PRODUCTION OF BIOFERTILIZER FROM BACTERIA ISOLATED FROM PLANT RHIZOSPHERE AND RELATIVE EFFECTS TO INORGANIC FERTILIZER

BY

ATTAH-OLOTTAH UFEDO RUTH

M.TECH/SLS/2017/7232

A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGER STATE, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTER OF TECHNOLOGY IN MICROBIOLOGY (ENVIRONMENTAL MICROBIOLOGY)

SEPTEMBER, 2021

ABSTRACT

Production of biofertilizer from bacteria isolated from plant rhizosphere and the relative effects to inorganic fertilizer was conducted by identifying the bacteria with the ability to solubilise phosphorous and fix nitrogen. *Alcaligene faecalis* and *Providencia vermicola* were identified as phosphate-solubilising and nitrogen fixing bacteria, respectively. Production of biofertilizer was conducted using fermentation process. Charcoal was used as a carrier material which was mixed with the bacterial consortium to form a powder. A pot experiment was conducted for seven (7) weeks using Maize (*Zea mays*) to compare the effectiveness of biofertilizer to inorganic fertilizer. The physicochemical parameters (Nitrogen 9.0 %, Phosphorus 33.7 mg/kg, Potassium 1.3 $Cmolkg^{-1}$) revealed that the organisms used for the production of biofertilizer had the potential to improve the soil quality. The growth parameters of the plant measured (Height of plant, Length of Leaf and Number of Leaf) and physicochemical parameters of the soil after planting revealed significant differences ($p < 0.05$) in biofertilizer effects on plant when compared with inorganic fertilizer. The study therefore, indicated that *Alcaligene faecalis* and *Providencia vermicola* can be employed as a potential Plant Growth-Promoting Rhizobacteria (PGPR), making the biofertilizer suitable for the growth of *Zea mays* as well as improving the soil quality.

TABLE OF CONTENTS

Content

Page

LIST OF TABLES

Table
Page

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

PGPR	Plant Growth-Promoting Rhizobacteria
BGA	Blue-Green Algae
SOB	Sulphur-Oxidizing Bio-fertilizer
NFB	Nitrogen-Fixing Bio-Fertilizers
PGPB	Plant Growth-Promoting Biofertilizer
KMB	Potassium-Mobilizing Biofertilizer
PMB	Phosphate-Mobilizing Biofertilizers
KSB	Potassium-Solubilizing Biofertilizer
PGPB	Plant Growth-Promoting Biofertilizer
AMF	Arbuscular Mycorrhizal Fungi
NPK	Nitrogen Phosphorous Potassium
FYM	Farm Yard Manure

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

The human population has grown over time, so has the demand for food. The inability to supply food to meet the demand has resulted in the current state of food insecurity in the world today. Food security is a global issue that is related to the environment, the economy, and the society (Matemilola and Elegbede, 2017). Plant nutrition is an important factor significant in food demand and supply. Although, over time crop output has increased due to the usage of commercial man-made fertilizers (Chemical fertilizers) to increase yield. The massive rise in Nitrogen (N) and Phosphorus (P) fertilization, along with the advent of highly productive and intensive agricultural systems, has allowed these advancements to take place at relatively low cost (Aggarwal *et al*., 2011).

The continuous use of chemical fertilizers and high-yielding systems has led to environmental issues such as soil quality degradation, biodiversity loss, surface and groundwater contamination, air pollution and reduced ecosystem function (Aggarwal *et al*., 2011). These hazards resulting from the prolonged use of chemical fertilizers affects man and the environment directly and indirectly. Misuse and excessive or poorly managed fertilizer usage can directly result in leaching, volatilization, acidification, eutrophication and denitrification. Indirectly, fertilizer manufacturing (use of fossil fuel in Haber Bosch process) and transportation (combustion of fossil fuel) result in airborne Carbon dioxide (CO_2) and Nitrogen (N) pollution, which is eventually deposited in terrestrial ecosystems (European Commission, 2013). Harpole *et al*. (2011) reported that the most limiting nutrients for plant growth are Nitrogen (N) and Phosphorous(P). The soil is a habitat of a vast number of organisms resulting in high organic matter content, one would wonder, how can elements possibly be limited when it should be in vast amount? The answer is simple, most nutrient is not readily available for plant use.

Majority of N in the soil is bonded to the soil organic matter. Plants compete with soil microorganisms for easily available soluble N even after fertilization. P precipitates with iron or aluminium in acidic soils, even when substantial amount of fertilizer is applied whereas, P precipitates as calcium phosphates in alkaline soils (Aggarwal *et al*., 2011). Due to phosphate fixation by mineral ions such as Iron (Fe), Aluminium (Al) and Calcium, or by organic acids, the accessible phosphate (Pi) level in the soil is significantly below plant demands. Chemical Pi fertilisers are also quickly immobilised in the soil, with less than 20 % being taken up by plants (Withers *et al*., 2014). As a result, overcoming P limitation with the addition of P-containing fertilizers can be challenging. To address N and P deficiency using chemical fertilizer, large application rates will be required, which might result in (underground) runoff, where soluble nutrients can easily reach surface or groundwater. In industrial countries, for example, P loss in agricultural systems is a primary cause of hypoxia and eutrophication in lakes and estuaries (Withers *et al*., 2014). Hence, Phosphate-Solubilizing Bacteria (PSB) or Phosphate Biofertilizers have been offered as a remedy to the environmental deterioration caused by the depletion of Pi resources on the one hand, and the manufacturing and application of Pi fertilizers on the other (Debnath *et al*., 2019). Biofertilizers contain microorganisms that can help restore soil's natural nutrient cycling and build organic matter. The use of biofertilizers promotes the growth of healthy plants as well as the long-term sustainability and the health of the

soil. The recommended scientific term for these beneficial bacteria is "Plant Growth-Promoting Rhizobacteria" (PGPR) because of the various activities they perform. As a result, microorganisms and their by-products are particularly effective for increasing soil fertility and delivering organic nutrients to meet the nutritional needs of plants. Biofertilizers does not include any soil-harming chemicals (Al-Erwy *et al*., 2016).

Biofertilizers provide "eco-friendly" organic agro-input. *Rhizobium*, *Azotobacter*, *Azospirilium* and Blue-green algae (BGA) have long been utilised as biofertilizers. *Rhizobium* inoculants is used for leguminous crops. *Azotobacter* inoculants are recommended for wheat, maize, mustard, cotton, potato and other vegetable crops. *Azospirillum* inoculants are recommended for sorghum, millets, maize, sugarcane and wheat (Shree, 2020). *Cyanobacteria species*, (such as *Nostoc, Anabaena, Tolypothrix and Aulosira*) are Blue-green algae that fix atmospheric nitrogen and are utilised as inoculants for upland and low-land rice crops (Shree, 2020). Anabaena in symbiotic association with water fern *Azolla* can contribute up to 60 kg/ha/season of nitrogen as well as organic matter to the soil (Shree, 2020). Other types of bacteria, so-called phosphate-solubilizing bacteria, such as *Pantoea agglomerans* strain P5 or *Pseudomonas putida* strain P13, are able to solubilize the insoluble phosphate from organic and inorganic phosphate sources. In order for developing countries to produce adequate food for their rising populations at low cost, efficient and sustainable processes are required (Tuğrul, 2019). Microorganisms that can utilize nutrients more efficiently or enhance their availability can give long-term solutions to existing and future agricultural practises, alleviate environmental issues connected with plant nutrient loss, and boost crop yields without the use of costly fertilizers (Tuğrul, 2019).

1.2 Statement of The Research Problem

Inorganic fertilizers are applied on soil broadly and excessively to achieve high yield, but the application of these chemicals over a long period of time has led to environmental hazards such as increased soil acidity, degraded soil fertility (making it unsuitable for raising food crops), soil erosion, water contamination, falling ground water table, water logging, depletion of biodiversity and greenhouse effect. The inorganic fertilizer also has a small but cumulative effect on the health of humans and animals that consume food crops produced using chemical fertilizers. Some of these health challenges are malnutrition, cancer, waterborne diseases. The damages caused by chemical fertilizers are long-term and cumulative, it may be wiser to consider alternative and sustainable methods of fertilizing the soil, hence the necessity for this research.

1.3 Justification for the Study

According to Meenakshi (2016), Biofertilizers maintains the soil environment in its rich properties of micro and macro nutrients through activities of phosphate mineralization or solubilisation, nitrogen fixation, potassium mineralization or solubilisation, stimulating the release of plant growth regulating substances, improved nutrient uptake, biodegradation of organic matter, overcoming moisture and drought stress through increased tolerance and production of antibiotics. It poses little to no effect on man and animal. Most biofertilizers are crop specific. This research focused on alternative bacterial consortium in which its synergistic potential of Phosphorous solubilisation and nitrogen fixation supports the growth of *Zea mays*.

1.4 Aim and Objectives of the study

1.4.1 Aim

The aim of this study was to produce biofertilizer from bacteria isolated from plant rhizosphere and relate its effects with inorganic fertilizer

1.4.2 Objectives

The objectives of the study were to:

i. Isolate, characterize and identify bacteria from plant rhizosphere

ii. Produce biofertilizer with the isolated bacteria

iii. Characterize the biofertilizer produced

iv. Investigate the effects of biofertilizer produced and inorganic fertilizer on the growth of maize plant.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biofertilizer

Bio-fertiliser is a combination of two words **Bio-** meaning living or organic, and **Fertilizer-** a substance applied to the soil to increase its nutritional contents. A bio-fertilizer is a biological fertilizer devoid of chemical and it is a perfect green solution in improving food safety in the world (Bhat *et al.,* 2020); Hari *et al*. (2010) states that bio-fertilizer is normally discussed as a source of selected strains of beneficial soil microorganisms cultured in the laboratory with suitable carriers. Bio-fertilizers are important components of integrated nutrient management. Bio-fertilizer is a substance

constituting of microorganisms that inhabits the rhizosphere and interior of a plant, through the application to plants surfaces, soil or seeds, and increases the availability of primary nutrient and promotes growth to the plant (Itelima *et al.*, 2018b).

2.2 History of Biofertilizers

The history of organic farming is also termed the history of the organic movement, which began as an insider's group of agricultural scientists and farmers, and later expanded to become a grassroots consumer cause. Initially, organics focused on the methods, as a definite reaction against the industrialization of agriculture, and remained below the awareness of the food buyer (Bouyoucos, 2014). Only when the contrasts between organics and the new conventional agriculture became overwhelming, did organics rise to the attention of the public, creating a distinct organic market. World War II marks the two phases. Pre-World War II - The first 40 years of the 20th century saw simultaneous advances in biochemistry and engineering that rapidly and profoundly changed farming. Research in plant breeding led to the commercialization of hybrid seed, and a new manufacturing process made nitrogen fertilizer first synthesized in the mid -1800s affordably abundant. These factors changed the labour equation: there were some 600 tractors in the US around 1910, and over 3,000,000 by 1950; in 1900, it took one farmer to feed 2.5 people, where currently the ratio is 1 to well over 100. Fields grew bigger and cropping more specialized to make more efficient use of machinery (Barton, 2018).

In England in the 1920s, a few individuals in agriculture began to speak out against these agricultural trends consciously, organic agriculture (as opposed to the agriculture of indigenous cultures, which always employs only organic means) began more or less simultaneously in Central Europe and India. The British botanist Sir Albert Howard is often referred to as the father of modern organic agriculture. From 1905 to 1924, he worked as an agricultural adviser in Pusa, Bengal, where he documented traditional Indian farming practices, and came to regard them as superior to his conventional agriculture science. His research and further development of these methods is recorded in his writings, notably, his 1940 book, An Agricultural Testament, which influenced many scientists and farmers of the day (Ceballos *et al.*, 2013).

In Germany, Rudolf Steiner's development, biodynamic agriculture, was probably the first comprehensive organic farming system. This began with a lecture series Steiner presented at a farm in Koberwitz (now in Poland) in 1924. This lecture series, published in English as Spiritual Foundations for the Renewal of Agriculture, was the very first publication anywhere on organic agriculture. A number of farmers interested in finding a healthier approach to farming attended the course, and several farms began working with a biodynamic/organic approach. Steiner emphasized on the farmer's role in guiding and balancing the interaction of the animals, plants and soil. Healthy animals depended upon healthy plants for their food, healthy plants depended upon healthy soil for nutrients, and healthy soil upon healthy animals for the manure (Ceballos *et al.*, 2013). In the early 1900s, American agronomist F.H. King toured China, Korea, and Japan, studying traditional fertilization, tillage, and general farming practices. He published his findings in Farmers of Forty Centuries (Fattah, 2013). King probably did not view himself as part of a movement, organic or otherwise, but in later years his book became an important organic reference. In 1939, influenced by Sir Howard's work, Lady Eve Balfour launched the Haughley Experiment on farmland in England. It was the first scientific, side-by-side comparison of organic and conventional farming. Four years later, she published The Living Soil, based on the initial findings of the Haughley Experiment. Widely read, it led

to the formation of a key international organic advocacy group, the Soil Association (Abbott and Johnson, 2017). The coinage of the term organic farming is usually credited to Lord Northbourne, in his book, look to the Land (1940), wherein he described a holistic, ecologically-balanced approach to farming (Abbott and Johnson, 2017). In Japan, Masanobu Fukuoka, a Microbiologist working in soil science and plant pathology, began to doubt the modern agricultural movement. In the early 1940s, he quit his job as a research scientist, returned to his family's farm, and devoted the next 30 years to developing a radical no-till organic method for growing grain, now known as Fukuoka farming (Abbott and Johnson, 2017).

Post-World War II - Technological advances during World War II accelerated post-war innovation in all aspects of agriculture, resulting in big advances in mechanization (including large-scale irrigation), fertilization, and pesticides. In particular, two chemicals that had been produced in quantity for warfare, were repurposed to peace-time agricultural uses. Ammonium nitrate, used in munitions, became an abundantly cheap source of nitrogen. And a range of new pesticides appeared: DDT, which had been used to control disease-carrying insects around troops, became a general insecticide, launching the era of widespread pesticide use (Abbott and Johnson, 2017). At the same time, increasingly powerful and sophisticated farm machinery allowed a single farmer to work over larger areas of land. Fields grew bigger, and agribusiness as we know it today was well on its way (Abbott and Johnson, 2017). In 1944, an international campaign called the Green Revolution was launched in Mexico with private funding from the US. It encouraged the development of hybrid plants, chemical controls, large-scale irrigation, and heavy mechanization in agriculture around the world (Hinsinger *et al.*, 2012). During the 1950s, sustainable agriculture was a topic of scientific interest, but research tended to concentrate on developing the new chemical approaches. In the US, J.I. Rodale began to popularize the term and methods of organic growing, particularly to consumers through promotion of organic gardening (Mia *et al.*, 2010). In 1962, Rachel Carson, a prominent scientist and naturalist, published Silent Spring, chronicling the effects of DDT and other pesticides on the environment. A bestseller in many countries, including the US, and widely read around the world, Silent Spring is widely considered as being a key factor in the US government's 1972 banning of DDT. The book and its author are often credited with launching the worldwide environmental movement (Mia *et al.*, 2010).

In the 1970s, global movements concerned with pollution and the environment increased their focus on organic farming. As the distinction between organic and conventional food became clearer, one goal of the organic movement was to encourage consumption of locally grown food, which was promoted through slogans like "Know Your Farmer, Know Your Food" (Mia *et al.*, 2010).In 1972, the International Federation of Organic Agriculture Movements, widely known as IFOAM, was founded in Versailles, France, and dedicated to the diffusion and exchange of information on the principles and practices of organic agriculture of all schools and across national and linguistic boundaries (McGonigle *et al.*, 2013). In 1975, Fukuoka released his first book, One Straw Revolution, with a strong impact in certain areas of the agricultural world. His approach to small-scale grain production emphasized a meticulous balance of the local farming ecosystem, and a minimum of human interference and labour (McGonigle *et al.*, 2013) In the 1980s, around the world, various farming and consumer groups began seriously pressuring for

government regulation of organic production. This led to legislation and certification standards being enacted through the 1990s and to date (Ghazali *et al.,* 2020). Since the early 1990s, the retail market for organic farming in developed economies has been growing by about 20% annually due to increasing consumer demand. Concern for the quality and safety of food, and the potential for environmental damage from conventional agriculture, are apparently responsible for this trend (Ghazali *et al.,* 2020). 21st Century - Throughout this history, the focus of agricultural research, and the majority of publicized scientific findings, has been on chemical, not organic farming. This emphasis has continued to biotechnologies like genetic engineering. One recent survey of the UK's leading government funding agency for bioscience research and training indicated 26 GM crop projects, and only one related to organic agriculture (Ghazali *et al.,* 2020). This imbalance is largely driven by agribusiness in general, which, through research funding and government lobbying, continues to have a predominating effect on agriculture-related science and policy. Agribusiness is also changing the rules of the organic market. The rise of organic farming was driven by small, independent producers, and by consumers. In recent years, explosive organic market growth has encouraged the participation of agribusiness interests. As the volume and variety of "organic" products increases, the viability of the small-scale organic farm is at risk, and the meaning of organic farming as an agricultural method is ever more easily confused with the related but separate areas of organic food and organic certification (Mia *et al.,* 2010).

2.3 Types of Biofertilizer

The classification of biofertilizer is based on the type or group of microorganisms it is made up of; these classifications are as follows: Sulphur-Oxidizing Biofertilizer (SOB), Nitrogen-Fixing Biofertilizers (NFB), Plant Growth-Promoting Biofertilizer (PGPB), Potassium-Mobilizing Biofertilizer (KMB), Phosphate-Mobilizing Biofertilizers (PMB) and Potassium-Solubilizing Biofertilizer (KSB), (Itelima *et al.*, 2018a).

2.3.1 Nitrogen-fixing biofertilizers (NFB)

Examples include *Rhizobium* species, *Azospirillum* species and blue-green algae; these work by fixing atmospheric nitrogen and converting them to organic (plant usable) forms in the soil and root nodules of legumes, thereby making them available to plants. Nitrogen-fixing biofertilizers are crop specific biofertilizers (Itelima *et al.,* 2018a).

2.3.2 Phosphate-solubilizing biofertilizer (PSB)

Examples include *Bacillus* specie, *Pseudomonas* specie and *Aspergillus* specie. These work by solubilizing the insoluble forms of phosphate in the soil, so that plants can use them. Phosphorus in the soil occurs mostly as insoluble phosphate which cannot be absorbed by plants. However, several soil bacteria and fungi possess the ability to convert these insoluble phosphates to their soluble forms. These organisms accomplish this by secreting organic acids which lower the pH of the soil and cause the dissolution of bound forms of phosphate making them available to plants (Itelima *et al.,* 2018a).

2.3.3Phosphate-mobilizing biofertilizers (PMB)

Examples are *Mycorrhiza*. They work by scavenging phosphates from soil layers and mobilizing the insoluble phosphorus in the soil to which they are applied. Phosphate mobilizing biofertilizers are broad spectrum biofertilizers (Itelima *et al.,* 2018a).

2.3.4 Plant growth-promoting biofertilizer (PGPB)

Examples of plant growth rhizobacteria are *Pseudomonas* species, these work by producing hormones and anti-metabolites which promotes root growth, decomposition of organic matter which help in mineralization of the soil thereby increasing availability of

nutrients and improving crop yield. PGPB are crop specific biofertilizers (Itelima *et al.,* 2018a).

Table 2.1 Types of Biofertilizer and Microorganism used in Production

Groups	Example
	Nitrogen Fixing Biofertilizers
Free-living	*Azotobacter, Bejerinkia, Clostridium, Klebsiella, Anabaena, Nostoc*
Symbiotic	*Rhizobium, Frankia, Anabaena, Azollae*
Associative symbiotic	*Azospirillum*
	Phosphate Solubilizing Biofertilizer
Bacteria	*Bacillus megaterium var, Phosphaticum, Bacillus subtilis, Bacillus circulans*
Fungi	*Penicillum* Spp. *Aspergillus awamori*
	Phosphate Mobilizing Biofertilizers
Arbuscular Mycorrhiza	*Glomus* Spp., *Gigaspora* Spp., *Acaulospora* Spp. *Scutellospora* Spp. and *Sclerocystis* Spp.
Ectomycorrhiza	*Laccaria* Spp., *Pisolithus* Spp., *Boletus* Spp. and *Amanita* Spp.
Ericoid Mycorrhiza	*Pezizella ericae*
Orchid Mycorrhiza	*Rhizoctonia solani*
	Biofertilizers for Micronutrients
Bacillus Spp.	Silicate and zinc solubilizers
	Plant Growth Promoting Rhizobacteria
Pseudomonas	*Pseudomonas fluorescens*

Source: (Itelima *et al.,* 2018a).

2.3.5 Potassium-solubilizing biofertilizer (KSB)

Examples include *Bacillus* species and *Aspergillus niger*. Potassium in the soil occurs mostly as silicate minerals which are inaccessible to plants. These minerals are made available only when they are slowly weathered or solubilized. Potassium-solubilizing microorganisms solubilize silicates by producing organic acids which cause the decomposition of silicates and helps in the removal of metal ions thereby making them

available to plants. Potassium-solubilizing biofertilizers are broad spectrum biofertilizers (Itelima *et al.,* 2018a).

2.3.6 Potassium-mobilizing biofertilizer (KMB)

Example of potassium-mobilizing biofertilizer is *Bacillus* species. These work by mobilizing the inaccessible forms of potassium (silicates) in the soil. Some phosphate-solubilizing biofertilizers such as *Bacillus* species and *Aspergillus* species has been found to mobilize potassium and also solubilize phosphorus (Itelima *et al.,* 2018a).

2.3.7 Sulphur-oxidizing biofertilizer (SOB)

Example of sulphur-oxidizing microorganism is *Thiobacillus* species. These work by oxidizing sulphur to sulphates which are usable by plants (Itelima *et al.,* 2018a).

2.4 Production of Biofertilizer

Biofertilizers are the end result of the fermentation process, and they are made up of effective live soil microorganisms. The two primary forms of fermentation employed for the generation of biofertilizers are solid-state fermentation and submerged fermentation. Each type of biofertilizer is created by selecting an efficient microbial strain, cultivating it in a specific nutrient medium, scaling it up, and combining it with other ingredients (such as carriers and additives) to protect the microbial cell (Suthar *et al.,* 2017). In comparison to chemical fertilizers, the production of biofertilizer is inexpensive and simple (Adeleke *et al*., 2019). The methods involved in the production of biofertilizer are described further below.

2.4.1 Production of inoculants

The production of an effective inoculant is a multistep process that includes the attachment of one or more strains of microorganisms in a specific carrier, as well as sticking agents or other additives that protect the cells during storage and transportation. Because inoculants are frequently stored in less-than-ideal conditions (e.g., high temperature, light exposure), they must have a long shelf life, which means that the microbe must be either resilient or have a stronger potential to live in large numbers under severe conditions (Herrmann and Lesueur, 2013). To be easily accepted by farmers, an inoculant must be inexpensive and simple to handle and apply, ensuring that the microorganisms are delivered to the target plant in the most appropriate way and form. A proper formulation will offer effective introduction of microorganisms into the soil and will boost their activity in order to maximize the advantages after inoculation of the host plants (Koske and Gemma, 2013). However, there are some critical steps which must be precisely considered during the biofertilizers production (Kapulnik *et al.,* 2010). The choices made at these steps can lead to the success or the failure of the inoculation. The decision of the microorganisms to be inoculated is of crucial importance. Some of the most important desirable characteristics of the inoculant strain (bacterial or fungal) include its genetic stability, its ability to be beneficial for the target crops, to be competitive to the indigenous populations, to migrate from inoculation site to the hosts, and to survive in hostile soil without the presence of the host. Other important features sought during production is the ability of the strain to grow in laboratory conditions (exception is made for AMF (*Arbuscular mycorrhizal* Fungi) which cannot grow without a host plant), grow or survive in carriers (during curing or storage), on seeds and in soil and to be compatible with agrochemical products that might be applied on seeds. The live inoculant must also be able to overcome the various technological processes during production and maintain its functional properties (Kapal,

2010). Bacterial inoculants are generally cultivated in liquid medium to reach high biomass yields. The composition of the media and growth conditions (temperature, pH, agitation, aeration, etc.) are directly related to the physiology-biochemical properties of the particular strain and the kind of inoculant that is to be produced. Obtained bacterial cultures are then used to inoculate the different carriers (encapsulation or impregnation of peat and granules), or after addition of various additives liquid formulations could be produced. The large-scale production of bacteria in pure cultures using bioreactors is wildly spread common practice as shown in Plate I.

Plate I.: Mass production of Azolla
Source: (Debojyoti *et al.,* 2015)

2.4.2 Additives

Other materials added to the inoculant formulation include macro- and micronutrients, carbon or mineral sources, hormones, and even fungicides. The aim is to supply microorganisms with protective and/or a nutrient source, to assure better adhesion to seed thus improving the inoculant quality, to make the product more stable, to inactivate the toxins, or to enhance the strain(s) survival during storage and after exposure to environmental stress conditions (high temperature, desiccation) (Singh *et al.*, 2013). There is a critical interrelation between the strains survival rate and used additives. Some of them (such as glycerol) improve cell viability by protecting cells from desiccation through holding considerable amounts of water. Thus, the drying rate is significantly reduced. Each additive should be selected for individual strain in order to provide maximal performance. Moreover, their chemical nature should be complex to prevent them from rapid degradation. Several components have been tested, such as clay and skim milk, xanthan, or sodium alginate with variable results on strain(s) survival during storage and field application (Mukhongo *et al.,* 2016).

Furthermore, certain signalling molecules added in the growth media and inoculants have been shown to provoke desired physiological activities of used microorganisms. Recently, it was reported that some rhizobial metabolites enhance the performance of Bradyrhizobium spp. and *Azospirillum brasilense* inoculants when soybean and maize are treated. These metabolites include mainly lipochitooligosaccharides (LCOs also called Nod factors) but also exopolysaccharides and plant hormones. Nod factors were shown to be produced by most rhizobia and are mandatory for the root legume infection and nodule formation (Mukhongo *et al.,* 2016). The use of signalling molecules for improving the crop performance is still limited. However, several legume inoculants containing stimulators of nodulation (flavonoids or Nod factors) are commercially available in North and South America. Stimulators of the mycorrhizal symbiosis have also been identified. Strigolactones are of fundamental and practical interest as they are supposed to play a key role in the establishment of the mycorrhizal symbiosis. It was reported that they act as a hormone in plants, and they may also have a role in the pre-symbiotic growth of AMF. Application to crops could result in beneficial effects on plant development. However, more investigations are needed to assess the potential of these stimulators for the development of a new generation of mycorrhizal inoculants (Sharma *et al.,* 2013).

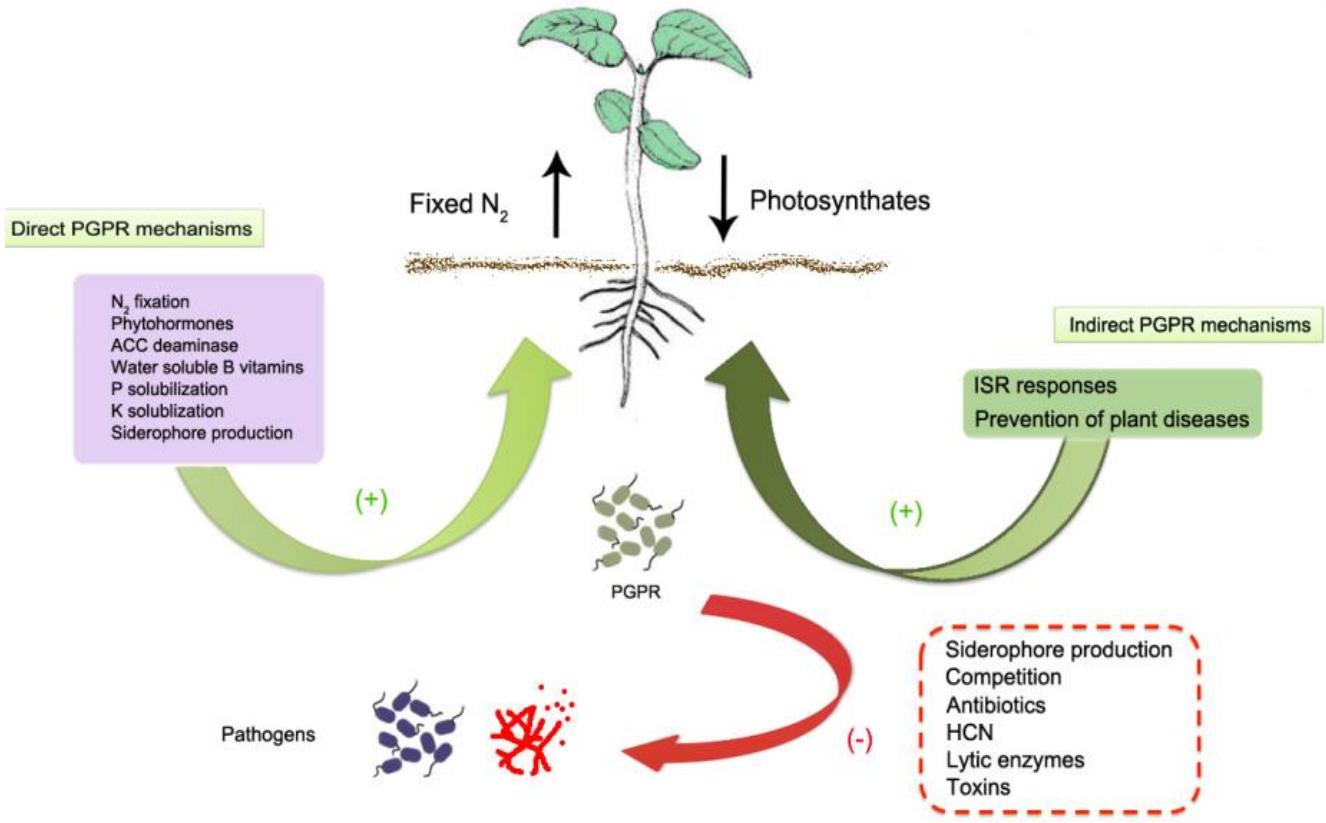
2.4.3 Packaging

Packaging material is another important issue to be consider when biofertilizer is produced as it can affect inoculant quality. It must allow some exchange of oxygen but restrict the passage of water. Particular care must be taken when choosing a material for a product that is supposed to be sterilized. Some materials are suitable for autoclaving but might break during irradiation and vice versa (Verbruggen *et al.,* 2012).

2.5 Mechanisms of Microbial Fertilizer Action

The mechanisms of microbial fertilizer are solely based on the interaction of the microorganisms with the environment and plant. Most of these interactions involve the release of metabolites from the microorganisms that act as growth enriching substances, antibiotics and stress resistance.

Figure 2.1: Mechanisms of Plant Growth-Promoting Rhizobacteria.
Source: (Uwah *et al.*, 2013)



2.5.1 Plant growth-promoting rhizobacteria

Rhizobacteria can promote plant growth through a broad variety of mechanisms, which can be grouped according to their mode of action in: (i) the synthesis of substances that can be assimilated directly by plants, (ii) the mobilization of nutrients, (iii) the induction of plant stress resistance and (iv) the prevention of plant diseases (Vanlauwe *et al.*, 2010).

2.5.2 Synthesis of substances that can be assimilated directly by plants.

Nitrogen, required for the formation of amino acids and proteins, is the most limiting nutrient for plants. The process by which atmospheric nitrogen is combined into organic forms that can be assimilated by plants is exclusive to prokaryotes (Taraken *et al.*, 2010). Some examples of free-living nitrogen-fixing organisms are *Azospirillum*, commonly associated with cereals in temperate zones and also reported to be able to improve rice crop yields; *Beijerinckia*, which seems to be associated with sugar cane plantations in tropical zones, and *Azotobacter*, which plays an important role in nitrogen fixation in rice crops and is used as a biofertilizer for wheat, barley, oat, rice, sunflowers, maize, line, beetroot, tobacco, tea, coffee and coconuts (Bailey *et al.*, 2016). Some species belonging

to the genera *Gluconacetobacter*, *Azospirillum* and *Herbaspirillum* are sugarcane endophytes and contribute to its nitrogen fertilization. *Herbaspirillum* has also been isolated from bean and rice. Some studies report how *Azoarcus*, *Azospirillum* and *Burkholderia* strains enter rice roots and increase the amount of nitrogen compounds in the crop. Nitrogen-fixing *Azorhizobium* strains have been isolated from wheat roots, and *Rhizobium* and *Bradyrhizobium* in rice roots (Schubert and Hayman, 2011). Moreover, certain diazotrophic bacteria establish truly mutualistic symbiosis with some plants through the formation of root nodules. These symbioses are found between rhizobia and legumes and *Frankia* and actinorhizal plants (Wang *et al.,* 2015).

Plant hormones are organic molecules involved in several plant growth and development processes. Phytohormone biosynthesis by some microorganisms is involved in pathogenesis in plants, but a wide spectrum of beneficial bacteria produces phytohormones that are involved in plant-growth promotion. Auxins act as key molecules, regulating most plant processes directly or indirectly (Liu *et al.,* 2010). Several bacteria secrete auxins, which seem to act as signaling molecules for bacterial communication in order to coordinate activities. Auxin-producing *Bacillus spp.* Have been reported to exert a positive effect in *Solanuntuberosum* development. Indole-3-acetic acid (IAA) is the best known and most active auxin in plants. The endophytic *Streptomyces* isolated from *Azadirachtaindica* produce IAA and are potential plant growth promoters (Verma and Gemma, 2014). Cytokinins promote cytokinesis, vascular cambium sensitivity, vascular differentiation and root apical dominance (Liu *et al.,* 2010). *Azotobacterchroococcum* and *Bacillus megaterium* strains were found to produce cytokinins and promote cucumber growth (Liu *et al.,* 2010). Gibberellins are involved in seed germination and emergence, stem and leaf growth, floral induction and flower and fruit development. The growth of red pepper plants was enhanced by treatment with a *Bacillus cereus* strain producing gibberellins (Rosendahl, 2012). Tomato plants inoculated with the gibberellin-producing *Sphingomonas* sp. LK11 strain showed a significant increment in several growth attributes.
Ethylene is a plant hormone known to regulate several processes such as the ripening of fruits, the opening of flowers or the abscission of leaves. However, it also promotes seed germination, secondary root formation and root-hair elongation. *Phyllobacterium brassicacearum* STM196 emits ethylene and contributes to root hair elongation in *Arabidopsis thaliana* (Rosendahl, 2012). High levels of ethylene, produced under stressed conditions, can inhibit certain processes such as root elongation or nitrogen fixation in legumes and contribute to premature senescence (Rosendahl, 2012).
Some bacteria produce the enzyme, 1-aminocyclopropane-1-carboxylate, to hydrolyse ACC, the precursor molecule of ethylene in plants, to obtain ammonia and α-ketobutyrate, which can be used as nitrogen and carbon sources. Therefore, these bacteria lower ethylene levels in plants and hence prevent some of the negative effects produced by high ethylene concentrations (Wen, 2015)
Microbial vitamin production promotes crop yields, affecting plant growth at different levels, enhancing plant-rhizobial symbiosis and plant mycorrhization. Plant growth promoting strains of *Azotobacter* have been described to be able to produce B-group vitamins (Revillas *et al.,* 2000).

2.5.3 Nutrient mobilization

After nitrogen, phosphorous (P) is the second essential nutrient in terms of necessary uptake amounts in plants. This element is fairly insoluble in soils and accordingly, traditional agriculture has been based on the application of chemical P fertilizers. Nevertheless, when applied as fertilizer to fields P passes rapidly to become insoluble and hence unavailable to plants (Wen, 2015). Accordingly, the use of P-solubilizing bacteria represents a green substitute for chemical P fertilizers (Wen, 2015). *Micrococcus, Pseudomonas, Bacillus* and *Flavobacterium* have been reported to be efficient phosphate solubilizers (Kavoo-Mwangi *et al.,* 2014). Phosphate-solubilizing rhizobial strains promote *Daucuscarota* and *Latuca sativa* growth and a *Phyllobacterium* starin able to solubilize phosphates improves the quality of strawberries. *Rhizobium leguminosarum*strainPETP01 and *R. leguminosarum* strainTPV08 solubilize phosphate and are PGPR for pepper and tomato plants (Kavoo-Mwangi *et al.*, 2014). Potassium (K) is the third essential nutrient necessary for plant growth. Some rhizobacteria are able to solubilize insoluble potassium forms (Etesami *et al.,* 2017). *Bacillus edaphicus* has been reported to increase potassium uptake in wheat and *Paenibacillus glucanolyticus* was found to increase the dry weight of black pepper. Sudan grass inoculated with the potassium-solubilizingbacterium *Bacillus mucilaginosus*had higher biomass yields. Also, *Bacillus mucilaginosus* in coinoculation with the phosphate-solubilizing *Bacillus megaterium* promoted the growth of eggplant, pepper and cucumber (Etesami *et al.,* 2017). Ahmed and Holmstrom (2014), described siderophores as organic compounds whose main function is to chelate the ferric iron (Fe (III)) from the environment. These researchers went further to explain that Microbial siderophores also provide plants with Fe, enhancing their growth when Fe is limiting, but the exact mechanisms of Fe supply to the plant are not well understood. Siderophores from endophytic *Streptomyces* promote *Azadirachtaindica* plant growth, Rhizobial strains able to produce siderophores have been reported to be potential biofertilizers, improving the production of carrots, lettuce, peppers and tomatoes, while one siderophore-producing *Phyllobacterium* strain promotes the growth and quality of strawberries (Ahmed and Holmstrom, 2014).

2.5.4 Induction of plant stress resistance

Abiotic stress in plants, originated in situations such as drought, water logging, extreme temperatures, salinity and oxidative stress, are the primary cause of crop loss worldwide (Jenkins, 2013). Liu *et* al. (2010) described *Pseudomonas* strains enhancing asparagus seedling growth and seed germination under water-stress conditions. *Pseudomonas fluorescens* MSP-393 acts as a PGPR for many crops grown in the saline soils of coastal ecosystems and *Pseudomonas putida*Rs-198 promotes cotton seedling grown under salt stress, increasing germination rates and protecting against salt stress by increasing the absorption of Mg^{2+}, K^{+} and Ca^{2+}, decreasing Na^{+} uptake, and improving the production of endogenous indole acetic acid. The inoculation of peanuts cultivated under salt-stress conditions with rhizobial strains showed comparable efficiency to the application of N fertilization in the same crop (Johnson *et al.,* 2010). Weber (2014) described that, strains of *Paenibacillus, alcaligenes, Bacillus polymyxa* and *Mycobacterium phlei* produce calcisol and improved maize growth and nutrient uptake under high temperature conditions as well as under salinity.

2.5.5 Prevention of plant diseases

The mechanisms of bacterial plant disease prevention may be direct, if pathogens are inhibited as a result from PGPR metabolism, or indirect, when the bacteria compete with the pathogens, reducing their ability to induce disease (Aderson and Ingram, 2014). Some

PGPR synthesize antibiotic substances that inhibit the growth of some plant pathogens. For instance, *Pseudomonas* sp. produces antibiotics that inhibit *Gaeumannomycesgraminis* var. tritici, the causal agent of take-all of wheat (Gao *et al.*, 2012). Most *Bacillus* ssp. produce antibiotics that are active against Gram-positive and Gram-negative bacteria, as well as many pathogenic fungi. *B. cereus* UW85 contributes to the biocontrol of alfalfa damping-off (Lamber *et al.*, 2011). Cyanogenic compounds are nitrogen-containing compounds that have been shown to repel leaf-chewing herbivores (Mwanga *et al.*, 2011b). Rhizobia-legume symbioses have been demonstrated to enhance the resistance of plants to herbivore attack. Presumably, an additional nitrogen provided by the bacterium allows the plant to synthesize cyanogenic defense compounds (Mwanga *et al.*, 2011b). Since chitin and β-glucan are the major fungal cell wall components, bacteria producing chitinases and β- glucanases inhibit fungal growth. Microbial siderophores supply Fe and control pl ant pathogens by limiting the Iron (Fe) available for the phytopathogens (Rakshit and Bh adoria, 2016). The *Fusarium* wilt produced by *Fuxarium oxysporum* in potato is controlled by *Pseudomonas* siderophores (Mwanga *et al.*, 2011a). *Pseudomonas* species and *Bacillus* species strains produce siderophores that inhibit fungal pathogens in maize, while siderophores from the *Chryseobacterium*sp C138 strain are effective in supplying Fe to iron-starved tomato plants (Ahmed and Holmstrom, 2014). Menendez and Garcia-fraile (2017) reported that a siderophore-producing strain identified as *Bacillus subtilis*exertsa biological control effect on *Fusarium* wilt and promotes pepper growth, and Verma *et al*. (2014) reported that endophytic *Streptomyces* isolated from *Azadirachtaindica* produce siderophores with biocontrol potential. Finally, the presence of PGPR in the rhizosphere and rhizoplane might prevent plant diseases by competing for available nutrients, reducing the contact surface between the pathogen and the plant root or by interfering with the mechanisms leading to plant disease (Bouyoucos, 2014).

2.6 Application of Biofertilizer

Microbial fertilizers can be inoculated on seeds as well as in the roots of different crop plants under ideal conditions. They can also be applied directly to the soil. There are certain approaches to the application of biofertilizers as described below.

2.6.1 Seed inoculation or seed treatment method

This is the most common practice of applying biofertilizers. In this method, the biofertilizers are mixed with 10% solution of jaggery (which is a traditional non-centrifugal cane sugar). The slurry is poured over the seeds spread on a cemented floor and mixed properly in a way that a thin layer is formed around the seeds. The treated seeds are dried in the shade overnight and ready for use. Generally, 750 g of biofertilizer is required to treat legume seeds for a one-hectare area (Anderson and Ingram, 2014).

2.6.2 Seedling root dip method

The seedling roots of transplanted crops are treated for half an hour in a solution of biofertilizer before transplantation into the field. In this method, the seedlings required for one acre are inoculated using 2–2.5 kg of biofertilizer. For this, a bucket having adequate quantity of water is taken and the biofertilizer is mixed properly. The roots of the seedlings are dipped in this mixture so as to enable the roots to get inoculum before the seedlings are transplanted. This method has been found very much suitable for crops like tomato, rice, onion, Cole crops and flowers (Itelima *et al.*, 2018b).

2.6.3 Main field application method

This method is mostly used for fruit crops, sugarcane and other crops where localized application is needed. At the time of planting of fruit trees, 20 g of biofertilizer is mixed with compost and added in the ring of one sampling. The same quantity of biofertilizer may be added in the ring soil of the seedling after it has attained maturity. Sometimes, biofertilizers are also introduced in the soil but this may require four to ten times more biofertilizers. Before use, the inoculants are incubated with the desired amount of well decomposed granulated farmyard manure (FYM) for 24 hours. The FYM acts as nutrition medium and adjuvant (carrier) for biofertilizers (Johnson *et al.,* 2010).

2.6.4 Self-inoculation or tuber inoculation

This method is exclusively suitable for application of *Azotobacter Biofertilizer.* In this method, 50 L of water is measured into a drum and 4–5 kg of *Azotobacter* biofertilizer is added and mixed properly. Planting materials required for one acre of land are dipped in this mixture. Similarly, this is used in treating potato tubers, the tubers are dipped in the mixture and planting is done after drying the materials in the shade (Lamber *et al.,* 2011).

2.7 Precautions in Biofertilizer Application

Biofertilizer packets are stored in a cool and dry place away from direct sunlight and heat. The following precautions are to be observed during application of biofertilizer:

i. Right combinations of biofertilizers are to be used

ii. As *Rhizobium* is crop specific, it should be use for the specified crop only

iii. Other chemicals should not be mixed with the biofertilizers

iv. When purchasing, one should ensure that each packet is provided with all necessary information such as the name of the product; the name of the crop for which it is intended; the name and address of the manufacturer; the date of manufacture; the date of expiry; batch number and instructions for use

v. The packet should be used before its expiry, only for the specified crop and by the recommended method of application

vi. Biofertilizers are live products and require care during storage

vii. Both nitrogenous and phosphate biofertilizers are to be used to get the best results

viii. It is important to use biofertilizers along with organic manures (they act as carriers and substrate to the microorganisms) (Itelima *et al.,* 2018a).

2.8 Limitations for Application of Biofertilizer

There are factors that to be considered by farmers before the application of biofertilizer. These factors also known as limitations are listed below:

i. Unavailability of suitable carrier resource constraint

ii. Market level constraints and lack of awareness of farmers

iii. Lack of quality assurance and limited resource generation for biofertilizers production

iv. Seasonal and unsure requirement

v. Soil and climatic factors and inadequately experienced staff

vi. Native microbial population, faulty inoculation techniques and mutation during fermentation (Bünemann *et al.*, 2016)

2.9 Advantages of Biofertilizers in Agriculture

The most important advantages of biofertilizer over the inorganic fertilizer are extensively described below:

2.9.1 Low cost and easy application techniques

Biofertilizers are cost effective compared to chemical fertilizers. They differ from chemical and organic fertilizers because they supply nutrients directly to crops and constitute cultures of special bacteria and fungi with requires low installation cost (Koske and Gemma, 2013). The use of biofertilizers can improve the productivity per unit area in a relatively short time. They have lower manufacturing costs and reduced use costs, especially regarding nitrogen and phosphorus use. Their easy way of application consumes smaller amounts of energy. In this sense, application of biological fertilizers can bring benefits from an economic point of view, since biofertilizers are a cost effective and renewable source of plant nutrients to substitute the chemical fertilizers for sustainable agriculture (Lambers *et al.*, 2014). Most commonly biofertilizers are in powder, carrier-based form. The carrier usually is lignite. The lignite has high organic matter content and holds more than 200 % water (McGonigle *et al.*, 2013). This high-water content enhances the growth of the microorganisms. The application method for this type of biofertilizers is preparation of slurry, which is applied to the seeds. At present, another method, dry complex fertilizer for direct soil application, has been developed. It consists of granules (1–2 mm) made from tank bed clay (TBC) and baked at 200 °C in a muffle furnace, which helps to sterilize the material and gives porosity to the granules (Rakshit and Bhadoria, 2016). The baked granules are soaked in a suspension of desired bacteria grown in a suitable medium overnight. The clay granules are air-dried at room temperature under aseptic conditions. They contain about 10^9 bacteria per gram of granules. These granules are suitable for field application along with seeds. However, the quantity of biofertilizer to be applied is slightly higher than that in seed application (Bailey *et al.*, 2016).

2.9.2 Increase yield with additional 15–35 % in most vegetable crops

Biofertilizer is a technological innovation that has the potential to increase crop yield, reduce production cost and improve soil condition (Wang *et al.,* 2015). Biofertilizers can be considered as supplementary to chemical fertilizers. When they are applied as seed or soil inoculants, they multiply and participate in the nutrient cycling, thus benefiting the crop productivity. Biofertilizers have great potential to improve crop yields through environmentally better nutrient supplies. They provide reserve plant nutrients. It is reported that biofertilizers increase crop yields by 20–30 % and stimulate plant growth (Shaji *et al.,* 2021). The efficiency of biofertilizer use is the key characteristic that ultimately contributes to the increase of the crop yield. There are numerous examples that biofertilizers positively affect the crop yield. For instance, Vital N®, an organic biofertilizer registered with the Philippine FPA, is a powder formulation that induces extensive growth in roots of crops like corn, rice, banana, garlic, orchids and onion. There are reports that the overall performance of potato crops is positively influenced by application of green manures (cowpea and *Crotolaria* sp.): 30 % yield improvements (Smith *et al.,* 2013). The increased productivity values verify the efficiency of biofertilizers in agricultural production. On the other hand, some physicochemical properties of the soil are improved and environmental impacts due to the prolonged use of chemical fertilizers are gradually mitigated (Taraken *et al.,* 2010). Furthermore, 10 % increases in the yield per hectare have been observed for crops treated with arbuscular mycorrhizal (AM) fungi, combined with increased resistance of the plants to the action of pathogenic microorganisms. A trial investigating the feasibility of biofertilizers prototypes based on native bacteria from rice crops reported 10 % increases in yield production by using the mixtures, from 7,625 kg/ha to 8,500 kg/ha. The application of the aquatic fern–cyanobacteria symbiotic association *Azolla–Anabaena* as a biofertilizer in rice paddies of northern Italy allowed obtaining yields close to 40 kg nitrogen/ha during a 3-month period and verifying increases in the growth rate of rice (Smith *et al.,* 2013).

2.9.3 Provision of nitrogen and several growth hormones

Biofertilizers contribute to the maintenance of stable nitrogen (N) concentrations in the soil. They replace chemical nitrogen by 25 %. Thus, nitrogen-fixing microorganisms play an important role in nitrogen supply by converting atmospheric nitrogen into organic forms usable by plants (Verbruggen *et al.,* 2012). Use of biological N_2-fixation technology can contribute to a decrease in the N fertilizer application and to the reduction of environmental risks. *Azotobacter* (free-living N_2-fixer) plays an important role in the nitrogen cycle in nature due to its diverse metabolic potential. In addition to N_2 fixation, this microorganism has the ability to synthesize and secrete considerable amounts of biologically active substances, among which the vitamins thiamine and riboflavin, nicotinic acid, pantothenic acid, biotin; the plant-growth hormones heteroxins, gibberellins. These biologically active substances help in modification of the nutrient uptake by the plants. Another free-living N_2-fixer, *Azospirillum,* is reported to produce plant-growth-promoting substances indole acetic acid (IAA) and indole butyric acid (IBA) and increase the rate of mineral uptake by plant roots, resulting in the enhancement of plant yield (Vanlauwe *et al.,* 2010). It is well known that most plants form symbiotic associations with the arbuscular mycorrhizal fungi (AMF) acting as bio-ameliorators (Sharma *et al.,* 2013). They have the potential to considerably enhance the rhizospheric soil characteristics. This, in turn, leads to improved soil structure and promotes plant growth under normal as well as stressed conditions. The results revealed that the AMF-induced enhancement in nutrient uptake promotes various

biologically important metabolites. Among them of special importance are the plant hormones, including gibberellin (GA) and auxin, which play a unique role in plant growth regulation under both normal and stress conditions. The activity of phytohormones like cytokinin and IAA is also significantly higher in plants inoculated with AMF. Higher hormone production results in better growth and development of the plant (Schubert and Hayman, 2011)

2.9.4 Does not cause atmospheric pollution but increase soil fertility

The use of biofertilizers is not only cost effective; it also augments the problem of environmental pollution. They are environmentally friendly because their use not only prevents damaging the natural resources but also helps to some extent to free the plants of precipitated chemical fertilizers. Biofertilizers promote the reduction of environmental impacts associated with the excessive use of chemical fertilization. Thus, their use in organic farming, sustainable agriculture, green farming and non-pollution farming contribute to implementation of healthy environment policies at national, regional and global level. All types of crops grown in different agro-ecologies can benefit from the use of biofertilizers. Continuous use of biofertilizers enables the microbial population to remain and build up in the soil and helps in maintaining soil fertility contributing to sustainable agriculture (Phogat *et al.,* 2015). Biofertilizers keep the soil environment rich in all kinds of micro- and macro-nutrients via nitrogen fixation, phosphate and potassium solubilization or mineralization, release of plant-growth-regulating substances, production of antibiotics and biodegradation of organic matter in the soil. Growing crops using biofertilizers is advantageous in protecting the soil from degradation. Biofertilizers can mobilize nutrients that favour the development of biological activities in soils. In this way, they prevent micro-nutrient deficiencies in plants and guarantee better nutrient uptake and increased tolerance to drought and moisture stress, all factors that strongly contribute to soil fertility (Pereira *et al.,*2020).

2.9.5 Excretion of antibiotics and act as pesticides

The use of biofertilizers can promote antagonism and biological control of phytopathogenic organisms. Thus, positive effect on soil microbiology is exerted: suppression or control through competition of pathogenic populations of microorganisms present on the soil (Adeleke *et al.,* 2019). Strategies for biological control of fungal species in crops include application of biofertilizers obtained from biological digestion to control target pests and pathogens. Through the siderophores and antibiotics produced by them, biofertilizers are antagonistic to foliar or rhizosphere pathogenic bacteria, fungi and insects (Mukhongo *et al.*, 2016). Arbuscular mycorrhizal fungi (AMF) have the potential to reduce damage caused by soil-borne pathogenic fungi, nematodes and bacteria. Meta-analysis has shown that AMF generally decrease the effects of fungal pathogens (Mwanga *et al.,* 2011b). A variety of mechanisms have been proposed to explain the protective role of mycorrhizal fungi. The major mechanism is nutritional, because plants with a good phosphorus status are less sensitive to pathogen damage. Non-nutritional mechanisms are also important, because mycorrhizal and non-mycorrhizal plants with the same internal phosphorus concentration may still be differentially affected by pathogens. Such non-nutritional mechanisms include activation of plant defence systems, changes in exudation patterns and concomitant changes in mycorrhizosphere populations, increased lignification of cell walls and competition for space for colonization and infection sites (Menendez and Garcia-Fraile, 2017). Recently, several fungal endophytes, like *Trichoderma* specie. (Ascomycota) and Sebacinales (Basidiomycota, with *Piriformospora*

indica as a model organism), which are distinct from the mycorrhizal species, have attracted scientific attention (Kavoo-Mwangi *et al.*, 2014). These fungi are able to live at least part of their life cycle away from the plant, to colonize its roots and to transfer nutrients to their hosts, using mechanisms that are not clear yet. *Trichoderma* spp. have been extensively studied and used for their biopesticidal (mycoparasitic) and biocontrol (inducer of disease resistance) potential, and have been exploited as sources of enzymes by biotechnological industries. Now it is speculated (on the basis of convincing evidence) that *Trichoderma* specie. also induce many plant responses (Menendez and Garcia-Fraile, 2017).

2.9.6 Improvement of physical and chemical properties of soil

Biofertilizers contribute to better physical conditions in the soil through improvement of structure and aggregation of soil particles, reducing compaction and increasing the pore spaces and water infiltration. They improve soil structure and allow better tilt; ensure better soil aeration and water percolation, reducing soil erosion. Biofertilizers serve as major food source for microbial populations thus keeping the soil alive. They also contribute to soil chemical conditions through improvement of nutrients availability in the soil, leaving free elements to facilitate their absorption by the root system; improved capacity of nutrients' exchange in the soil resulting in favourable effects on the physico-chemical stability of soils. As a result of the good structure and improved stability provided to the soil, root growth is promoted (Balasubramanian, 2017). The maintenance of good soil structure in all ecosystems is largely dependent on mycorrhizal fungi. Formation and maintenance of soil structure is influenced by soil properties, root architecture and management practices. The use of machines and fertilizers are considered to be responsible for soil degradation, which is a key component of soil structure. Mycorrhizal fungi contribute to maintain good soil structure through the following processes (Debnath *et al*., 2019);

i. growth of external hyphae into the soil creates a skeletal structure that holds soil particles together;
ii. external hyphae create conditions that are conducive to the formation of micro-aggregates;
iii. enlargement of micro-aggregates by external hyphae and roots to form macro-aggregates;
iv. Directly tapping carbon resources of the plant to the soils.

This process influences the formation of soil aggregates, because soil carbon is crucial to form organic materials necessary to cement soil particles. The hyphae of AM fungi are more important in this process than the hyphae of saprotrophic fungi due to their longer residence time in soil. In addition, AM fungi produce glomalin (12–45 mg/cm^3), a specific soil protein with still unknown biochemical nature (Debnath *et al*., 2019). Glomalin has a longer residence time in soil than hyphae, allowing for a long persistent contribution to

soil aggregate stabilization. The residence time for hyphae is considered to vary from days to months and for glomalin from 6 to 42 years. Glomalin is considered to stably glue hyphae to soil. The mechanism is the formation of a 'sticky' string-bag of hyphae which leads to the stability of aggregates (Debnath *et al*., 2019).

2.9.7 Enhance crop yield even under ill irrigated conditions.

Biofertilizers increase the water and nutrient holding capacity of the soil and also increase the drainage and absorption of moisture in soils, especially in those with structural deficiencies or lack of nutrients (Jenkins, 2013). They increase the tolerance towards drought and moisture stress. In this way, they increase the crop yield even in plantations that lack sufficient natural water supply or irrigation. For instance, AM association improves the hydraulic conductivity of roots at lower soil water potentials and this improvement is one of the factors contributing towards better uptake of water by plants. Moreover, leaf wilting after soil drying does not occur in mycorrhizal plants until the soil water potential is considerably lowered (approximately 1.0 MPa). Mycorrhiza-induced drought tolerance can be related to factors associated with AM colonization such as improved leaf water and turgor potentials and maintenance of stomatal functioning and transpiration, greater hydraulic conductivities and increased root length and development (Lambers *et al.,* 2014)

2.9.8 Eco-friendly and pose no danger to the environment

The most important and contributing function of biofertilizers is considerable reduction in environmental pollution and improvement of agro-ecological soundness. Biofertilizers are eco-friendly organic agro-input compared to chemical fertilizers (Ceballos *et al.,* 2013). They cause no harm to ecosystems and are valuable to the environment as they enable reduced use of chemical fertilizers in the production of crops worldwide. Namely due to their eco-friendly characteristics, the demand for biofertilizers is on the increase during the last decade. Their activities influence the soil ecosystem and produce supplementary substances for the plants. Providing continuous supply of balanced micronutrients to the plants and eliminating plantar diseases, biofertilizers enhance the maintenance of plant health and contribute to soil ecology (Lamber *et al.,* 2011). The provided food supply and impelled growth of beneficial microorganisms contribute to sustain the ecological balance. In the long run, biofertilizers are planned to complement and, where appropriate, replace conventional chemical fertilizers, resulting in economic and environmental benefits (Kalayu, 2019).

2.10 Inorganic Fertilizers

2.10.1 Types of inorganic fertilizers

Different types of inorganic fertilizers include nitrogen fertilizer, potash fertilizer, phosphorus fertilizer, fertilizer, compound fertilizers and leaves. The category of Nitrogen fertilizers includes the followings:

- ZA (Zwavelvuur Ammonium) 20.5 to 21 % nitrogen level
- Urea or $CO(NH_2)$ 45-46 % nitrogen content
- Chile saltpetre with 15 %nitrogen

- Ammonium nitrate or NH_4NO_3 who had higher levels of nitrogen by 35 %.

The category of phosphorous fertilizers includes the followings;

- Superphosphate multiple (DS=Double superphosphate) who had levels of 30 % P_2O_5.
- Triple superphosphate (TS=Triple Superphosphate) 45 % P_2O_5.

It is most widely used by the people. The category of potassium fertilizers includes the followings:

- Potassium chloride or KCl which had levels of 50 % K_2O
- Potassium Sulphate (ZK=Zwavelvuur time) with a grade of 50 % K_2O.

The category of Compound fertilizers includes NPK. Nitrogen phosphorus and potassium fertilizers are single because it contains only one type of primary nutrients whereas compound fertilizer contains more than one kind of primary nutrients. NPK fertilizers consist of Potassium Chloride (KCl), Ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and Ammonium nitrate (NH_4NO_3). For Example: fertilizer NPK 10-15-20 means it contains 10 % nitrogen, 15 % phosphorus (P_2O_5) and 20 % Potassium (K_2O). Another form is Leaf manure in which Foliar fertilizers are given to the plants by spraying the leaves and they are absorbed by osmosis or diffusion through stomata. Examples of foliar fertilizers are Wuxal, Baypolan and so many more (Ameeta and Ronak, 2017).

2.10.2 Advantages of inorganic fertilizers

There are certain advantages of inorganic fertilizers which makes them a potent candidate to enhance agricultural productivity. Some of the major benefits are listed below:

- There is no need of direct decomposition as the nutrients in mineral fertilizers are relatively high, and the release of these nutrients is quick.
- Inorganic fertilizers increase the growth rate and plant's overall productivity more rapidly. (Ameeta and Ronak, 2017).

2.10.3 Disadvantages of inorganic fertilizers

Despite the benefits there are serious major disadvantages of inorganic fertilizers which makes them less desirable. Some of these shortcomings are;

- They are carcinogenic to Humans: According to the EPA's Office of Pesticide Programs, most of the pesticides have ingredients which are known to cause cancer. Organic fertilizers are guaranteed to be safe for the environment, the body and are free of pesticides.
- Water pollution: Inorganic fertilizers accumulates salt which expend more energy to draw water from the soil and cause them to appear wilted or dried out and if there is a rainfall shortly after they are applied, the fertilizers wash away and can pollute streams, ponds and other water bodies causing Eutrophication in water bodies.
- It can also leach away from the root zone of the plant.
- Constant consumption of plants in which inorganic fertilizer has been applied on can accumulate in man and cause harm.
- Nutrient Imbalance: The reckless use of inorganic fertilizers can create nutrient imbalance that limits the uptake of other essential nutrients and cause soil acidity leading to low crop yields.
- Deficiency of secondary and micronutrients occur in soil and crop, if the common NPK type is consistently used.
- Total dependence on inorganic fertilizers leads to fall in soil organic matter, increased soil acidity, degradation of soil physical properties and structure and increased erosion.
- Dependence on fossil fuel: Agricultural chemicals have contaminated ground and surface waters, harmed fish and wildlife and greatly increased agricultural dependence on fossil fuel resources. Thus, there is a need to evolve to an alternative method by which we can reduce the use of chemicals (Ameeta and Ronak, 2017).

CHAPTER THREE
3.0 MATERIALS AND METHODS

3.1 Study Site

Biofertilizer production and comparison test were carried out at the Department of Microbiology, Federal University of Technology Minna, Bosso Campus (Latitude 9° 35' 0.96" N and Longitude 6° 32' 46.688" E). The inorganic fertilizer (compound fertilizer NPK 15:15:15) chosen for this study was acquired from an Agricultural store at Kasuwa Gwari Market, Minna, Niger State.

3.2 Collection and Processing of Samples

Soil samples used in this study were collected from the rhizosphere of groundnut plants, at a groundnut farm in Kampala, Minna, Niger state. The samples were collected in sterilized polythene bags and transported in an ice pack to the laboratory. 10 g of the soil sample was dissolved in 90 ml of sterile distilled water and mixed thoroughly to attain the dilution of 10^{-1}. The soil sample was serially diluted in 9 ml sterile distilled water up to 10^{-9}. After dilution, diluents 10^{-4}, 10^{-5}, 10^{-6} were inoculated in three (3) replicates on Nutrient Agar (NA) and Yeast-Mannitol Agar Medium (YEMA) (with Congo Red indicator) using spread plate technique. The mean microbial load was determined, and the organisms were further purified on NA for characterization and identification (Pepper *et al.*, 2009).

3.3 Characterization and Identification of Bacterial Isolates

The pure bacterial isolates were characterized and identified based on the Physiology and colonial morphology on selective and differential media, Gram's staining, Microscopic Appearance, Temperature Adaptability up to 65 ℃, and biochemical tests such as Motility, Production of catalase, urease, oxidase, citrate utilization test, Hydrogen Sulphide (H_2S) and Carbohydrate fermentation according to District Laboratory Practice in Tropical Countries (Cheesbrough, 2006). The bacterial isolates were further screened for Phosphate solubilising ability and Nitrogen fixing ability. All pure colonies were preserved on agar slants of Nutrient Agar medium at 4 ℃ until used. The bacterial isolates were identified by comparing their characteristics with those of known taxa using the schemes of Cowan and Steel (Phillips, 1993).

3.3.1 Gram staining

A smear of the pure isolate and distilled water was made on a clean and sterilised glass slide, and allowed to air dry. After, it was fixed with heat so the smeared organism remains permanently on the glass slide. The fixed smear was stained with crystal violet for 60 seconds. It was washed with distilled water, iodine was applied on the slide as a mordant for 1 minute, then washed with 95 % alcohol and washed with distilled water. Lastly, safranin was applied on the slides and allowed to stay for 30 seconds, then washed with distilled water and air dried. The dried stained slides were viewed under the microscope for the bacterial morphology using the oil immersion lens. (Anubrata and Rajendra, 2014)

3.3.2 Motility

This test was to identify isolates that were motile and non-motile. A semi-solid nutrient medium was prepared in test tubes in an autoclave at 121 ℃ for 15 minutes, and allowed to cool. A straight sterilised needle was used to pick a colony of a young (18 to 24-hour) culture growing on agar medium. The semi-solid medium was stabbed with the needle to a depth of only 1/3 to ½ inch in the middle of the tube. Extra caution was taken to keep the needle in the same line it entered as it was removed from the medium. The test tubes were incubated at 37 °C and examine daily for up to 7 days. The test tubes were observed for non- diffused (non-motile) and diffused zones of growth (Motile) flaring out from the line of inoculation (Cheesbrough, 2006).

3.3.3 Catalase

This test was carried out to differentiate between organisms that produce the enzyme catalase and organisms that cannot produce the enzyme. A sterilised inoculating loop was used to remove some colonies from the 24-hour test organism and smeared on a drop of 3 % hydrogen peroxide (H_2O_2) solution. Immediately there was bubbles which indicated positive for catalase, but no bubbles indicate a negative result. This procedure was carried out on all isolates (Cheesbrough, 2006).

3.3.4 Starch hydrolysis

This test was to determine the ability of the isolates to hydrolyse starch and differentiate the isolates based on their α- amylase enzyme activity. The starch medium was sterilised in an autoclave at 121 ℃ for 15 minutes, and allowed to cool. After cooling, it was poured into petri dishes and allowed to solidify. Each plate was inoculated aseptically with pure cultures and incubated at 37 ℃ for 24 hours. After incubation, the surface of the plates was flooded with iodine solution with a dropper for 30 seconds and the excess iodine was poured off. A clear zone was formed around the line of growth after the addition of iodine solution, this indicated that the organism had hydrolysed starch (Phillips, 1993).

3.3.5 Urease

This test was to determine the presence of urease enzyme activity in the isolates. Urea solution and Urea broth were sterilised in an autoclave at 121 ℃ for 15 minutes. The medium and solution was allowed to cool to 40 ℃. At 40 ℃ the urea solution was turned into the urea broth and dispensed into test tubes. When cooled, each test tubes were inoculated with the isolates and incubated at 37 °C for 24 to 48 hours. The change of colour of the broth from yellow-orange to bright pink was considered as positive (Cheesbrough, 2006).

3.3.6 Citrate

This test was conducted to identify the enterobacteria present and the ability of the organism to use citrate as a source of carbon. Simons citrate agar medium was sterilised at 121 ℃ for 15 minutes in an autoclave. The slants were inoculated and incubated at 37 °C for 24-hours. A positive slant was indicated by a change in colour from green to blue while a negative slant had no colour change (Cheesbrough, 2006).

3.3.7 Triple sugar iron (TSI)

This test was carried out to characterize bacteria based on the oxygen use (i.e., aerobic or anaerobic) and identify the isolates according to the ability to ferment glucose, lactose, sucrose and produce hydrogen sulphide. The Triple Sugar Iron medium agar was prepared in test tubes and sterilized in an autoclave at 121 ℃ for 15 minutes. The test tube slants

were inoculated with a colony from a 24 hours pure culture, using a sterilised inoculation loop. The colony was inoculated first by stabbing through the centre of the medium to the bottom of the tube and then streaking the surface of the agar slant. The test tube was incubated at 37 °C for 24 hours with a control. The change in colours and production of gas was observed (Cheesbrough, 2006).

3.3.8 Screening for phosphate-solubilising bacteria (PSB)

The pure bacterial isolates were streaked on Pikovskaya (PKV) agar media plates and incubated at 37 °C for 24 hours. After 24 hours, bacterial colonies showing a clear Halo zone formation were confirmed as Phosphate Solubilising Bacteria (PSB) species (Gupta *et al.,* 1994; Kalayu, 2019).

3.3.9 Screening for nitrogen-fixing bacteria (NFB)

Pure bacteria isolates were inoculated into Jensen's (Nitrogen Free) medium and incubated at 37 °C for 5 days with a control. The Jensen's medium was prepared as a broth using Phenol red as an indicator. Phenol red turned the media pink, a colour change to red confirmed the ability of the organism to fix nitrogen from the air (Callow and Vincent, 1971; Alam *et al.,* 2015).

3.4 Molecular Characteristics of Bacteria Isolates

The PCR cocktail mix consist of 2.5 µl of 10 x PCR buffer,1 µl of 25 mM $MgCl_2$, 1 µl each of forward primer and reverse primer, 1 µl of DMSO, 2 µl of 2.5 mMDNTPs, 0.1 µl of 5 µ/µl Taq DNA polymerase, and 3 µl of 10 mg/µl DNA. The total reaction volume was made up to 2 µl using 13.4 µl Nuclease free water. Primer sequence for Bacteria used was; 27F: **3' –** AGAGTTTGATCMTGGCTCAG - **5'** and 1525R: **5' –** AAGGAGGTGWTCCARC CGCA - **3**'. PCR cycling parameters used involved initial denaturation at 94 ˚C for 5 minutes, followed by 36 cycles of denaturation at 94 ˚C for 30 seconds, annealing at 56 ˚C for 30 seconds and elongation at 72 ˚C for 45 seconds. Followed by a final elongation step at 72 ˚C for 7 minutes and hold temperature at 10 ˚C forever. Amplified fragments were visualized on ethidium bromide-stained 1.5 % agarose electrophoresis gels. The size of the amplicon was about 1500 bp (Base pairs) and the DNA ladder used was 1 Kb (Kilobase) from NEB. The sequencing was performed using genetic analyser ABI 3500 from Thermo Fisher. The forward and reverse sequences were further analysed to acquire the consensus sequence for blasting using the Bioedit application. The consensus (fasta format) sequence generated using the Bioedit application was further blasted using the NCBI Nucleotide blast (Altschul *et al.,* 1990; Lodish *et al.,* 2004).

3.5 Biofertilizer Production

The biofertilizer was produced on a small scale in the laboratory using two bacterial isolates which were subjected to a fermentation process. The production process involved four (4) stages which are further explained below.

3.5.1 Production of starter culture

After biochemical and molecular identification of the bacterial species, the starter culture medium was prepared. The starter culture medium can also be called the production medium, i.e., a medium in which the bacteria species were able to grow rapidly for mass production. Nutrient broth was used as the starter culture. Two 100 ml conical flasks containing nutrient broth were inoculated with the bacterial cultures from the 24-hour pure cultures. The conical flasks were kept in a shaker incubator at 37 ℃ for 7 days at 100 rpm

(revolution per minute). After 7 days these starter cultures were introduced to large conical flasks for mass production (Debojyoti *et al.*, 2017).

3.5.2 Mass production of bacteria cultures

After one week in the shaker incubator, the starter cultures were transferred to two large conical flasks of 1,000 ml each for further viable cell production. Each 1,000 ml flasks contained Nutrient broth incubated with 10ml from the starter cultures. The larger conical flasks were kept inside the shaker incubator for mass production at 37 ℃, 100 rpm for 7 days. These processes were conducted aseptically. During fermentation, the conical flasks were checked constantly to prevent contamination (Debojyoti *et al.*, 2017).

3.5.3 Preparation of carrier material

The carrier material chosen for this study was charcoal. This is because, charcoal is cheap and easy to acquire, it prevents leaching of nutrients in the soil. Charcoal was pounded into fine texture, mixed with activated charcoal and sieved. It was sterilised at 121 ℃ for

A

B

C

M

ge of biofertilizer

This was the final stage of the biofertilizer production. After curing, the double composite biofertilizer was packed in a breathable polythene bag and stored away from sunlight, at room temperature maintaining the cool conditions before taking to the farm for application during cultivation of *Zea mays* in the pot experiment (Debojyoti *et al.*, 2017; Anubrata and Rajendra, 2014).

3.6 Physicochemical Analysis of Biofertilizer

The biofertilizer was analysed at the National Cereals Research Institute Badeggi, Niger State. The biofertilizer was analyzed for the content value of elements present in the biofertilizer. These elements include; Organic Carbon, Organic Matter, Nitrogen, Phosphorous, pH, Sodium, Magnesium, Potassium, Calcium, Exchangeable acidity, Electrical conductivity and Moisture. The physical properties were also determined. These parameters are described further below.

3.6.1 pH

The pH of the biofertilizer was determined using pH in H_2O (1:1) and pH in 1 M $CaCl_2$ (1:1) method. This experiment was carried out by weighing 10 g of the biofertilizer into an extraction cup dispensed with 10 ml of distilled water. It stood for 15 minutes before mixing on a mechanical shaker for 30 minutes at 150 rpm**.** After standing for 10 minutes, the pH meter was standardized using buffers 7.0 and 4.0. After recording the pH in H_2O, a drop of 1 M $CaCl_2$ solution was added to the biofertilizer water suspension, stirred for 15 minutes and stood for 25 minutes. The pH value of the biofertilizer was standardized on the pH meter using buffers 7.0 and 4.0, then the pH was read using the pH meter (Onyeonwu, 2000).

3.6.2 Organic carbon

Organic carbon estimation is a complete oxidation method. The biofertilizer sample was grounded further to pass through a 0.5 mm sieve before weighing. 500 mg of the biofertilizer was weighed into a 50 ml digestion tube, and a standard sucrose solution was prepared with 1 ml added into 5 digest tubes. 5 ml of Potassium dichromate ($K_2Cr_2O_7$) solution and 10 ml of concentrated H_2SO_4 was added to the biofertilizer and standard tubes, closed with a rubber stopper, and swirled on a vortex mixer until the biofertilizer sample was completely dispersed. The tubes were placed in a digestion block preheated to 150 ℃ for exactly 30 minutes and then allowed to cool, diluted to 50 ml, mixed, and let to stand overnight. The standards and sample were read on a spectrophotometer at a wavelength of 600 nm using a 1 cm cell. The standards contained 0, 2.50, 5.00, 7.50, and 10.00 mg of Carbon according to Heanes (1984) method. The result obtained is further calculated using the formula below to obtain the percentage of the organic carbon present in the biofertilizer and determine the amount from a standard curve.

$$\% \text{ OC} = \text{mg C} \div \text{mg of biofertilizer X } 10 \quad (3.1)$$

Where OC is Organic carbon.

3.6.3 Nitrogen analysis

The nitrogen analysis was conducted by three process methods, Digestion, Neutralisation and Titration. To digest the biofertilizer, 200 mg of biofertilizer sample was measured into a 50 ml digestion flask, 5 ml H_2SO_4 (Tetraoxosulphate IV Acid) and 5 ml of H_2O_2 were added and kept in a digestion chamber for 24 hours. After 24 hours, the digested sample was transferred to a 100 ml volumetric flask which was filled up to the mark with distilled water. 10 ml of 40 % NaOH (Sodium Hydroxide) was added to the volumetric flask to neutralise the solution to alkaline, a receiving flask containing 10 ml boric acid (As an indicator) was attached to the volumetric flask. After the addition of NaOH, nitrogen was released in the form of ammonia and collected in the receiving flask turning boric acid green. The ammonium borate solution was titrated against H_2SO_4. The percentage of nitrogen was calculated using the formula below (Boraste *et al.*, 2009): -

$$\% \text{ nitrogen} = \text{Burette reading x } 0.02 \text{ x } 0.014 \text{ x} \frac{100}{10} \text{x} \frac{100}{0.02} \quad (3.2)$$

3.6.4 Phosphorous

Stock standard solution (20 ppm Phosphors) - Reagent grade potassium di-hydrogen phosphate (KH_2PO_4) was dried at 105 ℃ for 2 hours and cooled in a desiccator. 0.0879 g

of KH_2PO_4 was weighed and dissolved in distilled water and diluted to 1000 ml. It was mixed by shaking.

Standard X Curve- 0, 1, 5, 10, 15 & 20 ml was pipetted out of the stock solution and each was kept in 50 ml volumetric flask. 10 ml of vanado-molybdate reagent was added to make up the volume to the mark with distilled water. This resulted to 0, 5, 10, 15 & 20 ppm of phosphors. The intensity was read using a colorimeter at 470 nm wavelength

Procedure - The estimation of phosphorous content began with digestion of the biofertilizer. The biofertilizer was digested in similar procedure as in nitrogen analysis. After 24 hours of digestion, the digested biofertilizer was transferred to a 100 ml volumetric flask which was filled up to the mark with distilled water. 10 ml of the sample aliquot and vanando-molybdate reagent was pipetted into a 50 ml volumetric flask and diluted to 50 ml volume with distilled water. It was further observed at 470 nm wavelength using a colorimeter. After using the colorimeter to measure, the parts per million (ppm) of the phosphorus of the biofertilizer from the standard X curves were worked out. Calculation of Phosphorus is illustrated in the formula below (Boraste *et al.,* 2009): -

$$\% \text{ phosphorous } = \frac{X}{1000{,}000} \text{ x } 50 \text{ x} \frac{100}{10} \text{x} \frac{100}{0.2} \quad (3.3)$$

3.6.5 Potassium

Preparation of standard curve: - 0, 1, 2, 4, 6, 8 and 10 ml of stock solution was pipetted into 100 ml volumetric flask. The volume was marked up to the mark with addition of distilled water, this resulted to 0, 10, 20, 40, 60, 80, and 100 ppm of potassium respectively.

Procedure: - The intensity of potassium at flame photometer was observed. An aliquot of the biofertilizer was subjected directly to a flame photometer. The ppm of potassium from the standard curve run and the blank reading were worked out. The percentage of potassium by Flame photometer method is calculated with the formula below (Boraste, *et al.,* 2009): -

$$\% \text{ potassium } = \frac{X}{1000{,}000} \text{x } 100 \text{ x} \frac{100}{0.2} \quad (3.4)$$

3.6.6 Exchangeable acidity

3 g of the biofertilizer was weighed (grind to pass a 2 mm sieve) into a folded filter paper and placed on an extraction cup. 50 ml of 1.0 *N* KCL (Potassium chloride) solution was poured gently into the biofertilizer on the filter paper while the leachate was collected in the extraction cup. 5 drops of phenolphthalein indicator were added to the leachate and titrated with 0.05 *N* NaOH to pink end point (Onyeonwu, 2000).

The volume (ml) of NaOH used was recorded. The reaction was further calculated using the following formula: -

$$\text{Exchangeable acidity (meg/100 g)} = \frac{v \times 0.05 \times 100}{w} = v \times 1 \cdot 67 \quad (3.5)$$

Were,

V = Titre volume of NaOH used (ml)

W = weight of soil sample used (3g)

3.6.7 Moisture

Moisture was determined by measuring 4 g of biofertilizer on a dry petri-dish. It was heated in an oven for about 5 hours at 65 ℃, constant weighing was done. Cooling was done in a desiccator and weighed (Onyeonwu, 2000). Percentage loss in weight was determined as moisture content of the biofertilizer in the calculation using the formula: -

$$\text{Moisture content} = \frac{(B-C)}{(B-A)} \times 100$$

(3.6)

3.6.8 Physical properties

The colour of the biofertilizer was determined using munsel colour chart, while the texture was determined by feel method as described by Brady and Weil (1999).

3.7 Physicochemical Analysis of Soil

The soil samples were air-dried for 72 hours. The air-dried samples were sieved using a 2 mm mesh. Parameters such as pH, Electrical conductivity, Organic Carbon, Phosphorus, Sodium (Na), Potassium (K), Calcium (Ca) and Magnesium (Mg) present in the soil were determined. The estimation of these parameters is further explained below.

3.7.1 pH

The pH of the soil was determined using Soil pH in H_2O (1:1) and Soil pH in 1 M $CaCl_2$ (1:1) method. This experiment was carried out by weighing 10 g of the soil into an extraction cup dispensed with 10 ml of distilled water. It stood for 15 minutes before mixing on a mechanical shaker for 30 minutes at 150 rpm**.** After standing for 10 minutes, the pH meter was standardized using buffers 7.0 and 4.0. After recording the pH in H_2O, a drop of 1 M $CaCl_2$ solution was added to the soil water suspension, stirred for 15 minutes and stood for 25 minutes. The pH value of the soil was standardized on the pH meter using buffers 7.0 and 4.0, then the pH was read using the pH meter (Onyeonwu, 2000).

3.7.2 Organic carbon

Determination of organic carbon is a complete Oxidation method. The soil sample was grounded further to pass through a 0.5 mm sieve before weighing. 500 mg of the soil was weighed into a 50 ml digestion tube, and a standard sucrose solution was prepared with 1 ml added into 5 digest tubes. 5 ml of Potassium dichromate ($K_2Cr_2O_7$) solution and 10 ml of concentrated H_2SO_4 was added to the soil and standard tubes, closed with a rubber stopper, and swirled on a vortex mixer until the soil sample was completely dispersed. The tubes were placed in a digestion block preheated to 150 ℃ for exactly 30 minutes and then allowed to cool, diluted to 50 ml, mixed, and let to stand overnight. The standards and sample were read on a spectrophotometer at a wavelength of 600 nm using a 1cm cell. The standards contained 0, 2.50, 5.00, 7.50, and 10.00 mg of Carbon according to Heanes (1984) method. The result obtained is further calculated using the formula below to obtain the percentage of the organic carbon present in the soil and determine the amount from a standard curve.

$$\%\ OC = \text{mg C} \div \text{mg of soil X } 100$$

(3.7)

Where OC is Organic carbon.

3.7.3 Nitrogen analysis

The nitrogen analysis was conducted by three process methods, Digestion, Neutralisation and Titration. To digest the soil, 200 mg of the soil sample was measured into a 50 ml digestion flask, 5 ml H_2SO_4 (Tetraoxosulphate IV Acid) and 5 ml of H_2O_2 were added and kept in a digestion chamber for 24 hours. After 24 hours, the digested sample was transferred to a 100 ml volumetric flask which was filled up to the mark with distilled water. 10 ml of 40 % NaOH (Sodium Hydroxide) was added to the volumetric flask to neutralise the solution to alkaline, a receiving flask containing 10 ml boric acid (As an indicator) was attached to the volumetric flask. After the addition of NaOH, nitrogen was released in the form of ammonia and collected in the receiving flask turning boric acid green. The ammonium borate solution was titrated against H_2SO_4. The percentage of nitrogen in the soil was calculated using the formula below (Boraste, *et al.,* 2009): -

$$\% \text{ nitrogen} = \text{Burette reading x } 0.02 \text{ x } 0.014 \text{ x} \frac{100}{10} \text{x} \frac{100}{0.02} \quad (3.8)$$

3.7.4 Phosphorous

Stock standard solution (20 ppm Phosphors) - Reagent grade potassium di-hydrogen phosphate (KH_2PO_4) was dried at 105 ℃ for 2 hours and cooled in a desiccator. 0.0879 g of KH_2PO_4 was weighed and dissolved in distilled water and diluted to 1000 ml. It was mixed by shaking.

Standard X Curve- 0, 1, 5, 10, 15 & 20 ml was pipetted out of the stock solution and each was kept in 50 ml volumetric flask. 10 ml of vanado-molybdate reagent was added to make up the volume to the mark with distilled water. This resulted to 0, 5, 10, 15 & 20 ppm of phosphors. The intensity was read using a colorimeter at 470nm wavelength

Procedure - The estimation of phosphorous content in the soil began with digestion of the soil. The soil was digested in similar procedure as in nitrogen analysis. After 24 hours of digestion, the digested soil was transferred to a 100 ml volumetric flask which was filled up to the mark with distilled water. 10 ml of the sample aliquot and vanando-molybdate reagent was pipetted into a 50 ml volumetric flask and diluted to 50 ml volume with distilled water. It was further observed at 470 nm wavelength using a colorimeter. After using the colorimeter to measure, the parts per million (ppm) of the phosphorus of the soil from the standard X curves were worked out. Calculation of Phosphorus is illustrated in the formula below (Boraste *et al.,* 2009):

$$\% \text{ phosphorous} = \frac{X}{1000{,}000} \text{x } 50 \text{ x} \frac{100}{10} \text{x} \frac{100}{0.2} \quad (3.9)$$

3.7.5 Potassium

Preparation of standard curve: - 0, 1, 2, 4, 6, 8 and 10 ml of stock solution was pipetted into 100 ml volumetric flask. The volume was marked up to the mark with addition of

distilled water, this resulted to 0, 10, 20, 40, 60, 80, and 100 parts per million (ppm) of potassium respectively.

Procedure: - The intensity of potassium at flame photometer was observed. An aliquot of the soil sample was subjected directly to a flame photometer. The ppm of potassium from the standard curve run and the blank reading were worked out. The percentage of potassium by Flame photometer method is calculated with the formula below (Boraste *et al.*, 2009): -

$$\% \text{ potassium } = \frac{X}{1000{,}000} \text{ x } 100 \text{ x } \frac{100}{0.2}$$

(3.10)

3.7.6 Exchangeable acidity

3 g of the soil was weighed (grind to pass a 2 mm sieve) into a folded filter paper and placed on an extraction cup. 50 ml of 1.0 *N* KCL (Potassium chloride) solution was poured gently into the soil on the filter paper while the leachate was collected in the extraction cup. 5 drops of phenolphthalein indicator were added to the leachate and titrated with 0.05 *N* NaOH to pink end point. The volume (ml) of NaOH used was recorded (Onyeonwu, 2000). The reaction was further calculated using the following formula: -

$$\text{Exchangeable acidity (meg/100 g)} = \frac{v \times 0.05 \times 100}{w} = v \times 1 \cdot 67$$

(3.11)

Were,

V = Titre volume of NaOH used (ml)

W = weight of soil sample used (3 g)

3.7.7 Moisture

Moisture was determined by measuring 4 g of soil sample on a dry petri-dish. It was heated in an oven for about 5 hours at 65 ℃, constant weighing was done. Cooling was done in a desiccator and weighed (Onyeonwu, 2000). Percentage loss in weight was determined as moisture content of the soil in the calculation using the formula: -

$$\text{Moisture content } = \frac{(B-C)}{(B-A)} \times 100$$

(3.12 **3.7.8 Physical properties**

The colour of the soil sample was determined using munsel colour chart. Soil texture was determined by feel method (Brady and Weil, 1999).

3.8 Pot Experiments on *Zea Mays* (Maize) for the Determination of Biofertilizer Potential in Comparison with Inorganic Fertilizer

The pot experiment was carried out to determine the relative effects of the biofertilizer produced with an NPK fertilizer using *Zea Mays* (Maize). This experiment was conducted using Complete Randomised Block Design (CRBD). The steps are further described.

3.8.1 Preparation of pot soil

Soil for the pot experiment was collected from a farm behind the Federal University of Technology, Minna, Bosso Campus. The soil was tilled, tilled soil was measured into15 pots. Five pots were designated as control (i.e., no application of any fertilizer only water), Five pots for treatment with inorganic fertilizer application and another Five pots for treatment with biofertilizer application, which sums up the fifteen pots. The soil was watered for a week as preparation before planting.

Plate III: Prepared Pots for Planting

ter to check its viability for planting. The not viable. The water was drained, and the

rn seeds (for five pots labelled MBF1, MBF g biofertilizer and 5ml of water for 24 hours. e pots consisting of three seeds each. The fter germination according to agricultural

3.8.2 Application of inorganic fertilizer

The NPK 15:15:15 was applied to five pots containing maize plant (labelled NPK1, NPK 2, NPK 3, NPK 4 & NPK 5) two weeks after germination according to agricultural practices for corn.

3.9 Experimental Design

The experimental design used was a Complete Randomised Block Design (CRBD), with one control, two treatments (T1 = Inorganic fertilizer application, T2 = Biofertilizer application), one cultivar and five replicates of each treatment in a pot experiment. Data were collected on growth parameters (germination date, leaf length, numbers of leaves per plant and plant length). The results attained were further analysed using a statistical application.

3.10 Statistical Data Analysis

Data generated were analysed statistically using SPSS (version 20). Differences and relationships of biofertilizer produced, physicochemical parameters of soil and plants were detected using One-way Analysis of Variance (ANOVA), followed by multiple

comparisons using Descriptive T-test, Bonferroni (Parametric test) and Games-Howell (Non-Parametric test). Significant level of (P<0.05) was used throughout the analysis.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Characterisation of Bacterial Isolates Biofertilizer Potential

The morphological characteristics and identification of bacterial isolates from the soil were conducted with more interest in the biofertilizer potential of the organism. A total of fourteen bacterial isolates were identified based on their physiological (on Nutrient Agar medium and Yeast-Mannitol Agar Medium (YEMA)) and morphological characteristics. The Total viable counts of microorganisms in the soil sample on Nutrient Agar and YEMA medium was 5.126×10^{-9} CFU/g and 3.256×10^{-9} CFU/g, respectively. Table 4.1 shows the biochemical characteristics and the organisms isolated from the which are all bacteria. According to Jacoby *et al.* (2017) microorganisms are part of a rich ecosystem in the soil, they are diverse, numerous and play important roles in plants by improving the nutrition of the soil. Hence, the total viable count determined from one gram (1 g) of the soil sample agrees with Jacoby *et al.* (2017), it also indicates that the soil was healthy with microorganisms. Two organisms with isolate code ISL 1 and NA 6 showed ability to solubilise phosphorous and fix nitrogen (Table 4.1). These organisms were identified as *Alcaligene faecalis* and *Providencia vermicola* respectively.

Table 4.1 BIOCHEMICAL TEST RESULTS

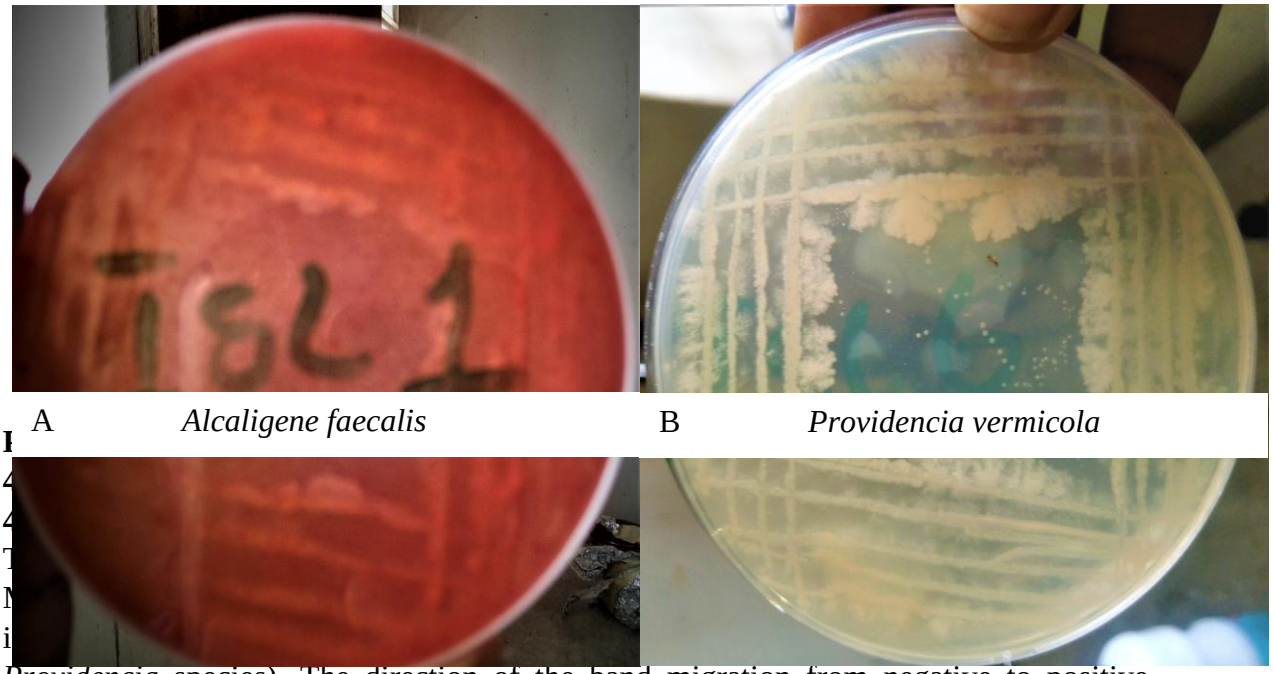
ISO Code	GRAM STAIN		TRIPLE SUGAR IRON				CAT	CIT	URE	STCH	MOT	TEM
	RXN	**SHAPE**	**BUTT**	**SLANT**	**GAS**	**H_2S**						**37 °**
ISL 1	-	Rods	Y	P	+	+	+	+	+	+	M	+
ISL 2	-	Rods	Y	P	-	+	+	+	+	-	M	+
ISL 3	-	Rods	Y	B	+	+	+	+	+	+	M	+
ISL 4	-	Rods	Y	Y	-	-	+	+	+	+	NM	+
ISL 5	-	Rods	Y	B	-	+	+	+	+	+	NM	+
ISL 6	+	Rods	B	P	-	+	-	-	+	-	M	+
ISL 7	-	Rods	Y	P	-	+	+	+	+	+	NM	+
ISL 8	-	Rods	Y	Y	-	-	+	-	+	-	NM	+
NA 1	-	Rods	Y	P	-	+	+	+	+	+	NM	+
NA 4	-	Rods	B	B	-	+	+	+	+	-	M	+
NA 6	-	Rods	B	B	-	+	+	+	+	-	M	+
NA 7	-	Rods	Y	Y	+	-	+	+	+	-	NM	+
NA 8	-	Rods	Y	B	+	+	+	+	+	+	M	+
NA 9	-	Rods	Y	P	-	+	+	+	+	+	NM	+

Y- Yellow, P- Pink, B – Black, M – Motile, NM – Non-motile, + Positive, - Negative, ISO Code – Isolate code, RXN – Reaction, H_2S Hydrogen Sulphide, CAT – Catalase, CIT – Citrate, URE – Urease, STCH – Starch, MOT – Motility, ℃ - Degree centigrade.

Table 4.2 Phosphate Solubilising and Nitrogen Fixing Potential of Bacterial Isolates

ORGANISM	PVK	N_2
Alcaligene faecalis	-	+
Providencia vermicola	-	+

+ Positive, - Negative.



A *Alcaligene faecalis* B *Providencia vermicola*

Providencia species). The direction of the band migration from negative to positive electrodes is due to the naturally occurring negative charge carried by their sugar phosphate backbone (Altschul *et al.*, 1990; Lodish *et al.*, 2004). Lane 3 is more prominent compared to Lane 1, while Lane 2 is blurring. The fragment pattern from Plate I shows that the two bands were 1500 bp in size with reference to the 1 Kb DNA Ladder.

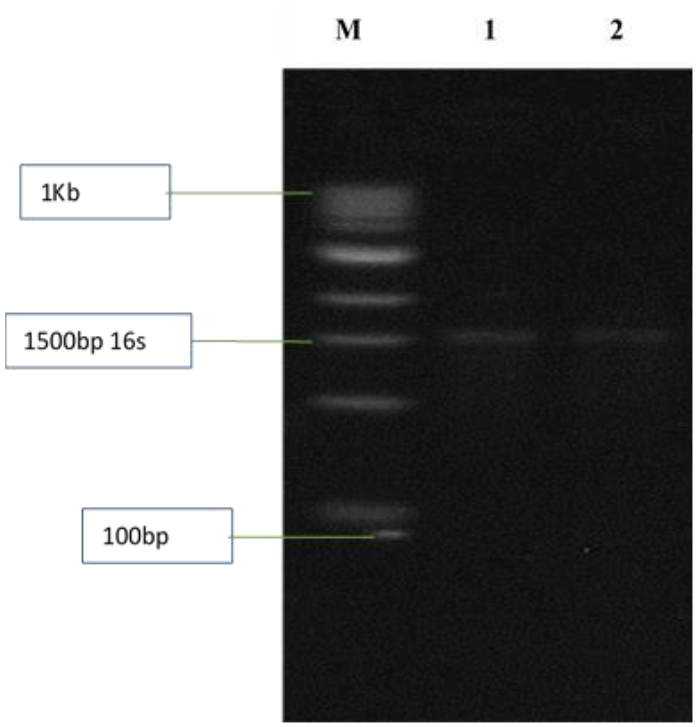


Plate V: Amplified image of the isolated bacterial DNA after electrophoresis

4.2.2 Sequencing of amplicons of bacteria isolates

The results (A-B) show the Forward, Reverse and Consensus sequenced amplicons of bacterial isolated for the production of biofertilizer as well as the resulting alignment of the concatenated nucleotides with other known (from 5' 3' and 3' 5') sequences previously blasted on National Centre for Biotechnology Information (NCBI) data base. The nucleotide sequence of Alcaligene faecalis is illustrated in Appendix A. The results in Table 4.3 shows the sequenced nucleotide of Alcaligenes species which has 81 % Query cover with known isolates and 95.4 % alignment with Alcaligenes faecalis strain NBRC 13111 16S rRNA gene. It also has a 94 % alignment with Alcaligenes faecalis strain IAM 12369 16S rRNA, Alcaligenes aquatilis strain LMG 22996 16S rRNA, Alcaligenes faecalis subsp. parafaecalis strain G 16S rRNA and Alcaligenes pakistanensis strain NCCP-650 16S rRNA.

Although, the decimal point of the percentage differs. *Alcaligenes faecalis* has more significant correlation with *Alcaligenes faecalis* strain NBRC 13111 16S rRNA gene.

Table 4.3 Sequence Alignment of *Alcaligenes* species with known Isolates

Description (16SrRNA gene partial sequenced)	Max Score	Total Score	QC (%)	E V	Ident (%)	Accession
Alcaligenes faecalis strain NBRC 13111	2019	2019	81 %	0.0	95.45 %	NR_113606.1
Alcaligenes faecalis strain IAM 12369	1982	1982	81 %	0.0	94.97 %	NR_043445.1
Alcaligenes aquatilis strain LMG 22996	1980	1980	81 %	0.0	94.80 %	NR_104977.1
Alcaligenes faecalis subsp. parafaecalis strain G	1964	1964	81 %	0.0	94.64 %	NR_025357.1
Alcaligenes pakistanensis strain NCCP-650	1947	1947	81 %	0.0	94.40 %	NR_145932.1
Alcaligenes endophyticus strain AER10	1905	1905	81 %	0.0	93.82 %	NR_156855.1
Alcaligenes faecalis subsp. phenolicus strain J	1899	1899	81 %	0.0	93.61 %	NR_042830.1
Paralcaligenes ginsengisoli strain DCY104	1799	1799	81 %	0.0	92.23 %	NR_148318.1
Parapusillimonas granuli strain Ch07	1799	1799	81 %	0.0	92.23 %	NR_115804.1
Paracandidimonas soli strain IMT-305	1783	1783	81 %	0.0	92.09 %	NR_156991.1

Source: Max Score: maximum, QC: Query cover EV: error value, Ident: identification.

The results in Table 4.4 shows the sequenced nucleotide of *Providencia* species which has 83 % (*Providencia rettgeri* strain NCTC 11801 16S rRNA and *Providencia sneebia* DSM 19967 strain A 16S rRNA) and 88 % Query cover with known isolates. No error value was observed. It has 89.05 % and 89.77 % identity alignment with *Providencia vermicola* strain OP1 16S rRNA, *Providencia rettgeri* strain NCTC 11801 16S rRNA and *Providencia sneebia* DSM 19967 strain A 16S rRNA. It also has 88.72 % and 88.39 % alignment with *Providencia rettgeri* strain DSM 4542 16S rRNA and *Providencia burhodogranariea* DSM 19968 strain B 16S rRNA. The nucleotide sequence of *Providencia vermicola* is illustrated in Appendix B.

Table 4.4 Sequence Alignment of *Providencia* species with known isolates

Description (16SrRNA gene partial sequenced)	Max Score	Total Score	QC (%)	E V	Ident (%)	Accession
Providencia vermicola strain OP1	1223	1223	88 %	0.0	89.05 %	NR_042415.1
Providencia rettgeri strain DSM 4542	1206	1206	88 %	0.0	88.72 %	NR_042413.1
Providencia burhodogranariea DSM 19968 strain B	1190	1190	88 %	0.0	88.39 %	NR_104914.1
Providencia rettgeri strain NCTC 11801	1186	1186	83 %	0.0	89.77 %	NR_115880.1
Providencia sneebia DSM 19967 strain A	1182	1182	83 %	0.0	89.77 %	NR_104913.1
Providencia rustigianii strain DSM 4541	1182	1182	88 %	0.0	88.29 %	NR_042411.1

Providencia rustigianii strain NCTC 11802	1179	1179	88 %	0.0	88.18 %	NR_115881.1
Providencia alcalifaciens DSM 30120 strain NCTC 10286	1177	1177	88 %	0.0	88.18 %	NR_115879.1
Providencia heimbachae strain NCTC 12003	1175	1175	88 %	0.0	88.07 %	NR_115882.1
Providencia heimbachae strain MUA 2-110	1168	1168	88 %	0.0	87.96 %	NR_042412.1

Max Score: maximum, QC: Query cover EV: error value, Ident: identification.

4.3 Physicochemical Properties of Biofertilizer Produced

The Physicochemical properties as shown in Table 4.5 revealed the physical and chemical properties of the biofertilizer. As illustrated, the pH of the biofertilizer was 7.01 which is almost neutral and within the optimal range (pH 6–8) required for microbial growth (Cho *et al.*, 2016). This makes it acceptable to be introduced to the soil to stimulate Plant Growth Promoting Microbes and enzymatic activities that will improve the yield of plants. According to Neina (2019), pH has a vast influence on the soil from the wide biological activities, biogeochemical processes to the physicochemical properties that affect the biomass and the plant yield. A high pH value could suggest that fermentation was adequate at the biofertilizer production stage. According to Onofre *et al.* (2017) pH ranging from 6.6 to 8.0 during biofertilizer fermentation suggests a complete and adequate fermentation process but, a lower pH indicates incomplete fermentation. The Nitrogen content (5.74 %), carbon-nitrogen ratio (3.61), sodium and potassium contents (2.34 & 1.96 $Cmolkg^{-1}$) appear low. The phosphorous content (20.2 mg/kg) is higher than the phosphorous content of NPK fertilizer which has phosphorous content value at 15.

Table 4.5 Physicochemical properties of Biofertilizer Produced

Properties	Value
pH	7.01
Nitrogen (N) (%)	5.74
Organic Carbon (%)	20.76
Organic Matter (%)	18.8
Carbon Nitrogen (C: N) ratio	3.61
Phosphorous (P) (mg/kg)	20.2
Sodium (Na) ($Cmolkg^{-1}$)	2.34
Potassium (K) ($Cmolkg^{-1}$)	1.96
Calcium (Ca) ($Cmolkg^{-1}$)	16.05
Magnesium (Mg) ($Cmolkg^{-1}$)	17.55
Exchangeable acidity ($Cmolkg^{-1}$)	3.12
Electrical Conductivity	149
Moisture (%)	8.66
Texture	Granular
Colour	Black

%- Percentage, mg/kg – Milligram per Kilogram, $Cmolkg^{-1}$ - Centimoles per kg.

Nitrogen, Carbon, Sodium, Phosphorous, Calcium, Magnesium and potassium are major elements required for the growth of plants, these elements are produced as chemical fertilizer, but they can be provided by microorganisms, especially Nitrogen (N), Phosphorous (P), and Potassium (K) making them easily accessible to plants (Nath *et*

al.,2018). Although there is no specific standard set for the quality of biofertilizer (which includes the physico-chemical standards), each country has standard specifications of biofertilizer which differ from country to country and may contain parameters like the microbial density at the time of manufacture, microbial density at the time of expiry, the expiry period, the permissible contamination, the pH, the moisture, the microbial strain, and the carrier. Except for some field research experiments, inoculant application among farmers in Nigeria is uncommon. However, in 2019, the National Agency for Food and Drug Administration and Control (NAFDAC) established a partnership with the COMPRO II project to create registration criteria and quality control mechanisms for biofertilizers in Nigeria (Raimi *et al*., 2021). The moisture content of the biofertilizer was low which could help to prevent the contamination by fungal growth, although, moisture content is very important for the survival of microorganisms.

4.4 Physicochemical Properties of Soil

The soil is a vast habitat where micro and macro-organisms are ubiquitous. It is a natural source that consist of solid minerals and organic matter (Balasubramanian, 2017). The quality of soil depends on the natural composition, the physicochemical properties of the soil and changes triggered by human activities (Oshoma *et al.,* 2017) Table 4.6 illustrates the Physical and chemical properties of the soil before planting. The soil was Grey in colour and moderately coarse in texture. The soil's blackness suggests a significant concentration of humus and organic materials (Phogat *et al*., 2015; Balasubramanian, 2017). The pH of the soil was acidic and the nitrogen content was low. The organic matter content of the soil was 5 %, which was consistent with Balasubramanian (2017)'s report that organic matter makes up 5 % of the soil. The organic matter of the soil is composed of macro elements such as organic carbon, nitrogen, phosphorus, sodium, potassium, calcium, and magnesium. However, Nitrogen and Potassium contents (0.52 % & 0.69 $Cmolkg^{-1}$) had low values in the soil (Table 4.6).

Table 4.6 Physicochemical Properties of Soil Before planting

PARAMETERS	VALUES
pH %	5.86
Nitrogen (N) (%)	0.52
Organic Carbon (%)	7.77

Organic Matter (%)	5.96
Phosphorous (P) (mg/kg)	30.29
Sodium (Na) ($Cmolkg^{-1}$)	1.450
Potassium (K) ($Cmolkg^{-1}$)	0.69
Calcium (Ca) ($Cmolkg^{-1}$)	6.85
Magnesium ($Cmolkg^{-1}$)	5.69
Exchangeable acidity ($Cmolkg^{-1}$)	1.80
Electrical Conductivity	56
Sand (%)	55.34
Clay (%)	26.60
Silt (%)	13.19
Soil Texture	Moderately coarse
Soil Type	Sandy Loam
Soil Colour	Grey

%- Percentage, mg/kg – Milligram per Kilogram, $Cmolkg^{-1}$ - Centimoles per kg.

4.5 Physicochemical Properties of Soil After Planting

Table 4.7 shows the physical properties of the soil after planting, soil treated with NPK fertilizer and water appeared Grey in colour and moderately coarse in texture. Compared to the physical properties of the soil before planting, there was no change in the colour and texture of these soils. Pots of soil treated with biofertilizer appeared Dark in colour and Coarse in texture, this might be due to the black colouration of the biofertilizer and its granular texture. Table 4.8 shows the chemical properties of the soil after planting.

Table 4.7 Physical properties of Soil After Planting

Sample	Soil Texture	Soil Type	Soil Colour
Control (MW)	Moderately coarse	Sandy Loam	Grey
Maize with Biofertilizer (MBF)	Coarse	Sandy Loam	Dark
Maize with NPK Fertilizer (MNPK)	Moderately coarse	Sandy Loam	Grey

In comparison to the soil before planting (Table 4.6) and after planting, pH had no statistically significant difference between treatment conditions as determined by one-way ANOVA ($F_{2,6} = 1.902$, $p = 0.229$). The Bonferroni post hoc test revealed that there was no statistical difference in pH between control (MW) (6.2±0.3, $p = 0.843$), Maize with Biofertilizer (MBF) (6.5±0.4, $p = 0.304$) and Maize with NPK Fertilizer (MNPK) (6.1±0.2, $p = 1.000$). Although, the soil in these pots increased from an acidic pH to a nearly neutral pH. There was statistical significance in the mean and standard deviation of nitrogen content in the soil. The biofertilizer treatment (MBF) had a higher mean and significant value (9.0±0.4, $p = 0.000$) than the control (MW) (3.5±0.8) and NPK treatment (4.9±0.9, $p = 0.002$). There was no statistically significant difference between the control and NPK treatment ($p = 0.170$). The same statistical significance in nitrogen can be observed in the mean values of Organic carbon, Sodium, Potassium, Magnesium and Electrical acidity across the treatment conditions with significant values lower than $p = 0.05$. Phosphorous

and Electrical conductivity were statistically significant in mean difference across the treatment conditions. Soil moisture had no significant difference between treatment conditions. These values were analysed for test of normalcy, although organic matter failed test of normalcy so it was analysed as a non-parametric test compared to the other values described above. A Kruskal-Wallis H test showed that there was a statistically significant difference in organic matter between the different treatment conditions, $\chi 2(2) = 6.489$, $p = 0.039$, with a mean rank of 2.00 for Control (MW), 7.67 for MBF and 5.33 for MNPK. Games-Howell post hoc test further reveals that the presence of organic matter in the control was statistically significant with both treatments (MBF & NPK), but organic matter present in MBF treated soil was not statistically different from NPK treated soil. The analysis table are described in Appendix B. Nitrogen is responsible for the cellular synthesis of chlorophyll and other components in plants to enhance plant growth (Hayat *et al*., 2010). Soil nitrogen status plays a role in determining the effect of the Plant growth promoting rhizobacteria (PGPR) inoculation on the nitrifying and denitrifying communities where the crop could affect the soil nitrogen dynamics within the rhizosphere, it also influences the level of mineral nitrogen available (Florio *et al*., 2017). Fan *et al.*, (2017) stated that the effectiveness of PGPR may vary based on the source of nitrogen and soil fertility.

Table 4.8 Chemical properties of Soil After Planting

Sample	pH	N	Organic Carbon	Organic Matter	P	Na	K	Ca	M
	%	%	%	%	Mg/kg	Cmolkg^{-1}	Cmolkg^{-1}	Cmolkg^{-1}	C
Control (MW)	6.2± 0.3	3.5±0.8	9.6±0.9	7.3±1.3*	14.5±20.6*	0.5±0.9	0.9±1.0	6.9±12.7	1
MBF	6.5±0.4	9.0±0.4*	17.4±0.8*	14.8±0.9*	33.7±36.3*	1.7±2.6*	1.3±1.9*	14.6±15.1*	1
MNPK	6.1±0.2	4.9±0.9*	12.2±1.5*	12.6±1.5*	26.1±29.6*	0.8±1.1*	0.9±1.1*	9.9±11.6	1

MW – Maize + water, MBF- Maize + biofertilizer, MNPK- Maize + NPK, N- Nitrogen, P- Phosphorous, Na- Sodium, K- Potassium, Ca- Calcium, Mg- Magnesium, E. acidity Exchangeable Acidity, Elec Cond- Electrical Conductivity, * Significant, *P*-Value - *P*<0.05

4.6 Effects of Treatments on the Growth Characteristics of Zea mays (Maize)

Table 4.9, 4.10 and 4.11 respectively shows the effects of the treatments on the growth characteristics of *Zea mays* such as, the number of leaves, height of shoot and Length of leaves, seven (7) weeks after planting (WAP). Below are further descriptions and discussions of these effects on the growth characters and appearance of *Zea mays*.

4.6.1 Number of Leaves

Table 4.9 shows the effect each treatment had on the number of leaves produced of the maize plant at the end of each week. Seven (7) weeks after sowing, some pots had 2 shoots while some had just a shoot. The data collected on the number of leaves from the treatments was statistically analysed using a non-parametric test. According to Kruskal Wallis Test, all treatments were significant on the number of leaves for seven weeks with the significant value lower than the *p* value (0.05). Games-Howell post hoc test revealed that in week one, number of leaves on *Zea mays* treated with biofertilizer (MBF) was significant to the number of leaves in the control and with leaves treated with NPK, but there was no significant difference between the number of leaves in the control and NPK treated plant. In week 2, 3, 6 and 7, there was significant difference between the number of leaves of the control maize and maize treated with biofertilizer. The increase in number of leaves in week 4 was significantly different between the control and the two treatments. In week 5, the number of leaves was significantly different among the control and treatments, with biofertilizer treatment having the highest mean.

Table 4.9 Effect of Biofertilizer on No of leaves of Maize Plant

TREATMENT	TIME (WEEK)						
	1	2	3	4	5	6	7
Control (MW)	2.7±0.6	3.3±0.6*	3.7±0.6*	5.3±0.6	6.7±0.6*	7.7±0.6*	9.0±1.0*
Maize with Biofertilizer (MBF)	7.7±0.6*	9.7±0.6*	11.7±0.6*	13.7±0.6	17.7±0.6*	21.7±0.6*	26.7±0.6*
Maize with NPK Fertilizer (MNPK)	3.0±11.0	5.7±1.5	8.7±2.3	10.7±1.5	12.7±1.5*	16.0±2.6	19.0±4.4

MW- Maize + water, * Significant.

The first week after sowing, all maize plant appeared indifferent, although the biofertilizer treated maize had a higher number of leaves irrespective of the number of shoots. This could be as a result of the application of biofertilizer before sowing, introducing the microorganisms into the rhizosphere to stimulate other microorganisms present and stimulate plant growth and exchange of nutrients. Maize plant treated with NPK fertilizer

showed an increase in the number of leaves every two weeks, this could be traced to the application of NPK every two weeks according to farming practice. Although, in the fifth and sixth week, the number of leaves were not increasing according to pace, this could be due to the sensitivity to heat and drought stress experienced by maize plant at this stage of maize leaf growth called the V14 or Eighth leaf stage (Ciampitti *et al.*, 2011). In the seventh week, there was no significant difference (P<0.05) between the treatments on the number of leaves. The control plant had lower number of leaves, although there could be a possibility of heat being a factor that contributed to the close similarity in the leaf number, because the weather during this period was dry and hot. According to Berihanu (2016), other factors such as sowing depth could also contribute to the number of leaves formed during cultivation of *Zea mays*.

4.6.2 Height of Plant

Table 4.10 shows the effect of each treatment on the height of the maize plant during the cultivation period. A non-parametric test was used to statistically analyse the data acquired from the treatments. Except for the second week, all treatments were significant in terms of plant height according to the Kruskal Wallis Test. The significant level in week two was more than the p-value, whereas the significant level in all subsequent weeks was less than the *P*-value (0.05). The Games-Howell post hoc test indicated that the mean height of *Zea mays* treated with biofertilizer in weeks 1 and 2 differed significantly from the control and NPK treatments, whereas the height difference between the control and NPK treatments was not significant. The height of the maize plant treated with biofertilizer increased significantly from the previous weeks in week 3 and was significantly different from the mean height of the control maize plant with a significant value $P = 0$. The mean height of *Zea mays* was significantly different in weeks 4, 5, 6, and 7, with the biofertilizer-treated plant having the highest mean value (Table 4.10).

Table 4.10 Effect of Treatments on Height of Plant (cm) per Week

TREATMENT	TIME (WEEK)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Control (MW)	8.4±0.25*	9.4±0.1*	11.1±0.1	14.4±0.1*	16.6±0.2*	19.6±0.2*	21.3±0.3*
Maize with Biofertilizer (MBF)	10.3±0.3*	11.3±0.4*	13.6±0.1*	17.7±0.4*	22.9±0.4*	27.1±0.3*	35.1±0.9*
Maize with NPK Fertilizer (MNPK)	8.1±0.3	9.3±0.3	11.7±0.8	16.1±0.4*	19.3±0.4*	22.8±0.1*	24.2±0.2*

MW- Maize + water, * Significant.

According to the results, biofertilizer had a positive effect on the height of *Zea mays* when compared to *Zea mays* treated with NPK. This could mean that *Alcaligene faecalis* and *Providencia vermicola* synergized as Plant growth promoting bacteria (PGPB) to produce phytohormones and exchange metabolites with the plant to stimulate maize growth. This is consistent with the findings of Vejan *et al*. (2016), who reported that PGPB plays a significant role in boosting plant development via a number of methods and has a good effect on the plant by reducing phytopathogenic bacteria. El-kholy *et al.* (2005) reported that microorganisms promote root development, which improves nutrient and water intake

from the soil which is beneficial for the growth of maize. According to Beyranvand *et al.* (2013), photosynthetic material exchange activity in plants is enhanced by symbiosis with microbes, which boosts the efficiency of photosynthetic phosphorus. As a result, the photosynthetic capacity of *Zea mays* treated with biofertilizer improved, resulting in increased height.

4.6.3 Length of leaves

At the conclusion of each week, the length of the leaves was measured to assess the impact of the two treatments on the growth of the maize plant. The data gathered was analysed using a one-way ANOVA and the Bonferroni post hoc test to determine the mean of leaf length across treatment conditions from Week 1 to Week 7, with the exception of Week 2 which was analysed using the Kruskal-Wallis H Test (non-parametric test). This is due to the fact that week 2 failed the normalcy test. On ANOVA the seven weeks were all significant, Bonferroni post hoc test revealed that biofertilizer had a significant effect on the length of leaf of *Zea mays* and it was significantly different from control and NPK treatment with significance $P = 0.000$. Although, the control had no significant difference to NPK treatment. Weeks 3, 4, 5, 6 and 7 had significant mean differences across the treatment conditions with biofertilizer having the most significant effect on the plant. The Kruskal-Wallis H test showed that there was no statistically significant difference in the length of leaf between the different treatment conditions, $\chi 2(2) = 5.647$, $P = 0.059$, with a mean rank length score of 3.00 for Control, 8.00 for Biofertilizer and 4.00 for NPK. The effect of Treatments on Length of leaves is shown in Table 4.11.

Table 4.11 Effect of Biofertilizer on Length of leaves of Maize Plant

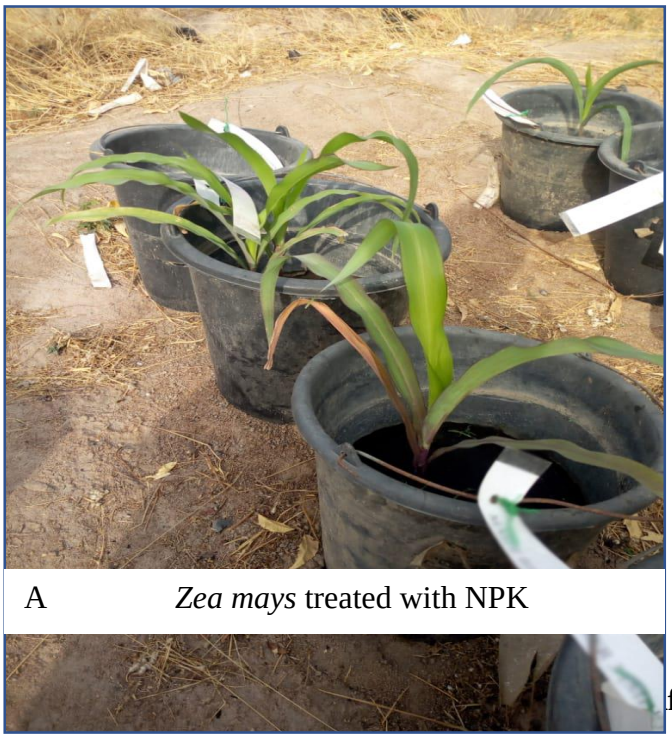
TREATMENT	TIME (WEEK)						
	1	2	3	4	5	6	7
Control (MW)	10.2±0.2	14.2±0.4	16.5±0.2*	17.1±0.1*	18.4±0.2*	20.4±0.2*	22.0±0.2*
Maize with Biofertilizer (MBF)	12.5±0.1*	17.4±0.2	21.9±0.2*	24.8±0.1*	29.6±0.1*	34.6±0.2*	41.4±0.5*
Maize with NPK Fertilizer (MNPK)	10.3±0.2	14.4±0.1	17.9±0.3*	21.0±0.2*	23.8±0.3*	27.5±0.58*	33.3±0.3*

MW- Maize + water, * Significant.

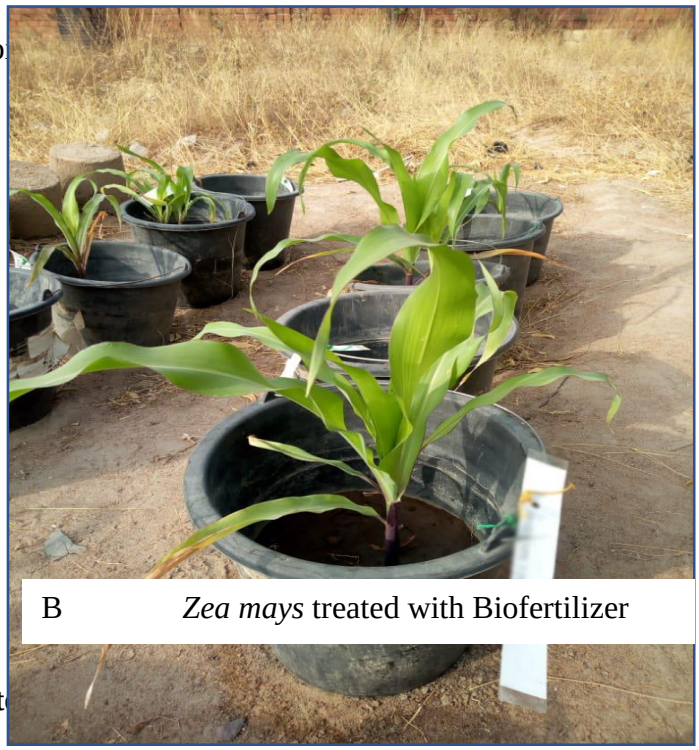
Pereira *et al.,* (2020) reported that Plant Growth Promoting Bacteria (PGPB) also influences aerial growth crops which can be observed in increasing plant height, shoot, weight and stem width, as well as increasing the number of leaves per plant. A similar finding was observed by Adiprasetyo *et al.,* (2014) where the multi-microbial biofertilizer was able to increase the height and the number of leaves of oil palm plants as compared to the sole treatment with chemical fertilizer.

4.7 Plant Appearance

During cultivation the maize plant showed no difference in appearance at germination stage. Two weeks after sowing, plants treated with biofertilizer and NPK appeared brighter in colour than the control, while the control appeared less green in colour. Plate VI: A, B and C show a pictorial description of the maize plant of the two treatments and



A *Zea mays* treated with NPK



B *Zea mays* treated with Biofertilizer

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Bacteria were isolated from the rhizosphere, characterised and identified as *Alcaligenes faecalis* and *Providencia vermicola* based on their ability to solubilise phosphorous and fix nitrogen. Biofertilizer was produced using the two bacteria with Charcoal as a carrier material.

The soil had a significant improvement in its physicochemical properties and nutrient value after the application of biofertilizer, compared to the properties of the soil before the application of biofertilizer. There were significant differences in the level impacts between

biofertilizer (26.7±0.6, 35.1±0.9 & 41.4±0.5) and NPK fertilizer (19.0±4.4, 24.2±0.2 & 33.3±0.3) on the growth of *Zea mays* based on the growth parameters measured

5.2 Recommendations

i. The use of biofertilizer, particularly that produced from *Alcaligenes faecalis* and *Providencia vermicola* should be encouraged to boost maize production.

ii. It is recommended that, further research should be undertaken to determine the biopesticide quality of *Alcaligenes faecalis* and *Providencia vermicola,* this is necessary to increase the market quality of the biofertilizer.

iii. Research on biofertilizer should be encouraged in the country as a cheap and eco-friendly way to improve food security in the country.

REFERENCES

Abbott, L. K., & Johnson, N. C. (2017). Introduction: Perspectives on Mycorrhizas and Soil Fertility. In *Mycorrhizal Mediation of Soil: Fertility, Structure, and Carbon Storage* (pp. 93-105). Amsterdam, Netherlands: Elsevier. doi:10.1016/B978-0-12-804312-7.00006-1

Adeleke, R. A., Raimi, A. R., Roopnarain, A., & Mokubedi, S. M. (2019). Status and Prospects of Bacterial Inoculants for Sustainable Management of Agroecosystems. In: B. Giri, R. Prasad, Q. S. Wu, & A. Varma (Eds.), *Biofertilizers for Sustainable Agricu lture and Environment* (pp. 137- 172). Basel, Switzerland: Springer International Publ ishing.

Adiprasetyo, T., Purnomo, B., Handajaningsih, M., & Hidayat, H. (2014). The Usage of BIOM3G-Biofertilizer to Improve and Support Sustainability of Land System of Independent Oil Palm Smallholders. *International Journal on Advanced Science Engineering and Information Technology*, 4(5), 43-46. Doi: 10.18517/ijaseit.4.5.431

Aggarwal, A., Kadian, N., Tanwar, A., Yadav, A., & Gupta, K. K. (2011). Role of Arbuscular mycorrhizal fungi (AMF) in Global Sustainable development. *Journal of Applied and Natural Science*, 3(2), 340-351. Doi: 10.31018/jans. v3i2.211

Ahmed, E., & Holmstrom, S. J. M. (2014). Siderophores in Environmental Research: Roles and Applications. *Microbial Biotechnology,* 7(3), 196 - 208. Doi: 10.1111/1751-7915.12117

Alam, F., Bhuiyan, M. A. H., Alam, S. S., Waghmode, T. R., Kim, P. J., & Lee, Y. B. (2015). Effect of Rhizobium specie BARIRGm901 Inoculation on Nodulation,

Nitrogen Fixation and Yield of Soybean (Glycine max) Genotypes in Grey Terrace Soil. *Bioscience, Biotechnology and Biochemistry*, 79(10), 1660 - 1668. Doi:10.1080/09168451.2015.1044931

Al-Erwy, A. S., Al-Toukhy, A., & Bafeel, S. O. (2016). Effect of Chemical, Organic and Bio Fertilizers on Photosynthetic Pigments, Carbohydrates and Minerals of Wheat (Triticum aestivum. L) Irrigated with Sea Water. *International Journal of Advanced Research in Biological Science*, 3(2), 296 - 310. Doi: 1.15/ijarbs-2016-3-2-40

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local Alignment Search Tool. *Journal of Molecular Biology*, 215(3), 403 - 410.

Ameeta, S. & Ronak, C. (2017). A Review on the Effect of Organic and Chemical Fertilizers on Plants. *International Journal for Research in Applied Science & Engineering Technology*, 5(2), 677 - 680. Retrieved from http://www.ijraset.com/fileserve.php.

Anderson, J. M. & Ingram J. S. I. (2014). TSBF: A Handbook of Methods of Analysis. Wallingford, UK: *CAB International*.

Anubrata, P., & Rajendra, D. (2014). Isolation, Characterization, Production of Biofertilizer & Its Effect on Vegetable Plants with And Without Carrier Materials. *International Journal of Current Research*, 6(8), 7986 - 7995. Retrieved from https://www.journalcra.com/

Bailey, J. S., Ramakrishna A., & Kirchof G. (2016). An Evaluation of Nutritional Constraints on Sweet Potato (Ipomoea Batatas) Production in The Central Highlands of Papua New Guinea. *Plant Soil*, 316, 97–106.

Balasubramanian, A. (2017). Chemical Properties of Soils. *Centre for Advanced Studies in Earth Science, University of Mysore*, 1 - 10. Retrieved from https://www.researchgate.net/publication/315669865

Barton, G. A. (2018). The Global History of Organic Farming. Oxford, England: Oxford University Press.

Berihanu, S. (2016). Effect of Different Sowing Depth on Germination and Growth Performance of Maize (Zea mays l.) at Jimma, Southwest Ethiopia. *International Journal of Research and Innovations in Earth Science*, 3(2), 2394-1375. Doi:10.2139/ssrn.3712003

Beyranvand, H., Farnia, A., Nakhjavan, S., & Shaban, M. (2013). Response of Yield and Yield Components of Maize (Zea mays L.) to Different Biofertilizers. *International Journal of Advanced Biological and Biomedical Research*, 1(9), 1068 - 1077.

Bhat, R. A., Hakeem, K. R., & Dervash, M. A. (2020). Bioremediation and Biotechnology, Vol 2 Degradation of Pesticides and Heavy Metals. In S. A. Raina, R. A. Bhat, H. Qadri, & A. Dutta (Eds.), *Values of biofertilizers for Sustainable Management in Agricultural Industries* (pp.121-137). Basel, Switzerland: Springer International Publishing.

Boraste, A., Vamsi, K. K., Jhadav, A., Khairnar, Y., Gupta, N., Trivedi, S., Patil, P., Gupta, G., Gupta, M., Mujapara, A. K., & Joshi, B. (2009). Biofertilizers: A Novel Tool for Agriculture. *International Journal of Microbiology Research*, 1(2), 23 - 31. Doi:10.9735/0975-5276.1.2.23-31

Bouyoucos, G. J. (2014). Hydrometer Method Improved for Making Particle Size Analyses of Soils. *Journal of Agriculture*, 53, 464 – 465.

Brady, N.C., & Weil, R.R. (1999). *The Nature and Properties of Soils*. (12th ed.). London, United Kingdom: Prentice Hall Publishers.

Bünemann, E., Bossio, D., Smithson, P., Frossard, E., & Oberson, A. (2016). Microbial Community Composition and Substrate Use in a Highly Weathered Soil as Affected by Crop Rotation and Plant fertilization. *Soil Biology Biochemistry*, 36, 889 – 901.

Callow, J. A., & Vincent, J. M. (1971). A Manual for the Practical Study of Root-Nodule Bacteria. *The Journal of Applied Ecology*, 8(3), 977. Doi:10.2307/2402718

Ceballos, I., Michael, R., Fernández, C., Penã, R., Rodríguez, A., & Sanders, I. R. (2013). The In-Vitro Mass-Produced Model Mycorrhizal Fungus, Rhizophagus irregularis, Significantly Increases Yields of The Globally Important Food Security Crop Cassava. *Ecosystem Environment*, 116, 72 – 84. Doi: 10.1371/journal.pone.0070633

Cheesbrough, M. (2006). *District Laboratory Practice in Tropical Countries, Part 2*. (2nd ed.). New York, USA: Cambridge University Press.

Cho, S., Kim, M., & Lee, Y. O. (2016). Effect of pH on Soil Bacterial Diversity. *Journal of Ecology and Environment*, *40*(1), 1- 9. Doi: 10.1186/s41610-016-0004-1j

Ciampitti, I. A., & Vyn, T. J. (2011). A Comprehensive Study of Plant Density Consequences on Nitrogen Uptake Dynamics of Maize Plants from Vegetative to Reproductive Stages. *Field Crops Research*, 121(1), 2 - 18. Doi: 10.1016/J.FCR.2010 .10.009

Debnath, S., Rawat, D., Mukherjee, A. K., Adhikary, S., & Kundu, R. (2019). Applications and Constraints of Plant Beneficial Microorganisms in Agriculture. In S. M. Mirmajlessi, R. Radhakrishnan (Eds.), *Rhizosphere and Soil Microbes - Utilization in Agriculture and Industry Under Current Scenario* (pp. 1-25). London, Kingdom: Intech Open. Doi:10.5772/intechopen.89190

Debojyoti, R., Manibrata, P., & Banerjee, S. K. (2017). Isolation Identification and Partial Characterization of Nitrogen Fixing Bacteria from Soil and Then the Production of Biofertilizer. *International Journal for Research in Applied Science and Engineering Technology*, *5*(11), 4021-4026. Retrieved from http://www.ijraset.com/fileserve.php.

Debojyoti, R., Manibrata, P., & Sudip, K. B. (2015). Isolation Identification and Character ization of Phosphate Solubilising Bacteria from Soil and the Production of Biofertilizer. *International Journal of Current Microbiology and Applied Sciences*, 4(11), 808 - 815. Retrieved from http://www.ijcmas.com.

El-Kholy, M.A., El-Ashry, S., & Gomaa, A.M. (2005). Biofertilization of Maize Crop and Its Impact on Yield and Grains Nutrient Content Under Low Rates of Mineral Fertilizers. *Journal of Applied Sciences Research*, 1(2), 117 - 121.

Etesami, H., Emami, S., & Alikhani, A. H. (2017). Potassium Solubilizing Bacteria (KSB): Mechanisms, Promotion of Plant Growth, and Future Prospects, A Review. *Journal of Soil Science and Plant Nutrition*, 17(4), 897 - 911. Doi:10.4067/s07189516201700 0400005

European Commission. (2013). Science for Environment Policy. In: *In-Depth Report, Nitrogen Pollution and The European Environment Implications for Air Quality Policy* (pp. 1-28). Bristol, England: University of the West of England (UWE). Retrieved from http://ec.europa.eu/science-environment-policy

Fan, X. H., Zhang, S., Mo, X. D., Li, Y., Fu, Y. Q., & Liu, Z. (2017). Effects of Plant Growth-Promoting Rhizobacteria and Nitrogen source on Plant Growth and Nitrogen and Phosphorous uptake by Tomato Grown on Calcareous Soils. *Pedosphere*, 27, 1027 – 1036. Doi:10.1016/S1002-0160(17)60379-5.

Fattah, O. A. (2013). Effect of Mycorrhiza and phosphorus on micronutrients concentration by soybean plant grown in acid soil. *International Journal of Plant Production*. 4, 429–437.

Florio, A., Pommier, T., Gervaix, J., Bérard, A., & Le Roux, X. (2017). Soil Carbon and Nitrogen Statuses Determine the Effect of Maize Inoculation by Plant Growth-Promoting Rhizobacteria on Nitrifying and Denitrifying Communities. *Scientific Reports Journal*, 7, 8411. Doi:10.1038/s41598-017-08589-4

Gao, X., Hoffland, E., Stomph, T., Grant, C. A., Zou, C., & Zhang, F. (2012). Improving Zinc Bioavailability in Transition from Flooded to Aerobic Rice. *Journal of Environment* 32, 465 – 478.

Ghazali, M. F., Wikantika, K., Harto, A. B., & Kondoh, A. (2020). Generating Soil Salinity, Soil Moisture, Soil pH from Satellite Imagery and its Analysis. *Information Processing in Agriculture*, 7(2), 294 - 306.

Gupta, R., Singal, R., Shankar, A., Kuhad, R. C., & Saxena, R. K. (1994). A Modified Plate Assay for Screening Phosphate Solubilizing Microorganisms. *Journal of General and Applied Microbiology*, 40(3), 255 - 260. DOI:10.2323/jgam.40.255

Hari, M., Seshadri, S. & Perumal, K. (2010). *Booklet on Biofertilizer (Phosphobacteria)*. Chennai, India: Shri AMM Murugappa Chettiar Research centre Taramani.

Harpole, W. S., Ngai, J. T., Cleland, E. E., Seabloom, E. W., Borer, E. T., Bracken, M. E., & Smith, J. E. (2011). Nutrient Co-limitation of Primary Producer Communities. *Ecology Letters,* 14(9), 852 - 862. Doi: 10.1111/j.1461-0248.2011. 01651.x

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil Beneficial Bacteria and Their Role in Plant Growth Promotion: A Review. *Annals of Microbiology*, 60, 579 – 598. Doi:10.1007/s13213-010-0117-1

Heanes, D.L. (1984) Determination of Total Organic-C in Soils by an Improved Chromic Acid Digestion and Spectrophotometric Procedure. *Journal Communications in Soil Science and Plant Analysis*, 15(10), 1191-1213. Doi: 10.1080/00103628409367551

Herrmann, L. & Lesueur, D. (2013). Challenges of Formulation and Quality of Biofertilizers for Successful Inoculation. *Applied Microbiology and Biotechnology*, 97(20), 8859 - 8873. Doi: 10.1007/s00253-013-5228-

Hinsinger, A. M., Kahangi, E. M., Ateka, E., Onguso, J., & Jefwa, J. M. (2012). Commercial Microbiological Products Affect Nutrient Concentration of Tissue Cultured Banana in Three Soil Types in Kenya. *International Journal of Agricultural Science*, 4, 344–355.

Itelima, J. U., Bang, W. J., Onyimba, I. A., & Egbere, O. J. (2018a). A Review: Biofertilizer - A Key Player in Enhancing Soil Fertility and Crop Productivity. *Direct Research Journal of Agriculture and Food Science,* 6(3), 73 - 83. Doi:10.26765/DRJAFS.2018. 4815

Itelima, J. U., Ogbonna, A. I, Onyimba, I. A., Nwadiaro, P. O., Nyam, M. A., & Agbala, C. C. (2018b). Comparative Study on the Effects of Bio-fertilizers, Organic and Inorganic Fertilizers on Growth and Yield Components of Cowpea (Vigna unguiculata). *Direct Research Journal of Biology and Biotechnology*, 4(2), 11 - 15. Doi: 10.26765/DRJBB .2018.5073

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., & Kopriva, S. (2017). The Role of Soil Microorganisms in Plant Mineral Nutrition—Current Knowledge and Future Directions. *Frontiers in Plant Science*, 8, 1617. Doi: 10.3389/fpls.2017.01617

Jenkins, W. R. (2013). A Rapid Centrifugal Flotation Technique for Separating Nematodes from Soil. *Plant Diseases*, 48, 692.

Johnson, N. C., Wilson G. W., Bowker M. A., Wilson J. A., & Miller R. M. (2010). Resource Limitation Is a Driver of Local Adaptation in Mycorrhizal Symbioses. *Nature,* 107, 2093 – 2301.

Kalayu, G. (2019). Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers. *International Journal of Agronomy,* 1-7. DOI: 10.1155/2019/4917256

Kapal, D. B., Taraken, I. T., & Sirabis, W. (2010). Soil Fertility Management Options in Sweet Potato Based Cropping Systems in The Highlands of Papua New Guinea. In: *19th World Congress of Soil Science, Soil Solutions for a Changing World* (pp. 144 - 146). Wien, Austria: International Union of Soil Sciences (IUSS).

Kapulnik, Y., Lahkim, L. T. L., Zipori, I., Hazanovsky, M., Wininger, S., & Dag, A. (2010). Effect of AMF Application on Growth, Productivity and Susceptibility to Verticillium Wilt Of Olives Grown Under Desert Conditions. *Symbiosis,* 52, 103 – 111.

Kavoo-Mwangi, A. M., Kahangi, E. M., Ateka, E., Onguso, J., Jefwa, J. M. (2014). Commercial Microbiological Products Affect Nutrient Concentration of Tissue Cultured Banana in Three Soil Types in Kenya. *International Journal of Agricultural Science*, 4, 344 – 355.

Koske, R. E., & Gemma, J. N. (2013). A Modified Procedure for Staining Roots to Detect VA Mycorrhizas. *Mycology Research*, 92, 486 – 489.

Lambers, H., Finnegan, P. M., Laliberté, E., Pearse, S. J., Ryan, M. H., Shane, M. W., & Veneklaas, E. J. (2011). Phosphorus Nutrition of Proteaceae in Severely Phosphorus-Impoverished Soils: Are There Lessons to Be Learned for Future Crops? *Plant Physiology*, 156(3), 1058 - 1066.

Lambers, H., Raven, J. A., Shaver, G. R., Smith, S. E. (2014). Plant Nutrient-Acquisition Strategies Change with Soil Age. *Trends Ecology Evolution*, 23, 95 – 103.

Liu, H., Finnegan, P. M., Laliberté, E., Pearse, S. J., Ryan, M. H., & Shane, M. W. (2010). Phosphorus Nutrition of Proteaceae in Severely Phosphorus-Impoverished Soils: Are There Lessons to Be Learned for Future Crops? *Plant Physiology*, 156, 1058 – 1066.

Lodish, H. B., Matsudaira, A., Kaiser, P., Krieger, C. A., Scott, M., Zipurksy, M. P. S. L., & Darnell, J. (2004). *Molecular Cell Biology.* (5th ed.). New York, USA: W.H. Freeman.

Matemilola, S. & Elegbede, I. (2017). The Challenges of Food Security in Nigeria. *Open Access Library Journal,* 4(12), 1 - 22. Doi: 10.4236/oalib.1104185

McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., & Swan, J. A. (2013). A New Method Which Gives an Objective Measure of Colonization of Roots by Vesicular-Arbuscular Mycorrhizal Fungi. *New Phytology,* 115, 495 – 501.

Meenakshi, S. (2016). Potential of Biofertilizers to Replace Chemical Fertilizers. *International Advanced Research Journal in Science, Engineering and Technology,* 3(5), 163-167. Doi:10.17148/IARJSET.2016.3534

Menendez, E., & Garcia-Fraile, P. (2017). Plant Probiotic Bacteria: Solutions to Feed the World. *AIMS Microbiology,* 3(3), 502 - 524. Doi: 10.3934/microbiol.2017.3.502

Mia, M. B., Shamsuddin, Z. H., & Mahmood, M. (2010). Use of Plant Growth Promoting Bacteria in Banana: A New Insight for Sustainable Banana Production. *International Journal for Agriculture Biology*, 12(3), 459 - 467.

Mukhongo, R. W., Tumuhairwe, J. B., Ebanyat, P., AbdelGadir, A. H., Thuita, M. & Masso, C. (2016). Production and use of Arbuscular Mycorrhizal Fungi Inoculum in Sub-Saharan Africa: Challenges and Ways of Improving. *International Journal of Soil Science*, 11(3), 108 – 122. Doi:10.3923/ijss.2016.108.122

Mwanga, R. O. M., Niringiye, C., Alajo, A., Kigozi, B., Namukula, J., Mpembe, I., Tumw egamire, S., Gibson, R. W., & Yencho, G. C. (2011). 'NASPOT 11', A Sweetpotato Cultivar Bred by A Participatory Plant-Breeding Approach in Uganda. *HortScience,* 46(2), 317 - 321.

Mwanga, R. O. M., Odongo, B., Ocitti p'Obwoya, C., & Turyamureba, G. M. (2011b). Sweet Potato (Ipomoea batatas (L.) Lam.), in Agriculture in Uganda, Vol. II. *Forestry Research,* 21, 45 – 52.

Nath, M., Bhatt, D., Bhatt, M. D., Prasad, R., & Tuteja, N. (2018). Microbe-Mediated Enhancement of Nitrogen and Phosphorus Content for Crop Improvement. In: *Crop Improvement Through Microbial Biotechnology* (pp. 293 – 304). Amsterdam, Netherlands: Elsevier. Doi: 10.1016/B978-0-444-63987-5.00014-1

Neina, D. (2019). The Role of Soil pH in Plant Nutrition and Soil Remediation. *Applied and Environmental Soil Science*, 2019(5794869), 1-9. Doi: 10.1155/2019/5794869

Onofre, S. B., Bertoldo, I. C., Abatti, D., & Refosco, D. (2017). Physico-Chemical Characteristics of the Biofertilizer Produced Anearobically in a Biodigester Using Poultry Litter as Substrate. *International Journal of Current Microbiology and Applied Sciences*, 6(2), 1830 -1839. Doi: 10.20546/ijcmas.2017.602.206

Onyeonwu, R. O. (2000). *Manual for Waste/Wastewater, Soil/ Sediment, Plant and Fish Analysis*. MacGill Environmental Research Laboratory. Benin City, 81.

Oshoma, C, E., Igbeta, B., & Omonigho, S. E. (2017). Analysis of Microbiological and Physiochemical Properties of Top Soil from Municipal Dumpsites in Benin City. *Journal of Applied Science and Environmental Management*, 21(5), 985 - 990. Doi: 10.4314/jasem. v21i5.28

Pepper, I. L., Gerba, C. P., Newby, D. T., & Rice, C. W. (2009). Soil: A Public Health Threat or Saviour? *Critical Reviews in Environmental Science and Technology*, 39(5), 416 - 432.

Pereira, S. I. A., Abreu, D., Moreira, H., Vega, A., & Castro, P. (2020). Plant growth-Promoting Rhizobacteria (PGPR) Improve the Growth and Nutrient Use Efficiency in Maize (*Zea mays* L.) under water deficit conditions. *Heliyon*, 6(10), e05106. Doi: 10.1016/j.heliyon. 2020.e05106

Phillips, I. (1993). Cowan And Steel's Manual for The Identification of Medical Bacteria. *Journal of Clinical Pathology*, 46(10), 975.

Phogat, V.K., Tomar, V.S., & Dahiya, R. (2015). Soil Physical Properties. In: R.K. Rattan, J.C. Katyal, B.S. Dwivedi, A.K. Sarkar, T. Bhattachatyya, J.C. Tarafdar, & S.S. Kukal (Ed.), *Soil Science: An Introduction* (pp.135-171). New Delhi, India: Indian Society of Soil Science.

Raimi, A., Roopnarain, A., & Adeleke, R. (2021). Biofertilizer Production in Africa: Current Status, Factors Impeding Adoption and Strategies for Success. *Scientific African*, 11, 1-19. Doi: 10.1016/j.sciaf. 2021.e00694

Rakshit, A., & Bhadoria P. B. S. (2016). Indigenous Arbuscular Mycorrhiza Is More Important for Early Growth Period of Groundnut (Arachis hypogea L.) for P Influx in an Oxisol. *Journal of Soil Science*, 91, 397– 406.

Revillas, J. J., Rodelas, B., Pozo, C., Martinez-Toledo, M. V., & Gonzalez-Lopez, J. (2000). Production of B-Group Vitamins by Two Azotobacter strains with Phenolic Compounds as Sole Carbon Source Under Diazotrophic and Adiazotrophic Conditions. *Journal of Applied Microbiology*, 89(3), 86 - 93.

Rosendahl, S. (2012). Communities, Populations and Individuals of Arbuscular Mycorrhizal Fungi. *New Phytology*. 178, 253 – 266.

Schubert, A., & Hayman, D. S. (2011). Plant Growth Responses to Vesicular–Arbuscular Mycorrhiza XVI. Effectiveness Of Different Endophytes at Different Levels of Soil Phosphate. *New Physiology*, 103, 79 – 90.

Shaji, H., Chandran, V., & Mathew, L. (2021). Controlled Release Fertilizers for Sustainable Agriculture. In: *Organic Fertilizers as a Route to Controlled Release of Nutrients* (pp. 231-245). Massachusetts, USA: Academic Press. Doi: 10.1016/B978-0-12-819555-0.00013-3

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate Solubilizing Microbes: Sustainable Approach for Managing Phosphorus Deficiency in Agricultural Soils. *Springer Plus*, 2(1), 587. Doi:10.1186/2193-1801-2-587

Shree, K. A. (2020). Engineering Cyanobacteria Nitrogen Biofertilizer for Rice Cultivation in Stressful Environment. *Mycobacterial Diseases*, 8(4), 1 - 2. Retrieved from https://www.longdom.org/mycobacterial-diseases.html

Singh, R., Soni, S. K., & Kalra, A. (2013). Synergy Between Glomus Fasciculatum and A Beneficial Pseudomonas in Reducing Root Diseases and Improving Yield and Forskolin Content in Coleus Forskohlii Briq. Under Organic Field Conditions. *Mycorrhiza*, 23(1), 35 - 44. doi: 10.1007/s00572-012-0447-x

Smith, F. W., Mudge S. R., Rae A. L., & Glassop, D. (2013). Phosphate Transport in Plants. *Plant Soil*, 248, 71–83.

Suthar, H., Hingurao, K., Vaghashiya, J. & Parmar, J. (2017). Fermentation: A Process for Biofertilizer Production. In: D. Panpatte, Y. Jhala, R. Vyas, & H. Shelat (Eds), *Microorganisms for Green Revolution* (pp. 229 - 252). Singapore: Springer.

Taraken, I. T., Kapal, D., Sirabis, W., & Bailey, J. (2010). Nutrient Deficiencies Limiting the Growth of Sweet Potato Vines on Important Soil Types in The Highlands of Papua New Guinea. In: *19th World Congress of Soil Science, Soil Solutions for a Changing World* (pp.32 - 35). Brisbane, Australia. Retrieved from https://www.researchgate.net/publication/287745345_Potassium_nutrition_of_sweet_potato

Tuğrul, K. M. (2019). Soil Management in Sustainable Agriculture. In M. Hasanuzzaman, M. C. M. T. Filho, M. Fujita & T. A. R. Nogueira (Eds.), *Sustainable Crop Production*. London, England: Intech Open. Doi: 10.5772/intechopen.88319

Uwah, D. F., Undie, U. L., John, N. M., Ukoha, G. O. (2013). Growth And Yield Response of Improved Sweet Potato (Ipomoea Batatas (L.) Lam) Varieties to Different Rates of Potassium Fertilizer in Calabar, Nigeria. *Journal of Agriculture Science*, 5, 61– 69.

Vanlauwe, B., Bationo, A., Chianu, J., Giller, K. E., Merckx, R., Mokwunye, U., Ohiokpehai, O., Pypers, P., Tabo, R., Shepherd, K.D., Smaling, E.M.A., & Sanginga, N. (2010). Integrated Soil Fertility Management: Operational Definition and Consequences for Implementation and Dissemination. *Outlook Agriculture*, 39(1), 17–24.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Boyce, A. N. (2016). Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability—A Review. *Molecules Journal*, 21(573), 1-17. Doi: 10.3390/molecules21050573

Verbruggen, E., Van der Heijden, M. G., Rillig, M. C., & Kiers, E. T. (2012). Mycorrhizal Fungal Establishment in Agricultural Soils: Factors Determining Inoculation Success. *New Phytolology*, 197, 1104 – 1109.

Verma, R. E., & Gemma, J. N. (2014). A Modified Procedure for Staining Roots to Detect VA Mycorrhizas. *Mycology Research*, 92, 486–489.

Wang, C., Li, X., Zhou, J., Wang, G., & Dong, Y. (2015). Effects Of Arbuscular Mycorrhizal Fungi on Growth and Yield of Cucumber Plants. *Soil Science*, 39, 499 – 509.

Weber, O. B. (2014). Biofertilizers with Arbuscular Mycorrhizal Fungi in Agriculture. In: *Mycorrhizal Fungi: Use in Sustainable Agriculture and Land Restoration* (pp. 45-66). Berlin, Heidelberg: Springer.

Wen, C. K. (Eds.). (2015). *Ethylene in Plants*. Netherlands: Springer. Doi: 10.1007/978-94-017-9484-8

Withers, P. J. A., Neal, C., Jarvie, H. P., & Doody, D. G. (2014). Agriculture and Eutrophication: Where Do We Go from Here. *Sustainability Journal*, 6, 5853-5875. Doi:10.3390/su6095853

APPENDICES

APPENDIX A: Nucleotide sequence of *Alcaligene faecalis*

Forward Sequence

AAATTATAGNTGCGCGNTATTCTTTTTAAGGCTGGGTGCCTGGATTTAAATGC
CCAATGCACAAGGCCGATGCCTGGAACACTCCTTAATACCGCATACGCCCTAC
GGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGG
ATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTT
TGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATC
CCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAA
AAGGTATCCCCTAATACGGGATACTGCTGACGGTATCTGCAGAATAAGCACCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAA
TCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGTATGTC
AGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGG
AATACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCAGACACGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACG
ATGTCAACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGA
AGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGAC
GGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAA
AACCTTACCTACCCTTGACATGTCTGGAAAGCCGAAGAAATTTGGCCGTGCTC
GCAAAAGAACCGGAACCCAGGTGCTGCATGGCTGTCCCCAGCTCCGGCCCGG
AAAAGTTGGGTTAAGTCCCGCACCGACGCAACCCTTGTCCTAATTGCTACCCA
AAACCCTCTAATGGGACTGGCCGGGGACAAACCGGAGGAAGGTGGGGATAAC
CCCCAGCCCCCTGGCCCTTATGGGGAG

Reverse Sequence

CCCGGAAACTAAAGAAGGGGAACCAGCGGCCACGGAGGGCCAGGGTCCACCT
CGTCTAGGTAGGACTGTAGCACGTGCTGGCTGTAGAATCGCCAATTGCCAAGG
CGATTTCGCGCTGAGCCATGATCAAACAACGTATTCACCGCGACATTCTGATC
CGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCG
GACTACGATCGGGTTTCTGAGATTGGCTCCCCCTCGCGGGTTGGCGACCCTCT
GTCCCGACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACT

TGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCT
CTTGCGTAGCAACTAATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC
ATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCCGGTTCTCT
TGCGAGCACGGCCAAATCTCTTCGGCTTTCCAGACATGTCAAGGGTAGGTAAG
GTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCC
CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTT
CACGCGTTAGCTGCGCTACTAAGGCCTAACGGCCCCAACAGCTAGTTGACATC
GTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG
TGTCTGAGCGTCAGTATTATCCCAGGGGGCTGCCTTCGCCATCGGTATTCCTCC
ACATATCTACGCATTTCACTGCTACACGTGGAATTCTACCCCCCTCTGACATAC
TCTAGCTCGGCAATTAAAAATGCAGTTCCAAGGTTGAGCCCTGGGATTTCACA
TCTTTCTTTCCGAACCGCCTACACACGCTTTACGCCCAGTAATTCCGATTAACG
CTTGCACCCTACGAATTACCGCGGCTGCTGGCACAAAGTAACCCGAGCCTAAT
TCTGCAAATACCGTCAGCAGCATCCCGCATTAAGGGATACCTTTTCTTCTCTGC
CAAAAGGACTTTACAACCCGAAGGCCTTCATCATACCCCCGGAATGGCTGGAT
CAGGGGTTTCCCCCATTGTCCAAAATTCCCCCCTGCTGCCCTCCCGGAAGA

Consensus

AAATTATAGTGCGCGTATTCTTTTTAAGGCTGGGTGCCTGGATTTAAATGAAC
CCAATGCACAAGGCCGATGCCTGGAACACTCCTTAATACCGCATACGCCCTAC
GGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCG
GATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGT
TTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCTCGGG
AGGGCAGCAGGGGGAATTTTGGACAATGGGGGAAACCCCTGATCCAGCCATC
CGGGTATGATGAAGGCCTTCGGGTTGTAAAGTCTTTTGGCAGAGAAGAAAAG
GTATCCCTAACGGGATCTGCTGACGGTATTGCAGAATAGCGGTACTGTGCCAG
CAGCCGCGGTAATCGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTT
GGAACTGCATTTTTAATGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCC
ACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGC
AGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGG
GCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGT
ACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGT
GGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACA
TGTCTGGAAAGCCGAAGAATTTGGCCGTGCTCGCAAAGAACCGGAACCAGGT
GCTGCATGGCTGTCCAGCTCGCGAAGTTGGGTTAAGTCCCGCACGAGCGCAAC
CCTTGTCATATTGCTACCAAGACCTCTAATGGACTGGCCGGGACAAACCGGAG
GAAGGTGGGGATACCAGTCCCTGGCCCTTATGGGAGGGCTTCACACGTCATAC
AATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAA
CCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATC
GCTAGTAATCGCGGATCAGAATGTCGCGGTGAATACGTTGTTTGATCATGGCT
CAGCGCGAAATCGCCTTGGCAATTGGCGATTCTACAGCCAGCACGTGCTACAG
TCCTACCTAGANCGAGGTGGACCCTGGCCCTCCGTGGCCGCTGGTTCCCCTTCT
TTNAGTTTNCCGGG

APPENDIX B: Nucleotide sequence of *Providencia vermicola*

Forward sequence

CGGGGACCCTAAAGTTTGAACATGGCTCAGGGGCCGCCTGGTATCACGTCCATG
GGGTTGACCGCGCTCGGGGGGGGGTGGGTCTGATTTCTTATACAGATAGAGGGGG
AGAACGCACGTGTGAAACGGCCGTCCTATATACCGCATAATCTCTTAGGAGCAA
AGCAGGGGAACTTCGGTCCTTGCGCTATCGGATGAACCCATATGGGATTAGCTA
GTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT
GATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGA
AGAAGGCCTTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGATGCTA
ATATCATCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCACGCAGGCGGTTGATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG
GGAATGGCATCTAAGACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCC
ATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCG
GCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGGAGTCCACGCTGTAAACGATGTCGATGAAGGTTGTTCCCT
TGGGAGTGGCTTTCGGAGCTAACGCGTTAAATCGACCGCCCGGGGAATACGGCC
GCAGGTTAAAACTCAAATGAATTGACGGGGGCCCCACAGCGGTGGAGCATGGG
GTTTAATTCGAGGCAACCGGGAGAAACCTTACCTACCTGGATCCAGGGAATTTA
CGAATGCTTTGGGGGCCTTGGGGAAACCTGAAAACGGGGGCG

Reverse sequence

GCCGCGGAAAAGGGCCCGCCGGTAAAAACCCGGCCGGCCACGTTATGGGAGCA
CTGTTACGCTAGCTTCGGCGTCGACTGCCCCCGTTCCGGGCCTCCCGAATAAGG
TTGGGTCCGCAACTGACAGCGCGCGGAGTTTTTTCTGAAGAGTTTTCGCCTGGG
CCCGATTCCTACGGTCGCGGGAGCC

Consensus

CGGGGACCCTAAAGTTTGAACATGGCTCAGGGGCCGCCTGGTATCACGTCCATG
GGGTTGACCGCGCTCGGGGGGGGGTGGGTCTGATTTCTTATACAGATAGAGGGGG
AGAACGCACGTGTGAAACGGCCGKCCTATAACCGCATAATCTCTTAGGAGCAAA
GCAGGGAATTMGGTCCTTSCGCTATCGGATGAACCCATATGGGATTAGCTAGTA
GGTGGGGTAAGGCTCACCTAGGCGACGATCCCAGCTGGTCGAGAGGATGATCAC
CACACTGCGGACTGAGACACGCCCAGACCCACGGGAGGCAGCAGTGGGGAATT
GCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCTTAG
GGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCTGATGCTAATATCATCAACGATT
GACGTTACCACAGAAGATGCACCGGCAACTCCGTGCAGCAGCCGCGGATAATAC
GGAGGGTGCAGAGCGTGTAATCAGGAATTCTGGCGTAAAGCGCACGCAGGCGCG
TGATTAAGTTAGATGTGAAATCCCGGGGCTTAACCTGGGAAGCGCATCTAAGAT
GGTCAGCTAGAGTCTTGTAGAGGGGGTGAATTCCATGTGCGGTGAAATGCGTAG
AGATGGGAGGAATACCGGTGGGAAGGGGCCCCCTGGACAAAGACGACGCCGGT
GCGAAAGCGTTTGGGACAAACGGATTAGAACTGGGGTCCACGCTGTTAAACTGA
TGCGAGCTGAAGGTTGTTCCTGNGGAGTGGCTTTCGGAGCTAACGCGTTAAATCG
ACCGCCCGGGGAATACGGCCGCNAGGTTAAAACTCAAATGAATTGACGGGGGCC
CCACNAGCGGTGGAGCATGGGGTTTAATTCGAGGCAACCGGGAGAAACCTTACC

TACCTGGATCCAGGGAATTTACGAATGCTTTGGGGGCCTTGGGGAAACCTGAAA
ACGGGGGCG