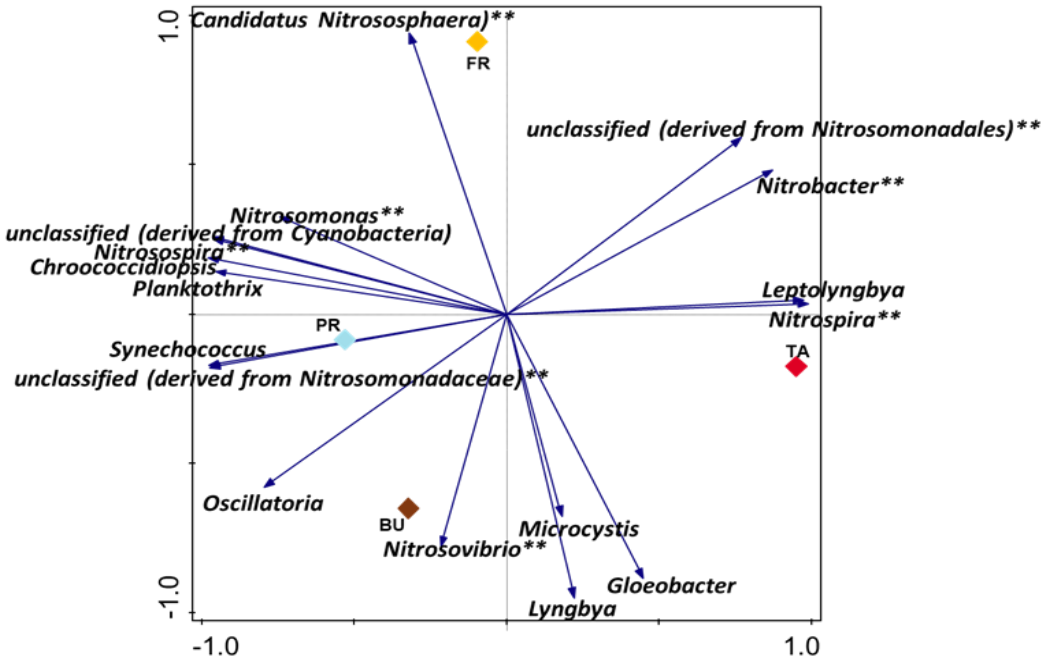

Figure 5.6: Principal Component Analysis (PCA) of nitrifying microorganisms and carbon fixing bacteria. The resultant vector showed the structural shift and the influence of nitrifying microorganisms and carbon fixing bacteria. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (81%), Axis 2 (14%). BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage. ** nitrifying microorganisms.

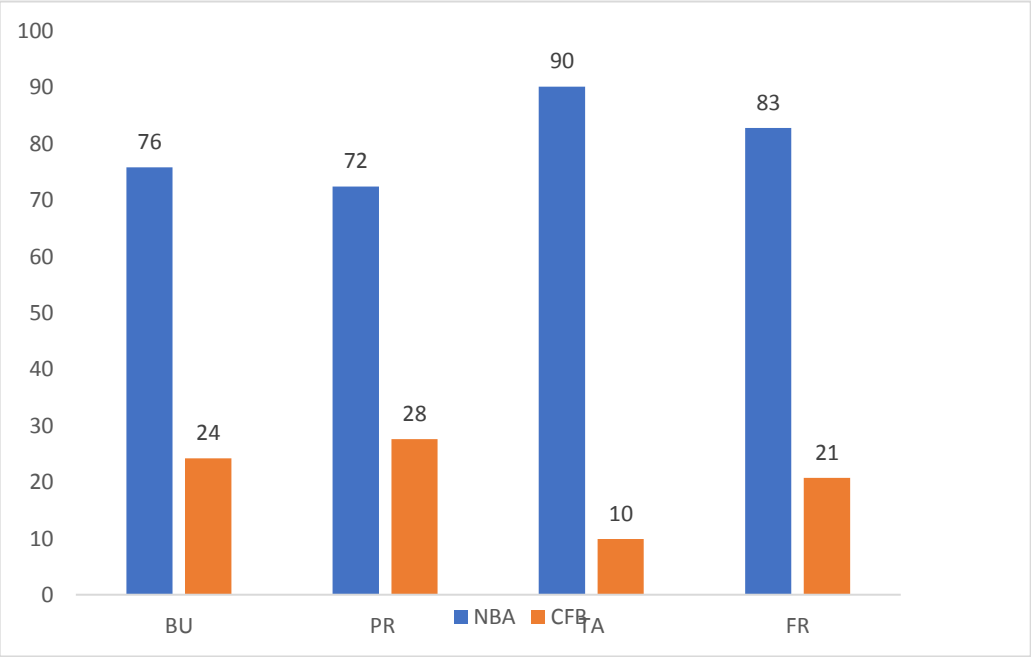

Figure 5.7: Relative abundance (%) of nitrifying microorganisms and carbon fixing bacteria across the different growth stages. NBA- Nitrifying Microorganisms, CFB- Carbon Fixing Bacteria, BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

Table 5.4: Pearson's correlation coefficient (r) matrix between nitrifying Microorganisms and carbon fixing bacteria

Carbon fixing bacteria / Nitrifying microorganisms	*Chroococcidiopsis*	*Gloeobacter*	*Leptolyngbya*	*Lyngbya*	*Microcystis*	*Oscillatoria*	*Planktothrix*	*Synechococcus*	Unclassified Cyanobacteria
Candidatus Nitrososphaera	0.48	-0.99	-0.27	-0.98	-0.75	-0.30	0.39	0.03	0.55
Nitrobacter	-0.77	0.03	0.90	-0.23	-0.23	-0.95	-0.82	-0.98	-0.71
Nitrosomonas	0.60	-0.56	-0.82	-0.32	0.11	0.24	0.90	0.66	0.59
Nitrosospira	0.95	-0.67	-0.95	-0.43	-0.25	0.59	0.96	0.86	0.94
Nitrosovibrio	0.23	0.49	-0.11	0.51	0.08	0.75	-0.05	0.35	0.18
Nitrospira	-0.97	0.45	0.95	0.22	0.18	-0.81	-0.91	-0.94	-0.95
Unclassified Nitrosomonadaceae	0.86	-0.23	-0.97	0.04	0.12	0.87	0.92	1.00	0.81
Unclassified Nitrosomonadales	-0.69	-0.14	0.72	-0.34	-0.17	-1.00	-0.60	-0.87	-0.62

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$

5.3.4 Relationship Between Nitrifying Microorganisms and Methane Oxidizing Bacteria

The distribution of the microorganisms across the different growth stages is shown using principal component analysis. The results showed that the fruiting stage had most of the microorganisms (Figure 5.8). The relative abundance of the microorganisms is presented in Figure 5.9. It was discovered that nitrifying microorganisms (> 80%) were more than methane oxidizing bacteria (< 20%). Pearson correlation matrix of nitrifying microorganisms and methane-oxidizing bacteria is presented in Table 5.5. Both positive and negative correlations were observed among them. Some were very high and some moderate (Table 5.5).

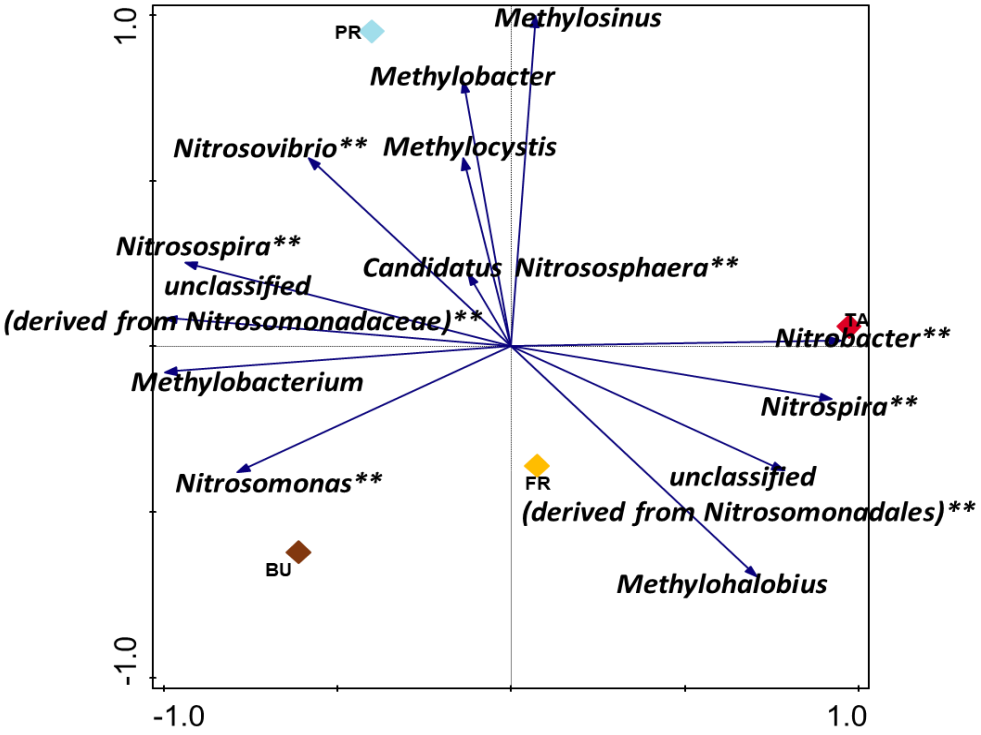

Figure 5.8: Principal Component Analysis (PCA) of nitrifying microorganisms and methane oxidizing bacteria. The resultant vector showed the structural shift and the influence of nitrifying microorganisms and methane oxidizing bacteria. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (66%), Axis 2 (23%). BU- samples from bulk soil, PR- samples from pretasselling growth stage, TA- samples from tasselling growth stage, FR- samples from fruiting growth stage. ** nitrifying microorganisms.

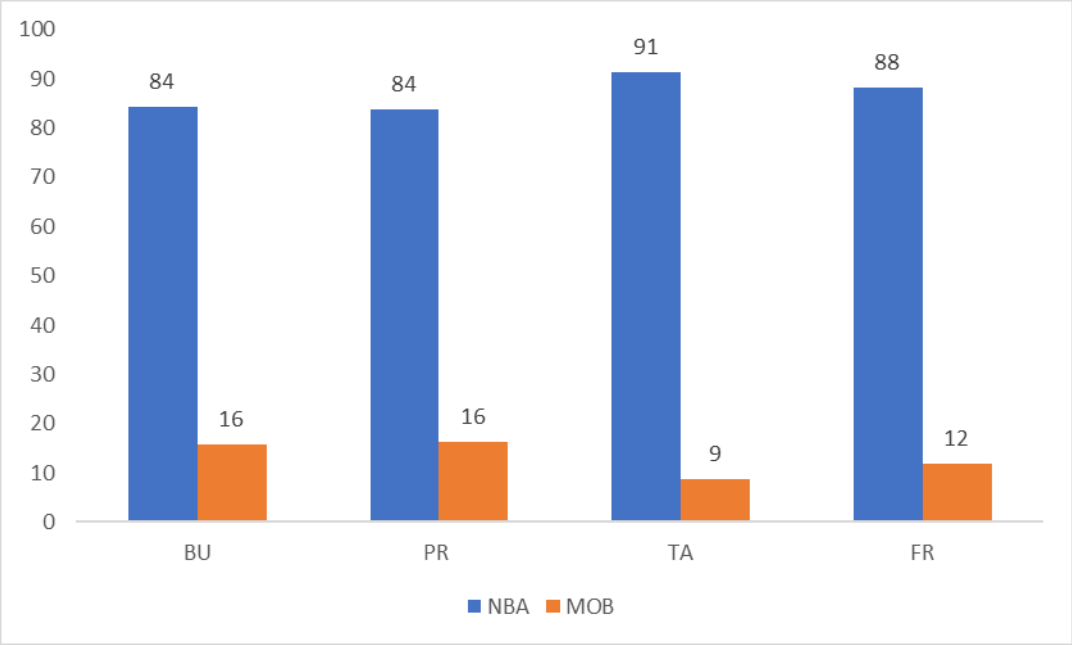

Figure 5.9: Relative abundance (%) of nitrifying microorganisms and methane oxidizing bacteria across the different growth stages. NBA- Nitrifying Microorganisms, MOB- Methane Oxidizing Bacteria, BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

Table 5.5: Pearson's correlation coefficient (r) matrix nitrifying microorganisms and methane oxidizing bacteria

Methane oxidizing bacteria / Nitrifying microorganisms	*Methylobacter*	*Methylobacterium*	*Methylocystis*	*Methylohalobius*	*Methylosinus*
Candidatus Nitrososphaera	-0.64	0.00	0.71	-0.36	0.17
Nitrobacter	-0.36	-0.98	-0.04	0.67	-0.03
Nitrosomonas	-0.52	0.76	0.07	-0.35	-0.42
Nitrosospira	0.13	0.87	0.52	-0.90	0.29
Nitrosovibrio	0.88	0.57	0.28	-0.73	0.60
Nitrospira	0.05	-0.86	-0.48	0.82	-0.15
Unclassified Nitrosomonadaceae	0.24	0.97	0.24	-0.78	0.12
Unclassified Nitrosomonadales	-0.73	-0.78	-0.17	0.74	-0.39

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$

5.3.5 Relationship Between Nitrifying Microorganisms and Sulfur Reducing Bacteria

Using principal component analysis, the microorganism distribution across the different growth stages is shown. The results showed that the fruiting stage had most of the microorganisms (Figure 5.10). The relative abundance of nitrifying microorganisms (50%) was greater than that of sulfur-reducing bacteria (50% in Figure 5.11). The Pearson correlation between nitrifying microorganisms and sulfur reducing bacteria is presented in Table 5.6. Highly positive and negative correlations, as well as moderately positive and negative correlations, were observed between the two groups of microorganisms (Table 5.6)

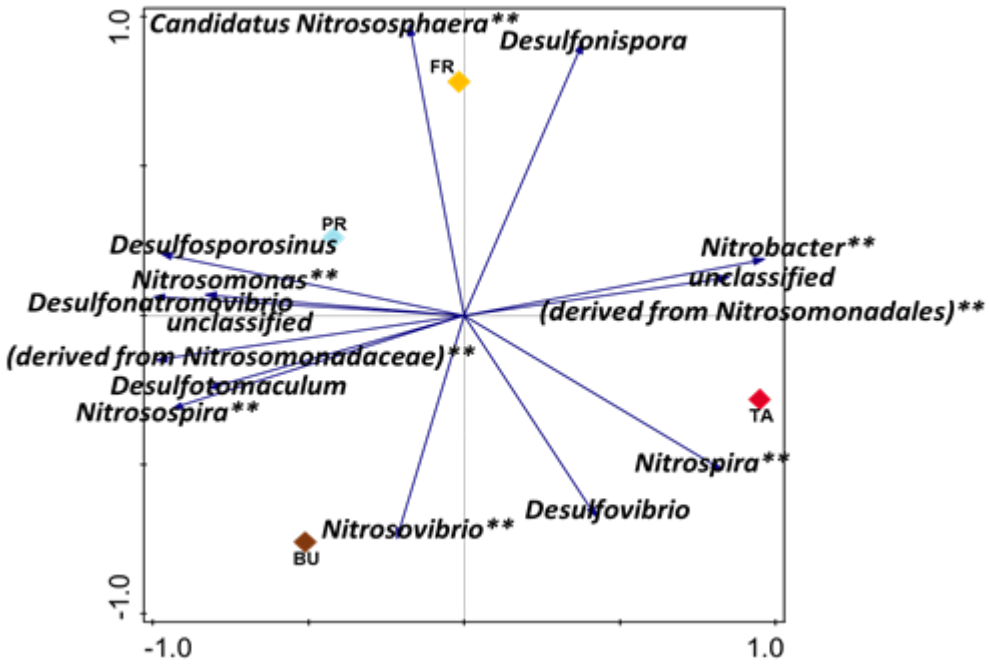

Figure 5.10: Principal Component Analysis (PCA) of nitrifying microorganisms and sulfur reducing bacteria. The resultant vector showed the structural shift and the influence of nitrifying microorganisms and sulfur reducing bacteria. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (84%), Axis 2 (12%). BU- samples

from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage. ** nitrifying microorganisms.

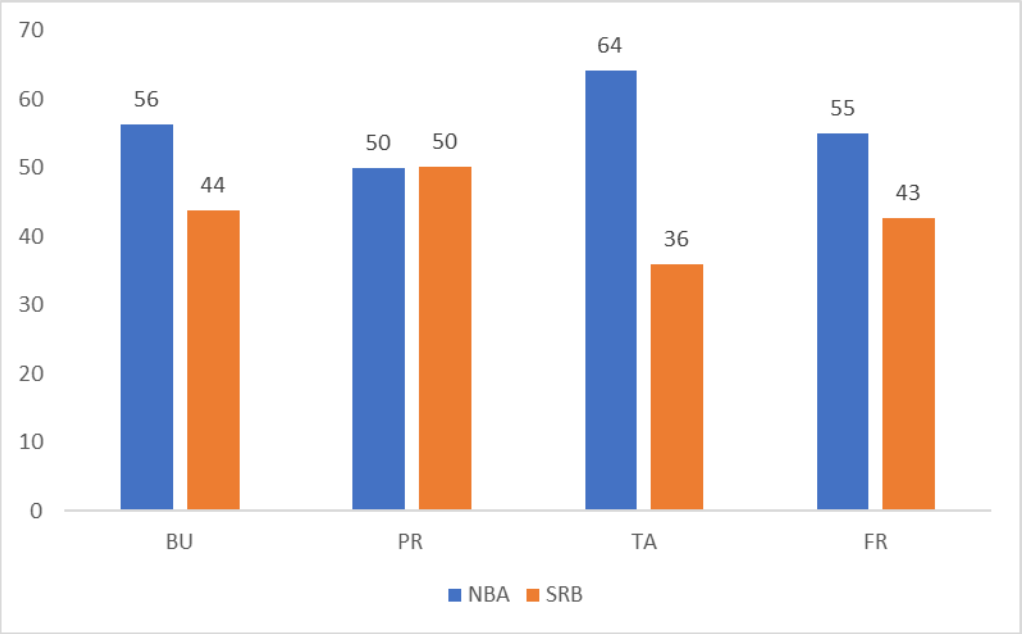

Figure 5.11: Relative abundance (%) of nitrifying microorganisms and Sulfur reducing bacteria across the different growth stages. NBA- Nitrifying Microorganisms, SRB- Sulfur reducing bacteria, BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage.

Table 5.6: Pearson's correlation coefficient (r) matrix between nitrifying microorganisms and Sulfur reducing bacteria.

Sulfur reducing bacteria / Nitrifying microorganisms	*Desulfonatronovibrio*	*Desulfonispora*	*Desulfosporosinus*	*Desulfotomaculum*	*Desulfovibrio*
Candidatus Nitrososphaera	0.06	0.85	0.32	-0.18	-0.82
Nitrobacter	-0.99	0.45	-0.94	-0.91	0.27
Nitrosomonas	0.80	-0.25	0.71	0.34	-0.75
Nitrosospira	0.96	-0.63	0.82	0.76	-0.29
Nitrosovibrio	0.32	-0.76	0.19	0.70	0.76
Nitrospira	-0.78	-0.21	-0.94	-0.66	0.62
Unclassified Nitrosomonadaceae	1.00	-0.52	0.91	0.85	-0.30
Unclassified Nitrosomonadales	-0.89	0.39	-0.90	-0.99	0.04

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. $p \leq 0.05$

5.4 Discussions

This study evaluated the relationship between nitrifying microorganisms and other energy metabolic functional groups across different growth stages. The predictive functional analysis carried out on Nephele revealed nitrogen metabolism, photosynthesis, sulfur metabolism, carbon fixation, and methane metabolism functions (Figure 5.1). The microorganisms associated with the metabolic processes were identified and categorized according to their functions. These were nitrifying microorganisms, nitrogen-fixing bacteria, carbon fixing bacteria, methane oxidizing bacteria and sulfur reducing bacteria. Except for nitrogen-fixing bacteria, the relative abundance of nitrifying microorganisms was higher when compared to the microorganisms associated with each of the energy metabolic functional groups (Figure 5.5, 5.7, 5.9 & 5.11). The soil environment must have been favorable to them as a result of the use of synthetic fertilizer on the studied site. To confirm this is the most numerous numbers of nitrogen-fixing bacteria. Enebe and Babalola (2021b), also confirmed the increase in abundance of nitrogen cycling genes under the influence of fertilizer.

The relative abundance of the microorganisms at the different growth stages showed that nitrogen-fixing bacteria, methane oxidizing bacteria and sulfur reducing bacteria were highest at the pretasseling stage (Figure 5.5, 5.9 and 5.11). While carbon fixing bacteria were highest at the tasseling stage (Figure 5.7). The tasseling stage must have been favorable to the proliferation of carbon fixing bacteria. Devi et al. (2020) observed in their study that the population of some certain species is known to increase several folds at the flowering and fruiting stages. Notable is the variation in the relative abundance of nitrifying microorganisms as they relate to other energy metabolic functional groups. In relationship with nitrogen-fixing bacteria, nitrifying microorganisms were highest at the pretasseling stage. However, with others, it was highest at the tasseling stage. Nitrifying microorganisms

have close relationships with sulfur reducing bacteria (Li et al., 2019a), carbon fixing (Liu et al., 2019) and methane oxidizing (Costa et al., 2019) microbial groups.

The findings showed that the distribution of the energy metabolic functions was mostly at the fruiting stage, although nitrogen metabolism was mostly in the bulk soil (Figure 5.2). This is noted in a study carried out on maize by Marag and Suman (2018), where microbial populations were found to be highest at the reproductive stage. Also, the study of nitrifying microorganisms with each of the energy metabolic microbial function groups showed that majority of the microorganisms concentrated at the fruiting stage. However, each showed a varying mode of distribution (Figure 5.3, 5.4, 5.6, 5.8 and 5.10). The metabolism and carbon fixation were most concentrated in the bulk soil. Methane metabolism was most observed at the pre-tasselling stage, while sulfur metabolism was at the tasselling stage. It has earlier been reported that microbial functional attributes are significantly affected by maize plant growth stages (Mashiane et al., 2018). Cavaglieri et al. (2009) stated that the development of maize plants does not affect microflora density; however, the community structure changes over time, favoring some peculiar groups.

The observed variation in the relative abundance and distribution of the studied group of microorganisms at different growth stages must have been because of the favorable condition of the rhizosphere at the time. Considering quantum signaling in plants (Venturi and Keel, 2016), it is most likely that the metabolic needs of the maize plant at those stages call for the functions of these groups of microorganisms. Metabolism in plants varies with their growth. Some species of microorganisms can increase at a certain growth stage (Devi et al., 2020). Wang et al. (2017a) also observed that the rhizosphere microbial community varies with plant growth stage.

Pearson correlation could be used to evaluate the relationship between microorganisms (Farina et al., 2012). The nitrogen metabolism has a strong positive correlation with methane

metabolism and carbon fixation and has a negative correlation with sulfur metabolism (Table 5.1). Also, the nitrifying microorganisms group showed both positive and negative correlations (Table 5.2). The correlation between the nitrifying microorganisms' group and each of the other energy metabolic groups showed a substantial number of microorganisms that were positively and negatively correlated together (Table 5.3, 5.4 and 5.5). A natural way of introducing abundant atmospheric nitrogen into the soil is through nitrogen fixation. They help improve soil fertility by incorporating ammonia into the soil. In a study carried out by Rocha et al. (2020) the abundance of nitrifying microorganisms was positively correlated with ammonium, a finished product of nitrogen fixation. The activities of nitrogen-fixing bacteria certainly increase the abundance of nitrifying microorganisms.

Nitrifying microorganisms had both positive and negative correlation with some methane oxidizing bacteria (Table 5.5). The oxidation of methane, the second most abundant greenhouse gas, enables the reduction of atmospheric methane (Cui et al., 2015). Sequestering methane while carrying out agricultural production can go a long way toward actualizing sustainable intensification. Negative correlation was also observed by Chan and Parkin (2001), Alam and Jia (2012). Chan and Parkin (2001) reported that there exists a negative correlation between methane oxidation and soil mineral nitrogen, nitrite, nitrate, and ammonia concentration. Also, Alam and Jia (2012) in a study carried out the inhibition of methane oxidation by nitrogenous fertilizers in paddy soil noted that the presence of nitrogen in soil significantly inhibits the activity of methane oxidation. They further explained that there exists a significant negative correlation between nitrification and methane oxidation.

Manipulating carbon dynamics below the soil in an agricultural system has been suggested by Gougoulias et al. (2014) to reduce carbon in the environment. During carbon fixation, inorganic carbon is incorporated into organic molecules. In this study, nitrifying

microorganisms had a positive and negative correlation with some carbon fixing bacteria (Table 5.4). These relationships could be harnessed and improved upon to successfully reduce carbon in the environment while maximizing the yield of crops sustainably.

The level of sulfur in agricultural soil is of importance for the healthy growth of plants (Lucheta and Lambais, 2012). Sulfur is important in determining the pH of soil. Where the sulfur level of the soil is high, it is usually accompanied by a low pH (Li et al., 2019c). An acidic environment increases the bioaccumulation of heavy metals (Ayangbenro et al., 2018). This study showed that there exists a positive and negative correlation between nitrifying microorganisms and sulfur reducing bacteria (Table 5.6). A previous study carried out by Wang et al. (2021) showed that a high correlation existed between the abundance of sulfate reductase gene (gene present in sulfur reducing bacteria) and nitrate (A final product of nitrification by nitrifying microorganisms). In the long run, these relationships could help adjust soil conditions biotechnologically and improve plant growth.

When comparing the bulk soil to the rhizosphere at different growth stages, the correlation between nitrifying microorganisms and each of the metabolic functional groups was majorly in the rhizosphere. The correlation between nitrifying microorganisms and nitrogen-fixing bacteria was obvious at the fruiting stages (Figure 5.4), while their correlation with carbon fixing, sulfur reducing and methane oxidizing bacteria were most observed at the pretasseling and fruiting stage (Figure 5.6, 5.8, 5.10). The variation in the correlation of nitrifying microorganisms with the different functional groups of microorganisms shows that the activities of microorganisms differ at varying growth stages.

This study showed that there exists a relationship between nitrifying microorganisms and other energy metabolic functional group that vary among the diverse microorganisms at different growth stages. In all, a shift was observed in the rhizosphere microbial community structure when compared to the bulk soil. Each group of microorganisms is closely related

to nitrifying bacteria in the rhizosphere. The rhizosphere's unique microbial structure has also been observed by several researchers (Ahkami et al., 2017, De Sousa et al., 2019, Devi et al., 2020, Emmett et al., 2020). The root exudate must have accounted for this.

5.5 Conclusions

The characteristics of the rhizosphere microbial population should have an overall synergistic effect on the plant. Many scientists have recommended using biotechnology to improve agricultural yields. For an organism to be successfully used, its effect on other soil organisms and vice versa must be understood. Our findings will help understand the diversity of nitrifying microorganisms, the relationships within them, and other energy metabolic functions. Also, insights into the dynamics of nitrifying microorganisms in association with other microbial groups of maize rhizospheres at different growth stages were provided. This will improve the growth and development of the maize through modification of the rhizosphere microbial community structure.

CHAPTER SIX

INFLUENCE OF NITRIFYING MICROORGANISM ON PLANT GROWTH PROMOTING BACTERIA AND COMMUNITY LEVEL PHYSIOLOGICAL PROFILE OF THE MAIZE RHIZOSPHERE

Abstract

The use of chemicals to inhibit nitrifying microorganisms is fast becoming the norm to reduce nitrous oxide emissions from farmlands. The purpose of this research is to see how nitrifying microorganisms affect plant growth by promoting rhizobacteria and microbial metabolic and functional processes. DNA was extracted from the maize rhizosphere at various stages of development and sequenced on an Illumina Miseq. The metabolic and functional activity was analysed using the Microresp Technique. Relative abundance showed variation among microorganisms and the utilized carbon substrate at the different growth stages. Principal component analysis showed that there exists a close association between some nitrifying microorganisms with some plant growth promoting rhizobacteria and with some utilized carbon substrate. Pearson correlation further showed a positive correlation between unclassified Nitrosomonadales and *Bacillus* (r = 0.59), unclassified Nitrosomonadales and *Azospirillum* (r = 0.52), *Nitrobacter* and *Azospirillum* (r = 0.54), *Nitrosomonas* and *Stenotrophomonas* (r = 0.54), *Candidatus Nitrosphaera* and *Rhizobium* (r = 0.68), *Nitrospira* and alanine (r = 0.52), lysine and *Nitrobacter* (r = 0.54). This signifies the influence of nitrifying microorganisms on plant growth promoting rhizobacteria and microbial metabolic and functional activity. Thus, the chemical inhibition of nitrifying microorganism would negatively affect the microorganisms and microbial metabolic activities that were influenced. Biotechnological means should be used to promote plant growth sustainably and reduce nitrous oxide emissions.

Keywords: Microresp technique; microbial metabolic activities, carbon substrates, plant growth; maize growth stage; nitrification inhibition

6.1 Introduction

The rhizosphere is a highly complex ecosystem that consists of the nutrient rich soil zone that surrounds the plant root (Venturi and Keel, 2016, Sugiyama, 2019). This increases the pool of microorganisms inhabiting it, and the high diversity of microorganisms has been attributed to plant root exudates (Li et al., 2019b). Rhizosphere components are unique to the host plant (Qiao et al., 2017). The characteristics of the maize rhizosphere soil have been observed by Xomphoutheb et al. (2020) to differ from bulk soil.

Maize is one of the important staple foods and it is important to understand its rhizosphere microbial network to effect better management. The holistic network of microbes in the soil can determine the development of a plant which can be evaluated using a community level physiological profile (Amoo et al., 2021, Enagbonma et al., 2021). The concept is that a microbial community would breakdown carbon sources peculiar to their environment (Enagbonma et al., 2021). Thus, respiratory activity of the overall microbial community in the soil would create a physiological profile as the carbon has been utilized.

The presence of microorganisms in agricultural soil could be of benefit to plants, directly or indirectly, increasing growth and yield. Some of the benefits of microorganisms in agricultural soil include tolerance to environmental stress (Ojuederie et al., 2019), suppressing pathogens (Omomowo and Babalola, 2019), biocontrol activities (Olanrewaju and Babalola, 2019), and improving soil quality (Fasusi et al., 2021). These group of microorganisms have been named plant growth promoters. They have recently been focused on in the pursuit of global food security in a sustainable way. Researchers have been able to identify some associated with the maize crop (Chukwuneme et al., 2021, Fadiji et al., 2021). Few have been isolated, characterized and inoculated to determine whether

they promote plant growth (Agbodjato et al., 2021, Chukwuneme et al., 2020). According to Olanrewaju et al. (2017) the mechanism in which they carry out their function include, phosphate solubilization, siderophores production, nitrogen fixation, production of gibberellin, production of ACC deaminase, quorum quenching and production of cell wall degrading enzyme.

Nitrogen is a macronutrient that is required for plant growth and development. It is often a limiting nutrient in many agroecosystems because of soil degradation. This influences the use of nitrogen-based synthetic fertilizer by many farmers (Zhai et al., 2017, Stewart and Lal, 2017). Plants preferences for nitrogen source differ (Byrnes et al., 2017). Some prefer ammonia as their source of nitrogen, while others prefer nitrate. Taylor and Bloom (1998) reported that maize's ability to assimilate NH_4 and NO_3 at the tip was found to be similar. However, Goulding (2016) and Lehtovirta-Morley (2018) stated that for the safety of other soil organisms, nitrate is usually preferred because ammonia is more acidic than nitrate and causes acidification of the soil. Acidic soil increases heavy metal bioavailability and has an impact on nutrient uptake (Ayangbenro et al., 2018, Shi et al., 2019). Plant yield, quality, and growth are optimized when the ammonia-to-nitrate ratio is low.

Nitrate affects the concentration of proteins in cereals (Dier et al., 2018), helps in embryo production (Yoneyama et al., 2016), and improves phytohormone signalling (Hachiya and Sakakibara, 2016). In a study carried out by Akinola et al. (2021b), nitrate was said to be the most influential factor that controlled microbial diversity. The transformation from NH_3 to NO_3 is carried out by a nitrifying process through nitrifying microorganisms (Heiss and Fulweiler, 2016). The use of chemical nitrifying inhibitors alongside nitrogen synthetic fertilizer is fast becoming the norm in agricultural soil. It is being used to reduce nitrogen loss by disrupting the activities of nitrifying microorganisms (Yang et al., 2016). Although the mechanism of the chemical nitrifying inhibitors is not fully understood, Zhou et al. (2018) explained a reduction in *amoA* genes as one of its effects.

Considering the complex network that exists between microorganisms (Nanjundappa et al., 2019), it would be necessary to evaluate the influence of nitrifying microorganisms on plant growth promoting microbes and overall microbial activities. This is necessary to discourage the use of chemical nitrifying inhibitors and promote biotechnological microbial adjustment of the soil. It would promote plant growth and yield in an environmentally friendly way. Also, the network between nitrifying microorganisms and others would be better understood. There is very little literature on the relationship between nitrifying microorganisms and plant growth promoting microorganisms. This study aims to evaluate the influence of nitrifying microorganisms on plant growth promoting microbes and community level physiological profiles.

6.2 Materials and Methods

6.2.1 Study Area and Sample Collection

Samples were collected at the North-West University maize plantation in Molelwane, Mafikeng, South Africa ('25.6188889 E 25.789989 S'; height 1012 m). The average annual temperature in the area ranges from 2^{o}C-21^{o}C in winter to 22^{o}C-35^{o}C in summer, with 450 mm of rainfall falling between October and April. The farm was watered and treated with NPK (20% nitrogen, 7% phosphorus, and 3% potassium) fertilizer prior to planting. The maize variety sown was QN.633. The development phases of the rhizosphere were identified as follows: pretasseling without silk (PR), tasseling with silk (TA), and fruiting with cobs (FR). The rhizosphere soil was gathered between 0-15 cm depth and 0-5 cm breath of each maize root. In addition, bulk soil (BU) was collected in a field away from the maize plantation. The soil was collected in triplicate into a plastic bag for each development stage and bulk soil, then transferred to the laboratory and kept at -20^{o}C.

6.2.2 Rhizosphere Physical and Chemical Analysis

Standard chemical analysis was used to measure physical and chemical properties. The particle size distribution (sand, silt, and clay) was determined using the Kroetsch (2008) method. Nitrate and ammonium were measured using the KCL extraction method described by Keeny and Nelson (1982). Organic matter was measured using the loss of ignition method (Nelson and Sommers, 1996). Total carbon was analysed using the dry combustion method (Santi et al., 2006). Organic carbon was measured using a method described by Walkley and Black (1934). Total nitrogen was analysed using the digestion method (Bremme and Mulvaney, 1982). Smittenberg (1951) HCl extraction method was used to determine the rhizosphere's sulphur content. After mixing 2 g rhizosphere soil with deionized water, the pH was measured with a Jenway 3520 pH meter (Cole – Parner instruments, Staffordshire, UK) (10 ml). The pH was measured with a Jenway 3520 pH meter (Cole – Parner instruments, Staffordshire, UK) after mixing rhizosphere (2 g) with deionized water (10 ml).

6.2.3 DNA Extraction, Sequencing, and Analysis

The rhizosphere DNA was extracted using the Nucleospin soil DNA extraction kit according to the manufacturer's instructions. The extracted DNA was sequenced using an illumina Miseq system. The reads were processed and analyzed using the MG-RAST server (http://www.mg-rast.org) with the default settings (Meyer et al., 2008). The microorganisms used in the research were identified to the genus level (Semedo et al., 2021).

6.2.4 Community Level Physiological Profile

The community level physiological profile was evaluated using the MicroResp technique following manufacturer’s instructions (AccuReader M965^{+}, Taipei, Taiwan). Ten (10) carbon substrates were used. Each substrate has four replicates, and a blank containing distilled water. The ten-carbon substrate used consisted of five carbohydrates (glucose, galactose,

fructose, maltose, cellobiose), three amino acids (lysine monochydrochloride, alanine, arginine) and two carboxylic acids (oxalic acid, malic acid) to determine the physiological profiles. The readings were taken at a 570 nm absorbance wavelength. The community level physiological profile for each of the samples was computed by subtracting the water response from the substrate response.

6.2.5 Statistical Analysis of Soil Physical and Chemical Parameter

The statistics of triplicate sample, the relative abundance of the microorganisms, and community level physiological profile were evaluated using Microsoft Excel software. Pearson correlation matrix was evaluated using XLSTAT. The principal component analysis (PCA) was carried out using Canoco 5 package.

6.3 Results

6.3.1 Statistics of Rhizosphere Physio-Chemical Parameter

Table 4.1 of Section 4.3.1 summarizes the statistical study of rhizosphere physical and chemical parameters. The pH, the focus of the physical and chemical parameters, ranges from 5.35 to 6.22, with a mean of 5.93. The read count for nitrifying bacteria varied from 282 to 673, with a mean of 424, whereas the read count for identified plant growth promoting bacteria ranged from 13320 to 8278, with a mean of 11518. The carbon-to-nitrogen ratio is around 9:1. The NH4 to NO3 ratio is around 1:1.4.

6.3.2 Relative Abundance of Nitrifying Microorganisms

The identified nitrifying bacteria have been previously mentioned in 4.3.4, they are *Nitrospira*, *Nitrosospira*, Unclassified Nitrosomonadales, Unclassified Nitrosomonadaceae, *Nitrobacter*, *Nitrosovibrio*, *Nitrosomonas* and an archaea, *Candidatus Nitrososphaera.* Although the microorganisms vary across the different growth stages, however, the fruiting stage had the most abundant (Figure 6.1).

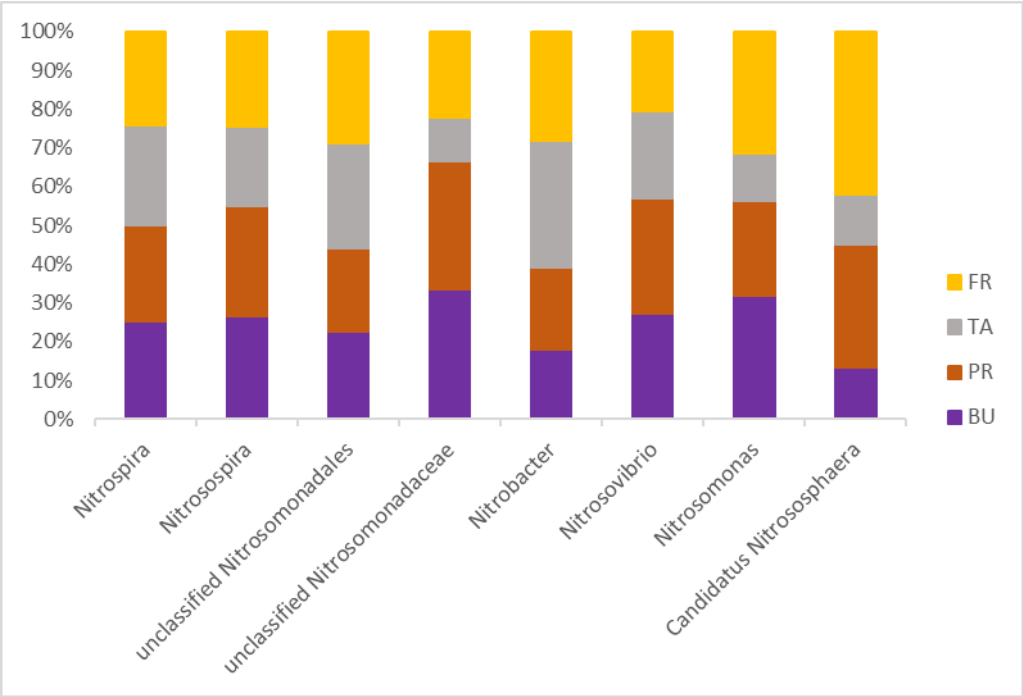

Figure 6.1: Relative abundance (%) of each nitrifying microorganism across the different growth stages. BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage

6.3.3 Relationship Between Nitrifying Microorganism and Plant Growth Promoting Microorganism

The identified plant growth promoting microorganism were, *Bacillus, Arthrobacter, Paenibacillus, Rhodococcus, Bradyrhizobium, Gordonia, Azospirilum, Burkholderia, Stenotrophomonas, Hrbaspirillum, Rhizobium, Alcaligenes, Chryseobacterium*, and *Pseudomonas*. They all showed variation across the different growth stages. While *Stenotrophomonas, Alcaligenes* and *Pseudomonas* were most abundant at the bulk soil, *Herbaspirillum* were most abundant at the pretasseling stage. *Bacillus* showed evenness across the different growth stage, *Rhizobium* was most obvious at the fruiting stage and the rest at the tasseling stage (Figure 6.2). In general, the rhizosphere had a higher amount of the microorganism when compared to the bulk soil (Figure 6.1).

The PCA was used to evaluate the association between the nitrifying microorganism and plant growth promoting rhizobacteria (Figure 6.3). The Euclidean dissimilarity matrix showed a variation of 74% on Axis 1 and 16% on Axis 2. A close relationship was observed between some group of nitrifying microorganisms and some specific plant growth promoting microorganism (Figure 6.3). A Pearson correlation further analysed the existing relationship between these microorganisms. Significant relationship exists between some of the nitrifying microorganism and few of the plant growth promoting microorganisms at $p \leq 0.5$ (Table 6.1). Notable is the moderate positive correlation between Unclassified Nitrosomonadales and *Bacillus* (r=0.59), *Azospirillum* (r=0.52). Also, between *Nitrobacter* and *Azospirillum* (r=0.54), *Nitrosomonas* and *Stenotrophomonas* (r=0.54), *Candidatus Nitrosphaera* and *Rhizobium* (r=0.68).

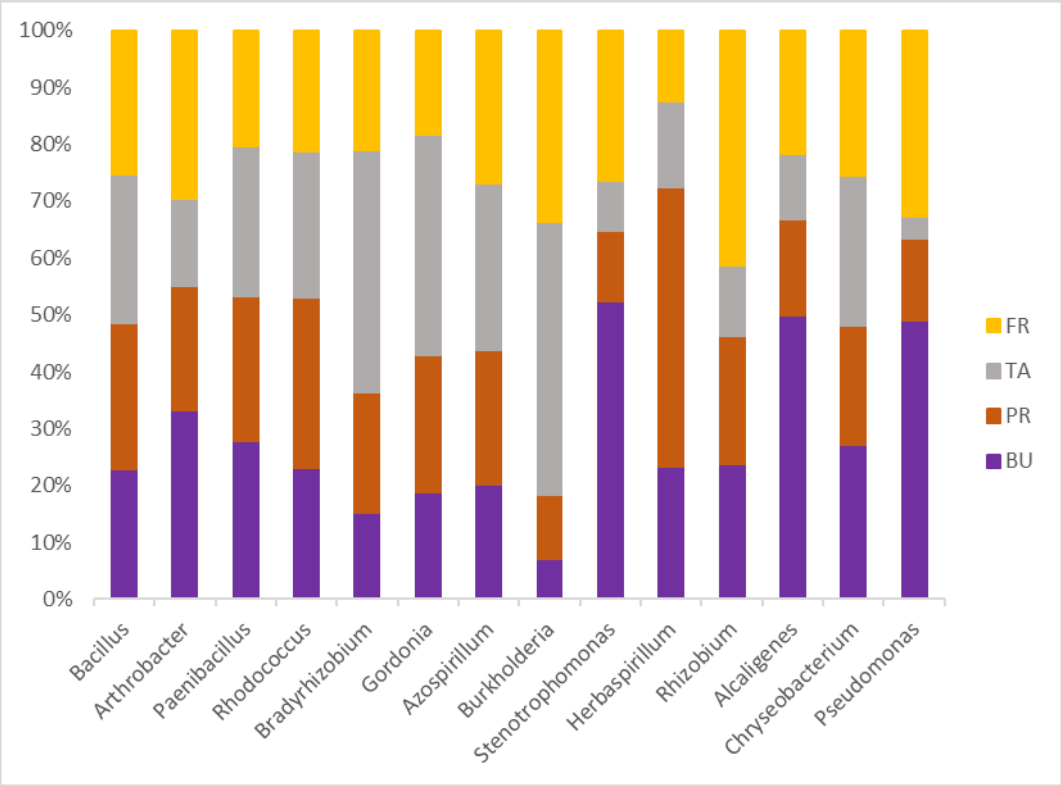

Figure 6.2: Relative abundance (%) of each plant growth promoting rhizobacteria across the different growth stages. BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage

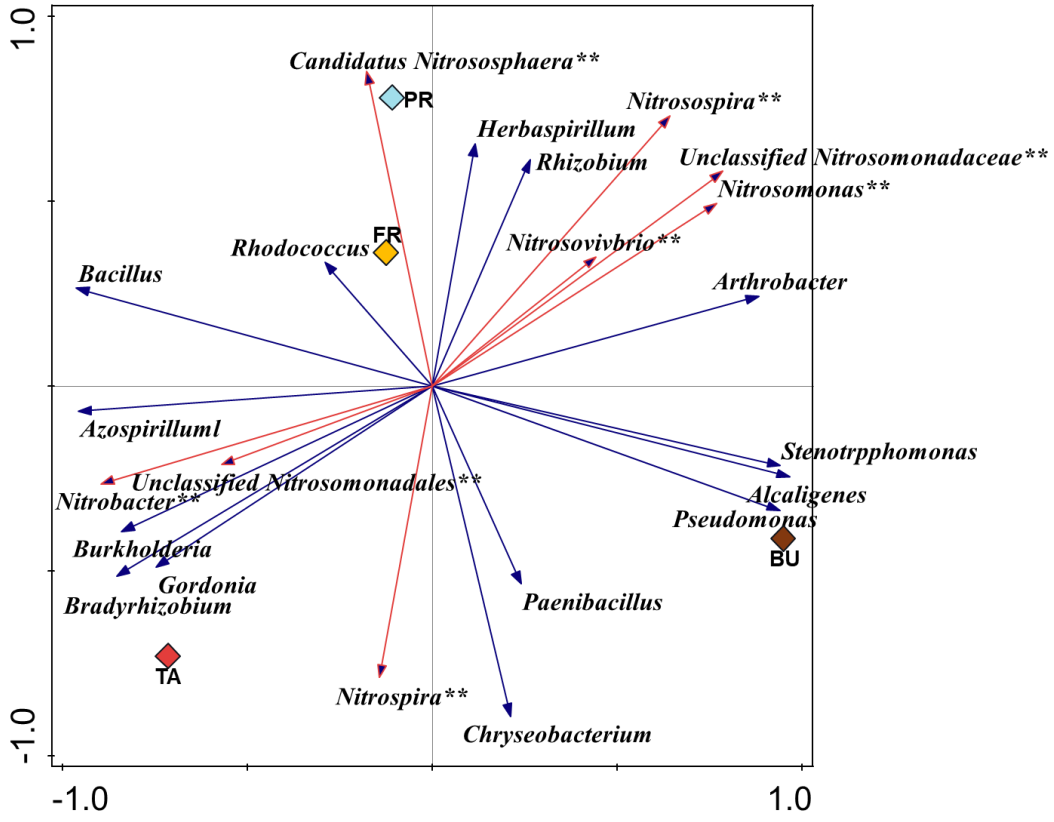

Figure 6.3: Principal Component Analysis (PCA) plot of nitrifying microorganisms and plant growth promoting rhizobacteria. The resultant vector showed the structural influence between nitrifying microorganisms and plant growth promoting rhizobacteria. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (74%), Axis 2 (16%). BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage. ** nitrifying microorganisms.

Table 6.1: Pearson's correlation coefficient (r) matrix between nitrifying microorganisms and plant growth promoting rhizobacteria.

Variables	*Nitrospira***	*unclassified Nitrosomonadales***	*Nitrosospira***	*unclassified Nitrosomonadaceae***	*Nitrobacter***	*Nitrosovibrio***	*Nitrosomonas***	*Candidatus Nitrososphaera***
Bacillus	-0.40	**0.59**	-0.06	0.03	0.23	0.13	-0.20	0.08
Arthrobacter	0.17	-0.41	0.20	0.17	-0.24	-0.17	0.19	0.09
Paenibacillus	0.33	-0.49	0.08	-0.14	-0.32	0.42	0.26	-0.26
Rhodococcus	0.17	-0.36	0.06	0.13	0.02	0.05	-0.22	-0.10
Bradyrhizobium	0.10	0.26	-0.38	-0.46	0.38	-0.15	-0.39	-0.04
Gordonia	0.10	0.07	-0.21	-0.35	0.46	-0.15	-0.48	-0.38
Azospirillum	-0.38	**0.52**	-0.07	-0.14	**0.54**	-0.10	-0.15	-0.06
Burkholderia	0.46	0.20	**-0.69**	**-0.60**	0.10	-0.49	-0.51	0.02
Stenotrophomonas	0.10	-0.10	-0.07	0.04	-0.16	-0.04	**0.54**	-0.23
Herbaspirillum	-0.10	-0.35	0.35	0.49	-0.09	0.47	0.03	-0.25

Rhizobium	-0.13	-0.06	0.16	0.03	0.02	-0.16	0.39	**0.68**
Alcaligenes	0.06	-0.22	0.08	0.28	-0.15	-0.02	0.12	-0.01
Chryseobacterium	0.37	0.16	**-0.63**	-0.28	-0.21	-0.11	0.21	-0.26
Pseudomonas	0.34	-0.48	-0.01	-0.09	-0.22	0.05	0.45	-0.24

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. Values in bold have a significant level $p \leq 0.05$. ** nitrifying microorganisms.

6.3.4 Relationship Between Nitrifying Microorganism and Community Level Physiological Profile

The community level physiological profile evaluated using the utilized carbon substrate showed that microbial community were most abundant at the fruiting stage (Figure 6.4). Table 6.2 showed there was no significant relationship between nitrifying microorganisms and the utilized carbon except between *Nitrospira* and alanine ($r = 0.52$, $p \leq 0.5$), lysine and *Nitrobacter* ($r = 0.54$, $p \leq 0.5$). Cellobiose and unclassified Nitrosomonadales also showed a positive correlation ($r = 0.42$, $p \leq 0.5$). However, PCA (Euclidean dissimilarity matrix showed variation of 51% on Axis 1 and 35% on Axis 2) showed close association between unclassified Nitrosomonadales and Malic acid, *Nitrobacter* with malic acid and alanine, *Nitrospira* and lysine monochydrochloride, *Candidatus Nitrosophaera* and oxalic acid.

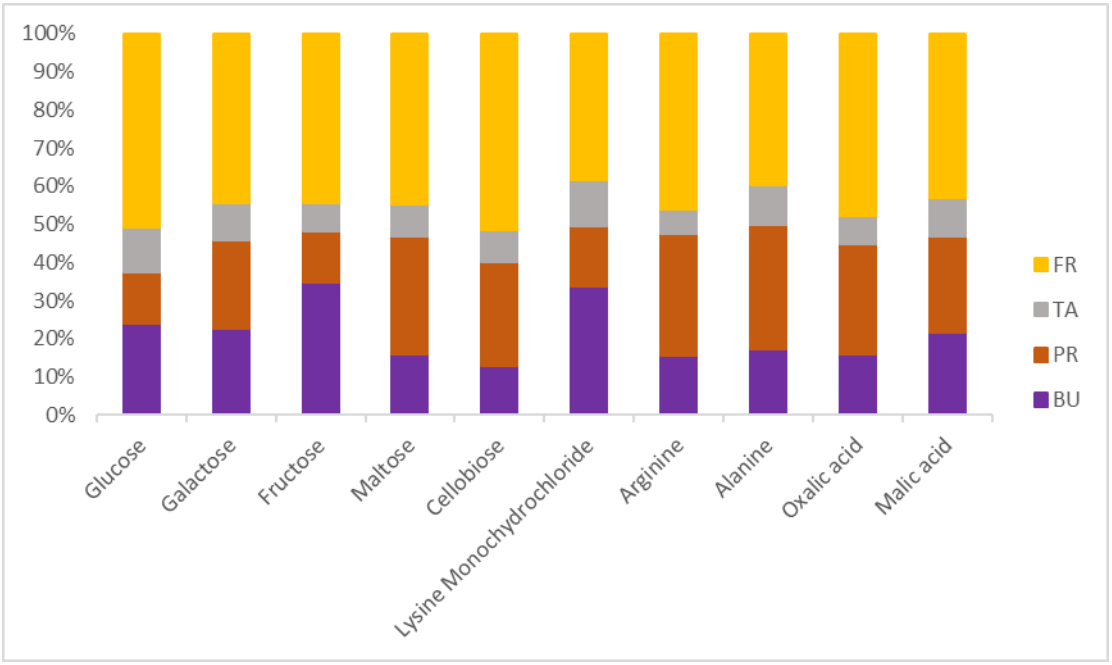

Figure 6.4: Relative abundance (%) of community level physiological profile across the different growth stages. BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage

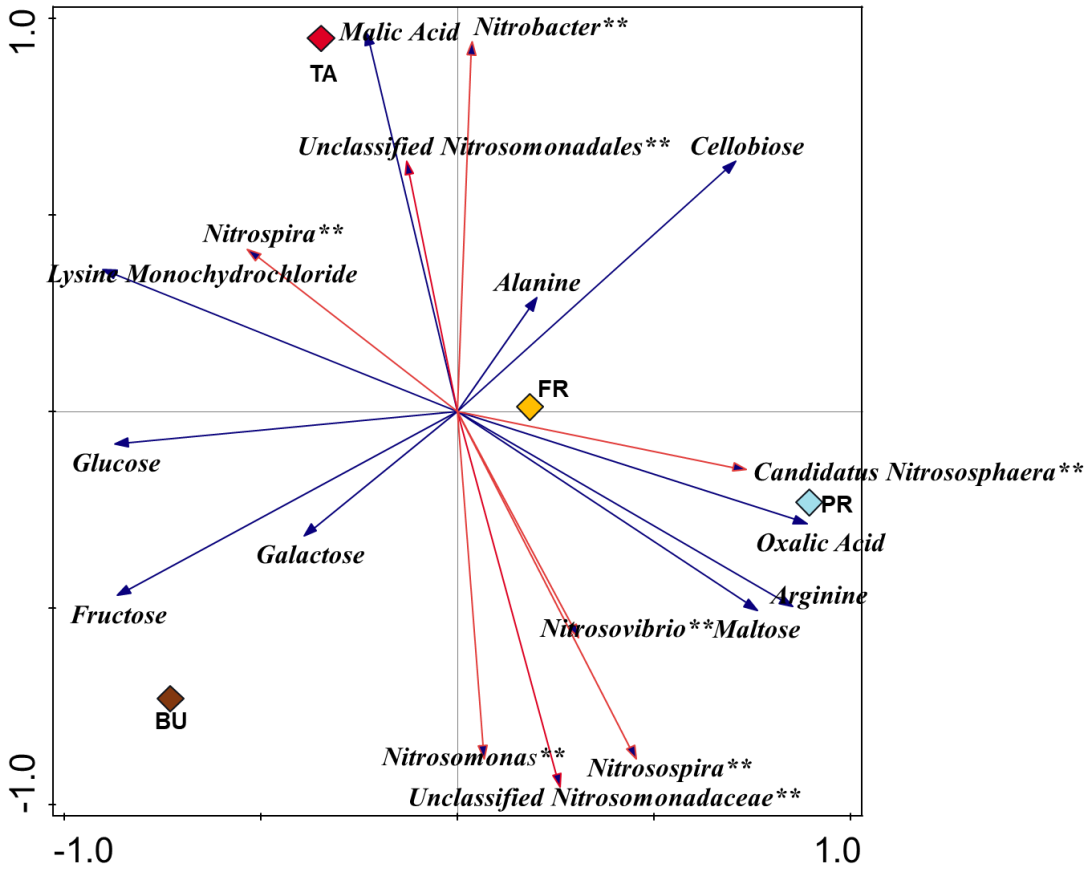

Figure 6.5: Principal Component Analysis (PCA) plot of nitrifying microorganisms and physiological abilities. The resultant vector showed the structural influence between nitrifying microorganisms and the carbon substrates. Axis 1 and 2 explained the observed variation based on Euclidean dissimilarity matrix. Axis 1 (51%), Axis 2 (35%). BU- samples from bulk soil, PR- samples from pretasseling growth stage, TA- samples from tasseling growth stage, FR- samples from fruiting growth stage. ** nitrifying microorganisms

Table 6.2: Pearson's correlation coefficient (r) matrix between nitrifying microorganisms and the utilized carbon substrates.

Variables	***Nitrospira*****	***unclassified Nitrosomonadales*****	***Nitrosospira*****	***unclassified Nitrosomonadaceae*****	***Nitrobacter*****	***Nitrosovibrio*****	***Nitrosomonas*****	***Candidatus Nitrososphaera*****
Glucose	-0.49	0.61	0.07	0.35	0.15	-0.05	-0.25	-0.08
Galactose	0.13	-0.37	0.24	-0.05	0.01	0.04	-0.32	0.18
Fructose	0.03	-0.05	0.07	0.36	-0.37	-0.10	0.10	-0.15
Maltose	0.20	-0.43	0.23	0.31	-0.46	0.19	-0.24	0.18
Cellobiose	-0.23	0.42	-0.17	-0.02	0.37	-0.20	-0.08	-0.18
Lysine Monochydrochloride	0.08	-0.06	-0.09	-0.48	**0.54**	-0.28	-0.08	-0.09
Arginine	0.23	-0.27	-0.03	0.08	-0.49	0.13	0.40	0.33

Alanine	**0.52**	**-0.54**	-0.12	0.07	-0.62	0.53	0.04	-0.24
Oxalic acid	-0.44	0.43	0.09	0.09	0.28	-0.18	0.22	0.19
Malic acid	0.19	-0.12	-0.15	-0.47	0.12	0.18	0.12	0.04

r; 0.90 to 1.00 (-0.90 to -1.00) = very highly positive (negative) correlation. r; 0.70 to 0.90 (-0.70 to -0.90) = highly positive (negative) correlation. r; 0.50 to 0.70 (-0.50 to -0.70) = Moderately positive (negative) correlation. r; 0.30 to 0.50 (-0.30 to -0.50) = Low positive (negative) correlation. r; 0.00 to 0.30 (-0.00 to -0.30) = insignificant correlation. Values in bold have a significant level $p \leq 0.05$. ** nitrifying microorganisms.

6.4 Discussions

The presence of nitrifying microorganisms in the soil reduces acidity caused by high levels of ammonia. Their other benefits include the development of lateral roots (Mantelin and Touraine, 2004), expansion of leaves, and induction of flowers (Hachiya and Sakakibara, 2016). It also supports the transport system (Plett et al., 2018). The nitrifying microorganisms in this study included two unidentified groups. One is from the class Nitrosomonadaceae and the other is from Nitrosomonadales. Lately, there has been increase in the discovery of novel nitrifying bacteria (Stein, 2019). This must have been because of the use of the new generation sequencing techniques adopted recently. The well-known nitrifying microorganisms in this study have also been recently associated with maize rhizosphere by other researchers. They are Nitrospira (Zhang et al., 2022), *Nitrosospira* (Shi et al., 2020), *Nitrobacter* (Meier et al., 2021), *Nitrosovibrio* (Peng et al., 2021), *Nitrosomonas* (Shi et al., 2022), and *Candidatus Nitrosophaera* (Enebe and Babalola, 2021a).

In this study, a positive influence was observed between nitrifying microorganisms and plant growth promoting microorganisms. Unclassified nitrosomonadales and *Bacillus* is one of them. *Bacillus* is well known for its plant growth promoting properties. Nitrogen-fixing (Singh et al., 2020), phosphorus solubilization (Saeid et al., 2018), siderophore production (Rizzi et al., 2019), and phytohormone production (Kang et al., 2019) are some of the mechanisms by which they promote plant growth. The presence of *Bacillus* in compost changed the composition of the bacterial community, favouring nitrifying microorganisms (Wang et al., 2019b).

Also, unclassified Nitrosomonadales and *Nitrobacter* each showed a positive correlation with *Azospirillum*. *Azospirillum* increases nitrogen use efficiency and improves biochemical characteristics that promote plant growth (Zeffa et al., 2019). It also improves plant

physiological characteristics (Latef et al., 2020). Aside from being a nitrogen fixer, Leite et al. (2019) observed its positive influence on nitrifying microorganisms. Besides, *Azospirillum* and *Nitrobacter* have been used together in a microbial consortium as a biofertilizer by Vatandoost et al. (2019). The physiological traits of the studied plant (rapeseed) were improved.

Furthermore, *Nitrosomonas* and *Stenotrophomonas*, showed a positive correlation in this study. Some species of *Stenotrophomonas* are plant growth promoter that produces volatile organic compound and have been used to promote crop yield under nitrogen deficient condition (Alexander et al., 2019). In addition, *Candidatus Nitrosphaera* and *Rhizobium* were also positively correlated. Although the relationship between *Candidatus Nitrosphaera* and *Rhizobium* have not been studied previously, Peng et al. (2021) observed that the presence of some plant growth promoting consortium consisting of *Pseudomonas* sp, *Peribacillus* sp, and *Streptomyces* sp, enriched *Candidatus Nitrosphaera*.

Microresp technique has been used previously to evaluate community level physiological profiles of microbial activity (Amoo et al., 2021). The amount of carbon source utilized corresponds to the amount of microbial metabolic properties and functional diversity (Deng et al., 2011). Also, Gao et al. (2022) observed that substrate induced respiration is dependent on the microbial community. In this study, some of the substrates utilized had a close association with some nitrifying microorganisms. Especially, between malic acid and unclassified Nitrosomonadales, malic acid and *Nitrobacter*, oxalic and *Candidatus nitrososphaera,* Lysine and *Nitrospira* (Figure 6.5). Also, positive influence was observed between some nitrifying microorganisms and some of the utilized carbon substrate. Specifically, cellobiose and unclassified Nitrosomonadales, lysine and *Nitrobacter*, alanine and *Nitrospira* (Table 6.2). This signifies that nitrifying microorganisms have an affiliation and a small contribution to the microbial metabolic process in the rhizosphere.

Variation has been seen in maize rhizosphere at different vegetative growth stages (Joshi et al. 2021). In this study, variation was observed among the rhizosphere microorganisms at different growth stages of maize. However, the community level physiological profile, which sums up the overall activities of the soil, was most abundant at the fruiting stage. Rocha et al. (2020) also affirmed that the rhizosphere soil of the fruiting stage possesses more microorganisms than the lower developmental stages. Shi et al. (2022) noted that the tasseling stage is the key period when nitrogen is needed. In this study, some nitrifying bacteria were noted at the tasseling stage. Overall, the rhizosphere contained more microorganisms than the bulk soil. The rhizosphere has an impact on the abundance of microorganisms (Devi et al., 2020, Emmett et al., 2020). This could have been a result of the root exudate.

6.5 Conclusions

This research shows that nitrifying microorganisms have an influence on some plant growth promoting microorganisms and the community level physiological profile of the microbial community. Thus, the absence of this organism could have a drastic effect on the rhizosphere soil microbial community and its functions. Therefore, the use of nitrification inhibitors and other agrochemicals that inhibit the proliferation of nitrifying microorganisms should be discouraged. Also, an environmentally friendly approach could be used in place of the agrochemicals. The findings of this study can be considered when carrying out a biotechnological process involving nitrifying bacteria.

CHAPTER SEVEN

SUMMARY, CONCLUSION AND RECCOMMENDATION

Nitrogen is one of the most important elements necessary for plant growth and development in plants. Nitrogen-based synthetic fertilizer and other agrochemicals used to provide it has adversely affected the environment and health. Thus, there is a need to achieve a sustainable solution by first studying the microorganisms associated with the natural sources of nitrogen. The microorganisms responsible for the conversion of ammonia to nitrate are nitrifying bacteria and archaea. Nitrate is preferred over ammonia as a nitrogen source in the soil environment because it is less volatile, more mobile, taken up by plants with higher efficiency, less acidic and synergistically promotes the uptake of cations.

The nitrifying bacteria and archaea associated with the maize rhizosphere were identified in this study, furthermore, their relationship with the soil's chemical properties and other microbes was examined. As observed in our result, the sequencing of DNA isolates collected from maize rhizosphere at different growth stages showed 10 genera; *Nitrospira, Nitrosospira, Nitrobacter, Nitrosovibrio, Nitrosomonas, Nitrosococcus, Nitrococcus,* unclassified (derived from Nitrosomonadales), unclassified (derived from Nitrosomonadaceae) and an archaeon, *Candidatus Nitrososphaera.* It was observed that physicochemical parameters of the maize rhizosphere had an influence on the abundance of nitrifying bacteria. Some of the parameters such as pH, orgainic carbon and total nitrogen were seen to be positively correlated with nitrifying bacteria while others are not.

The nitrifying bacteria and archaea were distributed differently across the different growth stages, with alpha diversity showing no significant difference while beta diversity showed a significant difference. Although there was no significant difference in the diversity of nitrifying bacteria and archaea across growth stages, however, they were most abundant during the tasseling stage. In addition, a significant number of nitrifying bacteria and archaea relate to

other microbial group both positively and negatively. The result also revealed that each of the functional groups in relation to the nitrifying community showed varying distributions across the different growth stages. More so, the nitrifying microbes showed a close association with some plant growth promoting microorganisms and some utilized carbon substrate used to evaluate the community level physiological profile.

Previously identified nitrifying bacteria and archaea and yet to be identified nitrifying bacteria were observed in the maize rhizosphere. Their presence in the soil does have an impact on the soil chemical properties, other soil microbial groups, plant growth promoting rhizobacteria, and soil microbial community level physiological profile. Further studies should be carried out on nitrifying bacteria and archaea inhabiting other crops and how they relate with other microorganisms to check for varying or likely patterns. Also, the production of the identified microbes in consortium or singly as a nitrogen-based biofertilizer and the possibility of incorporating them into organic materials rich in ammonia should be considered.

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APPENDICES

Appendix 1: Microresp community physiological profile detailed procedure

A. Preparation of Soil Samples

I. Sieve soils through a 2.0mm stainless steel sieve, removing roots and stones. A typical amount of soil required for a 96 well plate is 35–50g fresh weight. Store soil samples at 4°C when not in use. Determine soil moisture content using a sub-sample of 5–10 g soil.

N.B. Soils must not be too wet, as this restricts gaseous exchange, nor too dry, as this may adversely affect the microbial activity. For measuring microbial activity, an acceptable range for the moisture content is 30–60% of it's maximum water holding capacity. Soils with an ideal moisture content should fall easily through the filling device.

2. If the soil moisture has been adjusted, incubate as described above for 5 days prior to the soils being used, and regularly check that the wick remains moist. Incubation

Soil samples are incubated at 25°C for 3–5 days in a large, sealed box containing a dish of self-indicating soda lime and lined with wet paper towels, prior to carrying out the MicroResp method.

3. Fill the deepwell plates with soil samples as instructed and cover the plates with Parafilm. During incubation check that the Parafilm has not torn and replace if necessary.

B. Preparing Detection Plates

I. Prepare 3% Purified Agar (3 g per 100 ml) in d. H_2O and dissolve by heating in a microwave on a low setting. Ensure the lid is loose during microwaving and gently mix at intervals. Check the volume has not changed and allow agar to cool in a

water bath to 60°C.

II. Measure the required amount of indicator solution (2 times the amount of agar) into a bottle and warm in a water bath to 60°C.

III. Once the temperature of each solution has equilibrated, transfer the indicator solution and agar into a beaker and mix thoroughly, maintaining the heat at 60°C with constant stirring.

IV. Dispense 150µl aliquots into six columns of the microplate using an 8-channel pipette (discard the first dispense back

into the mixture). Repeat the procedure for the next 6 columns, and so on. N.B. Warm tips before use. When dispensing keep the pipette upright (not tilted). Place the tips in the wells so that when the agar mix is dispensed it rises up the end of the tips (immersing them in the agar mix). This reduces bubbles and also ensures the tip is “clean” when dispensing into the next well.

V. Store the plates, in the dark at room temperature, in a desiccator with self-indicating soda lime on the base and a beaker of water. Leave uncovered for 1–2 days to allow to equilibrate, then cover each plate with Parafilm® if they are not used soon after.

VI. Replace the soda lime when necessary and keep the atmosphere in the desiccator moist.

C. Preparing Deepwell plates

I. The substrates are prepared as 30mg per gram of soil water.

II. Soil samples are added and incubated in the deepwell plate(s) for 3–5 days prior to the addition of the carbon sources and detection plate

III. Insert the Perspex sheet into the filling device and place the filling device on top of the deepwell plate.

IV. Section off desired columns of the filling device with tape (if using more than one soil) before filling appropriate wells with soil. Sprinkle an excess of soil over the filling device

and gently brush the soil into the wells until evenly filled, tapping the whole system once to gently compact the soil before adding more soil. Level off the soil and brush away excess soil.

IV. Gently cover the section of soil filled, uncover the empty columns and fill with another soil as before. Once all the filling device is filled, remove all the tape.

V. Remove the Perspex sheet from between both plates, allowing the soil to fall through to the deep wells.

VI. Place the Perspex sheet on top of the filling device and, using the fingerholds, gently but firmly tap the assembly on the bench so that any remaining soil falls into the deep-well plate. Any soil particles that have stuck will need to be pushed lightly down into the wells using a clean wire or rod.

VII. Remove the filling device and for method (i) cover the deepwell plate with Parafilm for incubation.

VIII. To clean the filling device, wash by hand with detergent and rinse with deionised water, then dry.

D. Assembling components and experimental protocol

I. Switch on the spectrophotometer microplate reader.

II. Select your detection plates – check that the amount of agar in the wells of each detection plate is even and the colour consistent.

III. Allow the substrates to warm to room temperature. Use an 8-channel pipette to dispense 25 µl of each desired substrate into the appropriate wells of the deepwell plate.

IV. Apply the MicroResp seal to the deepwell plate(s).

V. Place the detection plate in the spectrophotometer and read the plate at absorbance wavelength 570 nm.

VI. Immediately place the detection plate onto the MicroResp seal by inverting the detection plate so that A1 corresponds to A12 on the deepwell plate. Apply firm, even pressure to seal correctly and secure the plates in a MicroResp clamp.

VII. Save the time “At0” results to file and check the % coefficient of variance. Discard the plate if the % CoV is >5% and read another plate.

VIII. Repeat steps 5–7.

IX. Incubate the plates for 6 hours at 25°C.

X. After incubation, carefully disassemble the clamp, remove the detection plate and peel off the seal.

XI. Immediately read the detection plate and save results “At6” to file as before.

XII. The deepwell plate should be disposed of appropriately at the end of the experiment. The detection plates can be re-used as long as the agar has not dried, and they have returned to their original colour and Absorbance reading.

XIII. MicroResp seals are cleaned with detergent and rinsed with deionised water, then dried.

XIV. Export the files from the spectrophotometer programme into an Excel spreadsheet and sort the absorbance (A570) data into a list format with the 0hr (At0) and 6hr (At6) data in single columns alongside each other.

Appendix 2: Article published from this study

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REVIEW
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Factors Influencing Soil Nitrification Process and the Effect on Environment and Health

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To meet the global demand for food, several factors have been deployed by agriculturists to supply plants with nitrogen. These factors have been observed to influence the soil nitrification process. Understanding the aftermath effect on the environment and health would provoke efficient management. We review literature on these factors, their aftermath effect on the environment and suggest strategies for better management. Synthetic fertilizers and chemical nitrification inhibitors are the most emphasized factors that influence the nitrification process. The process ceases when pH is <5.0. The range of temperature suitable for the proliferation of ammonia oxidizing archaea is within 30 to 37°C while that of ammonia oxidizing bacteria is within 16 to 23°C. Some of the influencing factors excessively speed up the rate of the nitrification process. This leads to excess production of nitrate, accumulation of nitrite as a result of decoupling between nitritation process and nitratation process. The inhibition mechanism of chemical nitrification inhibitors either causes a reduction in the nitrifying micro-organisms or impedes the amoA gene's function. The effects on the environment are soil acidification, global warming, and eutrophication. Some of the health effects attributed to the influence are methemoglobinemia, neurotoxicity, phytotoxicity and cancer. Biomagnification of the chemicals along the food chain is also a major concern. The use of well-researched and scientifically formulated organic fertilizers consisting of microbial inoculum, well-treated organic manure and good soil conditioner are eco-friendly. They are encouraged to be used to efficiently manage the process. Urban agriculture could promote food production, but environmental sustainability should be ensured.

Keywords: agricultural intensification, agroecosystems, environmental challenge, nitrification inhibitor, nitrifying microorganism, synthetic fertilizer

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INTRODUCTION

Nitrification process (NP) is an oxidation reaction that usually occurs under aerobic conditions. The process serves as an intermediate of oxidized and reduced forms of nitrogen in its cycling. The nitrate produced serves as a substrate for denitrification and a nutrient for plant growth. This has made it important to environmental sustainability and agricultural intensification. Compounds such as ammonium (NH_4), ammonia (NH_3), hydroxylamine (NH_2OH), nitrous oxide (NO), nitrite (NO_2-), and nitrate (NO_3-) are the major forms of nitrogen associated with the process. The soil nitrification process is divided into two major phases which are nitritation and nitratation,

Appendix 3: Article published from this study

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Sustainable Intensification of Maize in the Industrial Revolution: Potential of Nitrifying Bacteria and Archaea

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Sustainable intensification is a means that proffer a solution to the increasing demand for food without degrading agricultural land. Maize is one of the most important crops in the industrial revolution era, there is a need for its sustainable intensification. This review discusses the role of maize in the industrial revolution, progress toward sustainable production, and the potential of nitrifying bacteria and archaea to achieve sustainable intensification. The era of the industrial revolution (IR) uses biotechnology which has proven to be the most environmentally friendly choice to improve crop yield and nutrients. Scientific research and the global economy have benefited from maize and maize products which are vast. Research on plant growth-promoting microorganisms is on the increase. One of the ways they carry out their function is by assisting in the cycling of geochemical, thus making nutrients available for plant growth. Nitrifying bacteria and archaea are the engineers of the nitrification process that produce nitrogen in forms accessible to plants. They have been identified in the rhizosphere of many crops, including maize, and have been used as biofertilizers. This study's findings could help in the development of microbial inoculum, which could be used to replace synthetic fertilizer and achieve sustainable intensification of maize production during the industrial revolution.

Keywords: biotechnology, food security, bacteria, archaea, sustainable agriculture

INTRODUCTION

An agroecosystem where yields are increased without an adverse effect on the environment and a need for additional non-agricultural land is referred to as sustainable intensification (SI) (Pretty and Bharucha, 2014). The focus on agricultural intensification to increase yield for the growing population has escalated environmental degradation (Armstrong McKay et al., 2019). Furthermore, many agriculturists have yet to adopt environmental sustainability because the problem of low yields has not been addressed (**Figure 1**). Sustainable intensification can concurrently address environmental security and food security. This is because as agricultural production would be increased, environmental degradation would be reduced simultaneously without acquiring more land for farm use (Hunt et al., 2019). The components of SI (**Figure 1**) protect the process of an ecosystem and biological diversity while achieving an increase in food production. However, to achieve this aim, the development of suitable techniques for estimating both the

Appendix 5: Article published from this study

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ORIGINAL PAPER

Relationship between nitrifying microorganisms and other microorganisms residing in the maize rhizosphere

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Abstract

The microbial network of rhizosphere is unique as a result of root exudate. Insights into the relationship that exists with the energy metabolic functional groups will help in biofertilizer production. We hypothesize that there exists a relationship between nitrifying microorganisms and other energy metabolic functional microbial groups in the maize rhizosphere across different growth stages. Nucleospin soil DNA extraction kit was used to extract DNA from soil samples collected from maize rhizosphere. The 16S metagenomics sequencing was carried out on Illumina Miseq. The sequence obtained was analyzed on MG-RAST. *Nitrospira* genera were the most abundant in the nitrifying community. Nitrifying microorganisms were more than each of the studied functional groups except for nitrogen-fixing bacteria. Also, majority of the microorganisms were noticed at the fruiting stage and there was variation in the microbial structure across different growth stages. The result showed that there exists a substantial amount of both negative and positive correlation within the nitrifying microorganisms, and between them and other energy metabolic functional groups. The knowledge obtained from this study will help improve the growth and development of maize through modification of the rhizosphere microbial community structure.

Keywords Predictive functional analysis · Root exudate · Nitrogen fixing bacteria · Methane-oxidizing bacteria · Carbon fixation

Introduction

The complexity of the microbial network in rhizosphere has become unique over time when compared to the surrounding bulk soil as plants grow. Aside from exudates produced by plants, which is one of the causes (Peiffer et al. 2013), the secretion and detection of signaling compounds are usually produced between microbes, from plants to microbes, and from microbes to plants (Venturi and Keel 2016). These account for the different functional gene diversity between bulk soil and the rhizosphere, with the latter harboring many gene copies and different functional genes than bulk soils (Pascual et al. 2018). The signal from plants to microorganisms via small plant-secreted molecules allows microbial communities to form and synchronize (Venturi and Keel 2016). This has been implicated in several specialized relationships and most probably occurs frequently in other interactions.

There are several functional groups in the soil microorganisms associated with energy metabolism. These groups of microorganisms obtain energy through the absorption of nutrients. One of such group is the nitrifying microorganisms, which make use of ammonia to produce nitrate. Another is the nitrogen fixers, which converts atmospheric nitrogen into ammonia (Wagner 2011). The sulfur-reducing bacteria group makes use of sulfate to produce hydrogen sulfide (Myhr and Torsvik 2000). While carbon-fixing bacteria groups carry out oxygenic photosynthesis using carbon as an electron source (Stanier and Cohen-Bazire. 1977). Also among the functional groups are the methane-oxidizing bacteria, which make use of methane as their energy source (Anthony 1983).

Previous studies have proven that there is a form of relationship between these functional groups and nitrifying bacteria (Peng et al. 2020; Zhang et al. 2020; Cao et al. 2021). A significant correlation was observed between ammonia

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