Isolation and Characterization of Plant Growth Promoting Rhizosphere Bacteria and Arbuscular Mycorrhizal Fungi Associated with Coffee Rhizosphere from Qellem Wallaga, Ethiopia

By: Sagni Biranu

A Thesis Submitted to

The Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfilment of the Requirement for the Degree of Master's of Science Degree in Applied Biology (Microbiology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

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Candidate's Declaration

I hereby declare that this MSc thesis entitled as **Isolation and Characterization of Plant Growth Promoting Rhizosphere Bacteria and Arbuscular Mycorrhizal Fungi Associated with Coffee Rhizosphere from Qellem Wallaga, Ethiopia** is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis work have been acknowledged.

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Advisors' approval sheet

To: Applied Biology Department

Subject: Thesis Submission

This is to certify that the thesis entitled "**Isolation and Characterization of Plant Growth Promoting Rhizosphere Bacteria and Arbuscular Mycorrhizal Fungi Associated with Coffee Rhizosphere from Qellem Wallaga, Ethiopia**" Submitted in Partial Fulfilment of the Requirement for the Degree of Master's in Applied Biology (Microbiology), the Graduate Program of the Department of Applied Biology and has been carried out by **Sagni Biranu Gelata** Id. No: **GSR/0191/09**, under our supervision. Therefore, we recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

_____	_____	_____
Name of Advisor	Signature	Date
_____	_____	_____
Name of Co-advisor	Signature	Date

Approval of Board of Examiners

We, the undersigned members of the Board of Examiners of the final open defence by **Sagni Biranu Gelata** have read and evaluated his thesis entitled "**Isolation and Characterization of Plant Growth Promoting Rhizosphere Bacteria and Arbuscular Mycorrhizal Fungi Associated with Coffee Rhizosphere from Qellem Wallaga, Ethiopia**" and examined the candidate. Therefore, this is to certify the thesis has been accepted in partial fulfilment of the requirement of the Degree of Master's in Applied Biology (Microbiology).

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Advisor	Signature	Date
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External Examiner	Signature	Date

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ABBREVIATIONS AND ACRONYMS

ACC	1-Amino Cyclopropane-1-Carboxylic acid
AMF	Arbuscular Mycorrhiza Fungi
BTB	Bromothymol Blue
CFU	Colony Forming Unit
GA	Gibberellic Acid
HCN	Hydrogen Cyanide
IAA	Indole Acetic Acid
ISR	Induced Systematic Resistance
LB	Luria Bertani
LIA	Lysine Iron Agar
PGP	Plant Growth Promoting
PGPR	Plant Growth Promoting Rhizobacteria
PSB	Phosphate Solubilising Bacteria
RPM	Revolutions Per Minute
SAR	Systematic Acquired Resistance
TCP	Tri-calcium Phosphate
TSIA	Triple Sugar Iron Agar

ABSTRACT

Coffee is one of the most consumed and exported crop for income in the agriculture-based country like Ethiopia. However, the use of chemical fertilizers are not encouraged for coffee production due its cost, availability and environmental concerns. The uses of bio-fertilizers such as PGPR and AMF could serve as an alternative for enhancement of plant growth and sustainable agricultural production in eco-friendly ways. The purpose of this study was to isolate and characterize the PGPR and AMF associated with coffee rhizosphere. Rhizosphere soil samples and fine fibrous roots were collected from four different coffee variety of Coffea arabica species: 74110, Black Coffee (Buna Gurracha), Mana Sibu and Non-Shading coffee (Buna Aduu). The samples were collected from two different cultivation system, monoculture and agroforestry of Gawo Kebe District, Qellem Wallaga Zone, Ethiopia. The soil samples and fine coffee roots were utilized for isolation of rhizobacteria and determination of AMF root colonization potential. The isolates were tested for phosphate solubilisation ability, IAA production and antagonism against coffee wilt disease (Gibberella xylarioides) under in-vitro condition. From the total of five composite coffee rhizosphere soil samples, thirty-five bacteria (sixteen from monoculture and nineteen from agroforestry) were isolated. From the total rhizobacterial isolates, 42.9% were Gram positive while 57.1% of them were Gram negative bacteria. Based on their morphological and biochemical characteristics the rhizobacterial isolates were identified as genus Bacillus, Pseudomonas, Enterobacter, Corynebacterium, Citrobacter, Serratia, Brevibacterium, Micrococcus, Gordonia, Erwinia, Klebsiella and Burkholderia. The in-vitro screening of isolates showed that 40% exhibited phosphate solubilisation and 48.6% showed antagonism against Gibberella xylarioides. However, all the isolates failed to produce IAA without supplement of precursor Lysogeny-tryptophan. AMF spore density ranging from 1.66 to 3.68 g^{-1} of soil were recorded. The highest spore density was recorded from coffee rhizosphere soil of agroforestry. All of the coffee plant roots were colonized with AMF structures (hyphae, arbuscule and vesicle) with variable percentage. Total root length colonization was between 87.6% to 95% for agroforestry while monoculture was 61.9% to 81.5%. Generally, most of the coffee rhizobacterial isolates showed plant growth promotion ability. Therefore, the use of PGPR and AMF as bio-inoculant should be the future direction for agricultural productivity improvement of coffee.

Keywords: Biocontrol, Bio-inoculants, Coffee, Plant growth promotion, Mycorrhiza.

1. INTRODUCTION

The word coffee comes from the name of a place found in South Ethiopia, “Kaffa” which means the plant of God (Alemayehu, 2014). Coffee is classified under the family of Rubiaceae in the genus *Coffea*. The two most widely cultivated coffee species are *Coffea arabica* L. and *Coffea canephora* (Tekalign *et al.,* 2016). In Ethiopia, South-western and South-eastern parts are considered as the origin of *Coffea arabica* L (Diriba, 2007).

Of the total world coffee production, *Coffea arabica* L. and *Coffea canephora* accounts to 66% and 34% respectively (Alemayehu, 2014). Coffee is the most traded agricultural products around the globe next to oil (Tekalign *et al.,* 2016). *Coffea arabica* L. is the most important world commodity and is a principal source of revenue for the agriculture based Ethiopian economy (Diriba *et al.,* 2013).

PGPR are free-living soil bacteria that colonize the rhizosphere, enhance plant growth and yield of plants when applied to seed or crops (Glick, 2012). The plant growth promoting (PGP) effect of the PGPR is mostly explained by the release of metabolites that directly and indirectly stimulating growth. There are several mechanisms of PGPR to benefit the host plants (Nihorimbere *et al.,* 2011). These are the ability to produce phytohormones such as indole acetic acid (IAA), cytokinin and gibberellins, enhancing N_2 fixation, solubilising inorganic phosphate and mineralization of organic phosphate. They also promote plant growth indirectly by functioning as antagonistic against phytopathogenic microorganisms. This is due to production of siderophores, the synthesis of antibiotics and lytic enzymes (Majeed *et al.,* 2015).

Chemical fertilizers are used to supply essential nutrients to the soil plant system and increase plant growth and yield throughout the world. However, its price, availability and the environmental concerns are real issues of today’s agriculture. Therefore, it needs to find strategies that can ensure competitive crop yields, provide environmental safety and protect ecological balance in agro-ecosystem. The plant growth promoting rhizobacteria (PGPR) for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world (Majeed *et al.,* 2015).

Both Gram positive and Gram negative bacterial isolates from the coffee rhizosphere have plant growth promoting activity. Some of them have IAA producers while others have ability to degrade ACC (Diriba *et al.,* 2009). The bacterial genera of *Bacillus*, *Erwinia*, *Serratia* and *Pseudomonas* were reported to have plant growth promoting activities.

Some of the *Bacillus* BT_{42} species exhibited multiple plant growth promoting activity. They showed antagonism against fungal plant pathogen such as *Colletotrichum gloeosporioides* and *Fusarium oxysporum* with variable percentage of fungal mycelial inhibition (Tekalign *et al.,* 2016). They have ability to produce lytic enzymes such as chitinase, β-1,3-glucanase, protease and lipase. They have also phosphate and zinc solubilisation, ammonia, IAA and ACC production ability.

Arbuscular mycorrhizal fungi (AMF) are fungi active in the rhizosphere, take part in the cycles and transfer of mineral elements in the soil and into the roots (AL-Areqi *et al.,* 2013). They are grouped under phylum Glomeromycota that live in the soil and form symbiotic associations with the plant roots by forming symbiotic structures such as hyphae, arbuscule and vesicle (Smith and Read, 2008; Santhoshkumar and Nagarajan, 2017). They function as an extension of the root system of the plant and increasing absorptive area. AMF associations are of great importance in forest ecology, land rehabilitation, plant health, increase water and nutrient uptake (Smith and Read, 2008).

AMF increase the uptake of macro elements such as N, K specially P and micro elements such as Zn, Cu, S, Fe, Mg, Ca and Mn. Supplement of these nutrients enhance host resistance to root pathogens and abiotic stress such as drought and metal toxicity (Santhoshkumar and Nagarajan, 2017). Water deficiency is one of the most important abiotic factors limiting plant growth and yield. AMF decrease the effects of extreme variations in temperature, pH and water stress (AL-Areqi *et al.,* 2013).

1.1. Statement of the Problem

Coffee is one of the Ethiopian agricultural products that is consumed and also exported to foreign country for income (Alemayehu, 2014; Samuel and Ludi, 2006). Chemical fertilizers are industrially synthesized substances composed of known quantities of nitrogen, phosphorus and potassium. Their exploitation causes air and ground water pollution by eutrophication of water bodies (Bhardwaj *et al.,* 2014).

West Wallaga is one of the zone found in Ethiopia, which is known by coffee production. The farmers in this zone is mostly depend on the coffee production. However, Because of coffee is perennial plant, the cost of chemical fertilizer for coffee plant supplement is high, low availability and environmental concerns local farmers do not use chemical fertilizers for coffee farming. These lack of enough nutrient from the soil for coffee plant causes reduction in coffee yield and income from coffee market, and also exposing the plants to disease in the area. For this reason, coffee producers are not getting promising yield and income from coffee market. Therefore, it needs to find alternative strategy by which the coffee producers improve their yield quality and quantity, and income from coffee market which also improve income of the country from coffee export.

Besides, chemical fertilizers affect the beneficial microorganisms that can improve soil fertility and control soil borne pathogens. The use of bio-fertilizers and biocontrol can minimize the side effect of chemical fertilizers and minimize environmental pollution. A significant reduction in the use of phosphate fertilizer can be achieved if solubilisation of soil insoluble phosphorus is made available to crop plants by rhizosphere microorganisms. However, agrochemicals (fertilizers and pesticides) have greatly influenced natural rhizosphere microbes in agro-systems.

Plant beneficial microbial bio-resources are promising to replace many such destructive, high intensity practices and support eco-friendly crop production. In particular, the use of plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) for the benefits of agriculture and ecosystem functions is gaining worldwide importance and acceptance conditions.

1.2. Objectives of the Study

1.2.1. General Objective

The general objective of this study was to isolate and characterize the plant growth promoting rhizobacteria and arbuscular mycorrhizal fungi associated with coffee rhizosphere from Qellem Wallaga, Ethiopia.

1.2.2. Specific Objectives

- ✧ To isolate and determine the abundance and diversity of bacteria associated with coffee rhizosphere soil
- ✧ To screen and determine the plant growth promoting potential of plant growth promoting rhizobacteria (PGPR) isolated from coffee rhizosphere
- ✧ To evaluate the arbuscular mycorrhizal fungi spore density and root colonization potential of coffee trees.

1.3. Significance of the Study

The soil microorganisms constitute world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. Most of the rhizobacterial isolates are able to exert beneficial effects on plant growth. Inorganic forms of phosphate are solubilised by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic minerals or chelate cationic ion of the phosphate ions that is PO_4^{3-} directly.

Chemical fertilizers are expensive, less available and environmental concerns. In contrast, the use of PGPR and AMF can maintain soil fertility, plant health as well as increase plant resistance to biotic and abiotic stress. This study was addressed whether the beneficial rhizobacteria have potential to promote plant growth and potential of AMF root colonization. Exploring of rhizobacteria having useful plant growth promoting traits reduces the cost of chemical fertilizers and also reduces the risk of soil and water pollution from constant use of chemical fertilizers. This study provides the way to improve agricultural productivity by using microbial resources that have plant growth promoting activity.

2. LITERATURE REVIEW

2.1. Rhizosphere

Rhizosphere is a thin layer of soil surrounding plant roots and the soil occupied by the roots that supports large groups of microorganisms. Rhizobacteria are a group of bacteria competent in colonizing the root environment (Nandal and Hooda, 2013). Plant roots in the rhizosphere secrete most organic compounds such as amino acids and sugars. These compounds provide a food source for microorganisms, increasing microbial biomass and their activity in the rhizosphere (Geetanjali and Jain, 2016).

The root system provides anchorage and uptake of nutrients and water, and used to mediate numerous underground interactions. Plant roots also synthesize, accumulate and secrete different compounds that are used as a source of nutrients for diverse microorganisms present in the rhizosphere. Rhizosphere is the zone of soil influenced by roots through the release of substrates that affect microbial activity (Nandal and Hooda, 2013).

2.2. Plant Growth Promoting Rhizobacteria (PGPR)

There are enormous bacterial population ranging from 10^8 to 10^9 per gram of rhizosphere soil. They cover about 4-10% of the total root area occurring on the root hair region (Geetanjali and Jain, 2016). The different genera of bacteria that are found in the soil are involved in various biotic and abiotic activities of the soil ecosystem. The bacteria lodging in or on the plant roots are more versatile in transforming, mobilizing, solubilising the nutrients compared to those from bulk soils. Therefore, rhizobacteria are the dominant deriving forces in recycling of the soil nutrients and they are crucial for soil fertility (Nandal and Hooda, 2013).

PGPR are naturally occurring soil bacteria that colonize plant roots and benefit plants by stimulating plant growth (Shahab *et al.*, 2009). They belong to diverse bacterial genera of *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Rhizobium, Acinetobacter, Erwinia, Mycobacterium, Mesorhizobium, Flavobacterium, Alcaligenes, Arthrobacter and Serratia* (Nandal and Hooda, 2013; Shakeela *et al.*, 2017).

PGPR are defined by three intrinsic characteristics (Nandal and Hooda, 2013).

i. they must be able to colonize the root surface
ii. they must survive and multiply in microhabitats associated with the root surface and must also able to compete with other microbiota for plant protection from pathogenic microorganisms
iii. They must promote plant growth

2.3. Mechanisms of Plant Growth Promotion by PGPR

Microorganisms play a vital role in maintaining soil fertility and plant health. They can act as bio-fertilizers and biocontrol. PGPR can promote plant growth by two mechanisms (Figure 1). Directly, by production of phytohormones like auxins, cytokinin and gibberellins, enhancing plant nutrition by solubilisation of minerals like phosphorus and iron, fixing atmospheric nitrogen and lowering of ethylene levels (Shahab *et al.*, 2009). Indirectly benefit the plant by supressing plant pathogens that inhibit plant growth by production of antibiotics, siderophores, HCN which regulate availability of phosphate, competition for nutrients and niches within the rhizosphere, induction of systemic resistance (Munees and Mulugeta, 2013). They also produce extracellular enzymes such as chitinase and glucanase to hydrolyse the fungal cell wall and decreasing pollutant toxicity (Jha, 2012; Chauhan *et al.*, 2015).

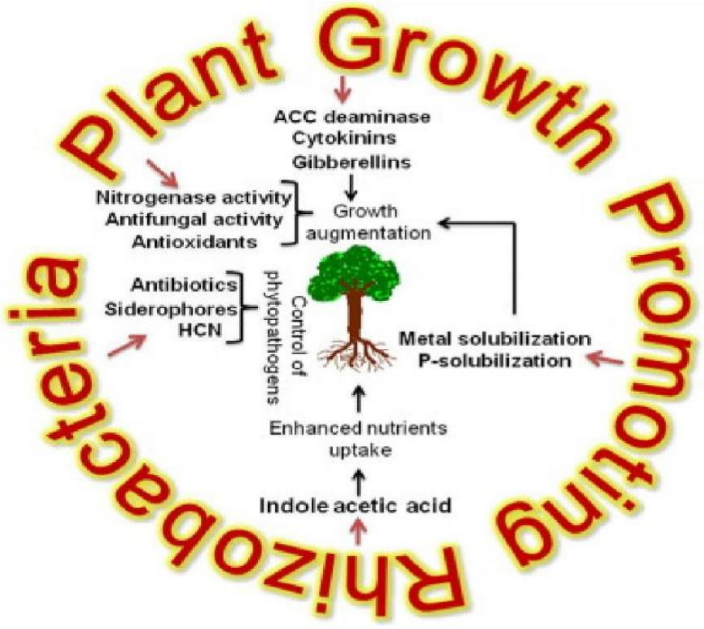


Figure 1: Mechanisms of plant growth promotion by rhizobacteria.

Source: (Nandal and Hooda, 2013).

2.3.1. Direct Plant Growth Promotion

2.3.1.1. Phosphate Solubilisation

Phosphorus (P) is one of the essential macronutrients next to nitrogen, that limit plant growth. Most soils in tropical and subtropical areas are predominantly acidic and extremely phosphorus deficient due to their strong fixation of phosphorus as insoluble phosphates of iron and aluminium.

The form of phosphorus that the plants need for growth and development is present in the inorganic form. Bacteria make P available to the plants by converting into soluble form by two mechanisms. These are: by releasing organic acids and affecting the mobility of phosphorus by means of ionic interactions and by means of phosphatase enzyme that help to unbind the phosphate groups from organic matter (Kundan *et al.,* 2015).

The release of phosphate by PSB from insoluble form is an important aspect regarding phosphate availability in soil. Microorganisms enhance the phosphate availability to plants by mineralizing organic phosphate in the soil and by solubilising inorganic phosphates (Figure 2). Thus, the use of soil microorganisms which can fix atmospheric nitrogen, solubilise phosphorus, stimulate plant growth through synthesis of growth promoting substances and antagonize plant pathogenic fungi have importance over the use of chemical fertilizers and fungicides (Shakeela *et al.,* 2017). Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth surroundings were reported to participate in phosphate solubilisation by PSB (Singh *et al.,* 2015).

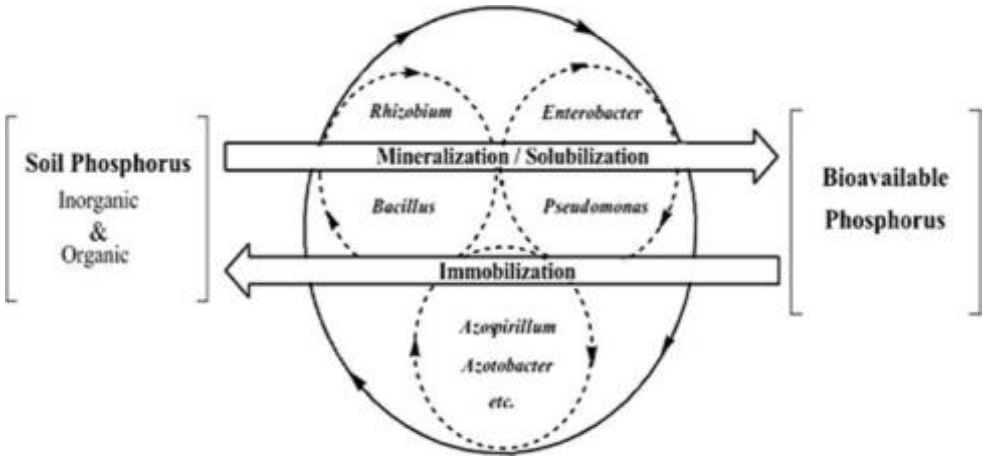


Figure 2: Solubilisation of soil phosphorus by rhizobacteria.

Source: (Jha, 2012).

Phosphate solubilisation ability was detected by the formation of clear zone around bacterial colony on Pikovskaya's agar medium supplemented with tri-calcium phosphate ($Ca_3(PO_4)_2$ as a source of insoluble inorganic phosphorus. Rahman *et al.* (2017) reported that, all the 104 bacteria isolated from wheat rhizosphere were able to produce transparent clear zone around the colony with different diameter. This indicates that, the isolates have exhibited phosphate solubilisation ability. They obtained phosphate solubilising ability of bacterial isolates ranging from 24.29 to 738.57 g/ml on National Botanical Research Institute Phosphate (NBRIP) liquid medium using tri-calcium phosphate as a source of insoluble phosphate.

Befekadu *et al.* (2017) isolated 169 bacteria from rhizosphere and root of *Coffea arabica* of southern Ethiopia, on nutrient agar. They evaluated ability to solubilise phosphate on Pikovskaya's agar plate. From this 54.7% of the identified bacteria were isolated from root, 27.9% from soil and the rest 17.4% were isolated from root washing solution. Members of the identified rhizobacteria were dominated by the genus *Pseudomonas* (44.1%) followed by *Bacillus* (11.6%), *Enterobacter* (10.5%) and *Stenotrophomonas* (10.5%). Out of these 32.5% of the isolates showed phosphate solubilisation ability on Pikovaskya's agar medium. Members of the phosphate solubilising bacteria were dominated by the genus of *Pseudomonas* (14.5%) *Citrobacter* (3.6%), *Rhodococcus* (9.1%), *Stenotrophomonas* (7.3%), *Gordonia* and *Bacillus* (3.6%) respectively.

Rodrigues *et al.* (2016) isolated endophytic bacteria from leaf, stem, root and rhizosphere of commercial sugarcane variety and screened for IAA production, ability to solubilise phosphate, fix nitrogen, produce HCN and ammonia, the enzymes pectinase, cellulase and chitinase. Out of 136 bacteria isolated, 83 of them showed plant growth promotion. From these, 47% phosphate solubilisers, 26% nitrogen fixers, 57% IAA producers, 0.7% HCN and chitinase, 45% ammonia, 30% cellulase and 8% were pectinase producers. They showed that seven isolates have highest PGR activity under *in-vitro* condition in maize. They reported that, from these, five isolates promoted plant growth in greenhouse experiments by showing potential as bio-fertilizers. The tested isolates were identified into the genera of *Klebsiella*, *Enterobacter* and *Pantoea.*

Delelegn and Fassil (2011) isolated rhizobacteria from rhizosphere of *Eragrostis tef*. Fifty-two percent of the isolates showed the ability of phosphate solubilisation, HCN and antibiotic production. They reported that, most of the bacterial isolates with phosphate

solubilisation ability were a Gram positive of genus *Bacillus*. In another study, Rawat and Mushtaq (2015) isolated plant growth promoting rhizobacteria from legume rhizosphere and identified as *Bacillus*, *Pseudomonas and Corynebacterium* sp. They characterized the isolates under *in-vitro* condition for plant growth promotion activities. Their result revealed that, these isolates exhibited significant plant growth promotion by phosphate solubilisation, siderophore and protease production.

PSB isolated from wheat rhizosphere of saline soil in western region of Algeria were tested for their plant growth promoting traits such as IAA, HCN, siderophore, ammonia production and their ability to fix nitrogen (Rahman *et al.,* 2017). They reported that, among the total 104 bacterial isolates, 41 were selected for their phosphate solubilising activity using tri-calcium phosphate (TCP) as a sole inorganic phosphorus source. IAA was produced by most of the isolate. Twelve isolates were positive for HCN production, 32 produced siderophore and 31 were able to fix nitrogen. The most dominant PSB were identified as *Pseudomonas* followed by *Aeromomas hydrophila*, *Bacillus* sp. and *Burkholderia cepacia*. It was reported that, the PSB plant inoculations resulted in 10-15% increases in crop yields and phosphate uptake in ten out of thirty-seven experiments in India (Adesemoye and Egamberdieva, 2013).

2.3.1.2. Production of Phytohormones (Indole Acetic Acid)

Phytohormones are also called plant growth regulators. They are well known for their regulatory role in plant growth and development, and work extremely at low concentrations. IAA is the most common hormone produced by PGPR and physiologically most active hormone in plants (Shahab *et al.,* 2009; Bhardwaj *et al.,* 2014).

Lysogeny-tryptophan, an amino acid is serves as a precursor for biosynthesis of auxins in higher plants and in microbes. Root exudates are natural sources of tryptophan for the rhizosphere microflora, which can enhance auxin biosynthesis in the rhizosphere. It has many functions such as xylem and root growth, and also participate in plant cell division, differentiation and elongation (Kundan *et al.,* 2015).

IAA is known to stimulate both a rapid response (increased cell elongation) and a long term response (cell division and differentiation) in plants. Specifically, IAA is a phytohormone that is known to be involved in root initiation, cell division and cell enlargement. A significant activity of PGPR is the production of auxin type phytohormones that affect root morphology and thereby improve nutrient uptake from soil.

IAA producing PGPR increase root growth and root length, resulting in greater root surface area, which enables the plant to access more nutrients from soil (Figueiredo *et al.*, 2010).

Microorganisms inhabiting rhizosphere of various plants are likely to synthesize and release auxin as secondary metabolites. This is due to the rich supplies of substrates supplied from the roots compared with non-rhizosphere soils. Diverse soil microorganisms including bacteria, fungi and algae are capable of producing physiologically active quantities of auxins, which may exert beneficial effects on plant growth and establishment (Shahab *et al.*, 2009). They reported that, *Azotobacter paspali* secreted IAA into culture media and significantly increased the dry weight of leaves and roots of mung beans following root treatment.

The beneficial characteristics of PGPR were reported in Malaysia on potato (Adesemoye and Egamberdieva, 2013). They screened 15 PGPR strains for IAA production with and without addition of the precursor Lysogeny-tryptophan. Phosphate solubilising activity, nitrogen fixing, antagonistic activity against fungal pathogens, siderophore production and antibiotic resistance were tested. All the isolates were produced IAA and grew in nitrogen free media. They found that *Pseudomonas fluorescens* significantly increased plant height (16%), the number of grains per spike (11.7%) and grain yield (39%) compared to non-inoculated control. They reported that, bacterial based fertilizer increased the yield of wheat and maize, and protected plants from fungal disease.

Phosphate solubilising bacteria isolated from wheat rhizosphere were tested for their ability to produce IAA in Lysogeny broth medium supplemented with Lysogeny tryptophan as precursor. The appearance of pink colour after addition of Salkowski reagent to the culture was reported as IAA production. IAA production was revealed by almost all the bacterial isolates and quantities were ranged from 10.9 to 63.35 g/ml (Rahman *et al*., 2017). Shahab *et al.* (2009) isolated 28 PSB bacteria from soil and identified them on the basis of API kit. They tested the isolates for IAA production and obtained IAA production in PSBs isolates with tryptophan was 57 to 288 g/ml.

2.3.1.3. Nitrogen Fixation

Nitrogen (N) is the most vital nutrient for plant growth and productivity. Although, there is about 78% of atmospheric N, it is unavailable to plants free. The atmospheric nitrogen is converted into plant utilizable form by biological nitrogen fixation (BNF). Nitrogen fixing

(diazotrophic) bacteria fix atmospheric nitrogen by means of nitrogenase enzyme. It is a two component metalloenzyme composed of dinitrogenase reductase (Fe protein) and the dinitrogenase (MoFe protein) component that contains metal cofactors. The dinitrogenase reductase gathers electrons from low redox carriers (ferrodoxin, flavodoxin) and transfers them to dinitrogenase. Nitrogen is bond to the dinitrogenase protein where it is reduced. The process requires ATP (Nandal and Hooda, 2013).

Nitrogen fixing bacteria can occur as obligate symbionts of leguminous plant *Rhizobium* and in non-leguminous plant *Frankia*; while bacteria such as *Azotobacter, Azospirillum* and *Acetobacter* are free living (Chauhan *et al.,* 2015). Microorganisms belongs to genera of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azotobacter*, *Azospirillum* and *Enterobacter* isolated from rhizosphere of *Sorgastrum nutans* were reported to enhance plant growth because of their nitrogen fixing ability (Singh and Jha, 2015).

The visual detection of nitrogen fixing activities of the selected isolates were observed by using nitrogen-free malate-mannitol medium (NF-MM), containing bromothymol blue (BTB) as an indicator. After the medium was inoculated by the bacterial isolates and incubated at 28^0C for 24 hours, blue coloured zone producing isolates were marked as nitrogen fixers in the solid culture conditions (Rahman *et al.,* 2017). They tested PSB isolated from wheat rhizosphere for their ability to fix nitrogen on nitrogen free medium (NFM). The medium was supplemented with bromothymol blue as colour indicator to detect the release of ammonium in the culture. Among the 41 bacterial isolates, 31 showed nitrogen fixing ability by changing medium colour from green to blue. They recorded diameter of coloration zone ranged from 7 to 26 millimetre.

2.3.1.4. Production of Aminocyclopropane-1-carboxylate (ACC) Deaminase

Ethylene is an essential metabolite for the normal growth and development of plants. However, it also established as a stress hormone under stress conditions like those generated by salinity, drought, water logging, heavy metals and pathogenicity. The endogenous level of ethylene is significantly increased which affects the overall plant growth in such conditions. For instance, high concentration of ethylene induces defoliation and other cellular processes that may lead to reduced crop performance. PGPR which possess 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme facilitate plant growth and development by optimizing ethylene levels (Aarab *et al.,* 2015).

This enzyme induces salt tolerance and reduce drought stress in plants (Munees and Mulugeta, 2013). It presents in strains from different rhizobacterial genera and hydrolysis ACC, ethylene precursor that inhibits root elongation, thereby promote root development.

Bacterial strains exhibiting ACC deaminase activity were identified in a wide range of genera such as *Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia and Rhizobium* (Munees and Mulugeta, 2013). Aarab *et al.* (2015) detected ACC deaminase enzyme in *Pseudomonas* sp. isolated from rhizosphere soil of rice.

Such rhizobacteria take up the ethylene precursor ACC and convert it into 2-oxobutanoate and ammonia. Several forms of stress are removed by ACC deaminase producers. They also, function in resistance to stress from heavy metals, wounding, high salt concentration, extremes of temperature and high light intensity. The seed or root inoculation with ACC deaminase producing rhizobacteria were reported to promote plant root elongation, shoot growth and enhancement in rhizobial nodulation. They also play important role in N, P and K uptake as well as mycorrhizal colonization in various crops (Munees and Mulugeta, 2013).

2.3.2. Indirect Plant Growth Promotion

2.3.2.1. Production of Siderophores

Siderophores are low molecular weight iron chelating agents produced by microorganisms (Nandal and Hooda, 2013). They comprise lateral chains and functional groups that confer ligands a strong affinity to coordinate with the ferric ion. Some siderophores (e.g., aerobactin) have lower and others (e.g., enterobactin) have higher affinities. Siderophores are produced by bacteria, fungi and monocotyledonous plants in response to iron stress (Munees and Mulugeta, 2013).

Siderophores are Fe^{3+} binding agents, that can prevent the harmful effects caused by phytopathogenic organisms on plant. When iron concentration is getting low, many bacteria produce siderophore (Ali and Vidhale, 2013). Siderophore chelate iron and supply to bacterial cell by outer membrane receptors (Rodrigues *et al.*, 2016).

Iron is an essential micronutrient that serves as a cofactor of many enzymes with redox activity. It is required in a number of major physiological processes like N_2 fixation, photosynthesis and respiration for plants. Microorganisms and plants have evolved specific

mechanisms to chelate insoluble iron through the release of Siderophores. They uptake iron siderophore complexes through specific outer membrane receptor proteins to meet their iron requirements (Nandal and Hooda, 2013).

Siderophores have three different types: hydroxamates, catecholates and carboxylates (Chauhan *et al.,* 2015). The synthesis of siderophores in bacteria is induced by the low level of ferric ion (Fe^{3+}). Microbial siderophores in the rhizosphere are frequently associated with biocontrol activities than plant nutrition (Nandal and Hooda, 2013).

In aerobic environment, iron occurs principally as Fe^{3+} and form insoluble hydroxides and oxyhydroxides. This, makes it inaccessible to both plants and microorganisms. PGPR can acquire iron by the secretion of siderophores which have high association constants for complexing iron (Munees and Mulugeta, 2013). Thus, PGPR improve plant growth and development by increasing iron accessibility in the rhizosphere (Chauhan *et al.,* 2015).

Siderophore production is an important feature in the suppression of plant pathogens. They act as determinants of induction of systematic resistance (ISR) under iron starved conditions (Dey *et al.,* 2014). They function mainly in the solubilisation, transport and storage of iron. The mechanisms by which siderophore producing bacteria contribute to the promotion of plant growth are example, *Pseudomonas fluorescens* species suppress the soil borne plant diseases and restrict the growth of pathogens.

2.3.2.2. Inhibition of Coffee Wilt Disease (*Gibberella xylarioides*) by PGPR

Coffee wilt disease (tracheomycosis) induced by *Gibberella xylarioides* (anamorph: *Fusarium xylarioides*) is one of the major fungal disease contributing to reduced coffee yield in Ethiopia. It is prevalent in most of the coffee growing areas with average incidence and severity of 28% and 5% respectively (Kifle *et al.,* 2016). Bacteria that reduce the incidence of plant diseases are often referred to as biocontrol agents. PGPR have antagonistic activity against plant pathogen through synthesis of hydrolytic enzymes such as chitinase, glucanase, protease and lipases that can lyse pathogenic fungal cells (Beneduzi *et al.,* 2012).

PGPR are not only promote plant growth directly; they also play important effect against plant disease. *Gibberella xylarioides* is a fungal pathogen that inhibit coffee plant. It is soil inhabiting fungus which penetrates coffee tree through wounds in the aerial parts and the

roots. After slow incubation period, the pathogen finally invades the vascular tissues resulting in rapid wilt and cause death of the tree (Girma, 2004).

According to Sihen *et al.* (2012) study, incidence of *Gibberella xylarioides* varied from field to field. The incidence was greater during dry season than wet season. They reported that, 90.6% of *Gibberella xylarioides* was isolated from wood samples of infected coffee trees from southwest and southeast forest coffee site of Ethiopia. Coffee wilt disease is causing significant losses to coffee trees in forest coffee. It kills coffee plant at any growth stage in all production system (Girma, 2004).

Toppo *et al.* (2015) tested antagonism of bacterial isolates from soil of agriculture based area against plant fungal pathogen *Fusarium* species by using dual culture plate method. Their result revealed that, from total of fourteen bacterial isolates on nutrient agar six isolates that showed antagonistic activity against *Fusarium oxysporum*. By performing biochemical tests, the isolates showed antagonistic activity were identified as genus *Bacillus.* A study done by Geetha *et al.* (2014) showed, among the twenty isolates from rhizosphere of green gram, seven of them exhibited antagonism activity against plant fungal disease *Fusarium oxysporum* and *Macrophomina phaseolena* by inhibiting mycelial growth.

2.3.2.2.1. Mechanisms of Antagonism

PGPR can suppress plant pathogen by producing antibiotics (pyrrolnitrin, pyocyanin), HCN and siderophores. They also limit availability of iron necessary for the growth of pathogen by production of pseudobactin, and degrade fungal cell wall by production of lytic enzymes such as chitinase and glucanase (Chauhan *et al.*, 2015). Prashar *et al.* (2013) isolated *Bacillus* species from rhizosphere of tomato and reported that *Bacillus* species have antagonism against fungal plant disease *Fusarium oxysporum* by production of HCN and ammonia. Hydrogen cyanide production is one of the way by which PGPR can suppress plant pathogens. Microbial production of HCN is reported to have antifungal activity (Chauhan *et al.*, 2015).

2.3.2.3. Induced Systematic Resistance (ISR)

Induced resistance is an enhancement of the plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection is called induced systematic resistance (ISR) or systematic acquired resistance (SAR). The induction of systemic resistance by

rhizobacteria, which are non-pathogenic is referred as ISR, whereas that by other agents is called systematic acquired resistance (Van Loon and Bakker, 2005). SAR is commonly triggered by the elicitors of a virulent pathogens, such as microbial associated molecular patterns, but it can also be induced by chemical compounds (Dey *et al.*, 2014).

SAR can be triggered by exposing the plant to pathogenic and non-pathogenic microbes. Depending on the plant and elicitors, a set period of time is required for the establishment of SAR wherein accumulation of pathogenesis related proteins (chitinase and glucanase), and salicylic acid takes place. ISR is produced by PGPR such as stains belonging to genus *Pseudomonas* that does not cause damage to the plant's root system. Unlike SAR, ISR does not involve the accumulation of pathogenesis related proteins or salicylic acid, but instead, relies on pathways regulated by jasmonate acid and ethylene (Devendra *et al.*, 2007).

Plants can resist pathogen by direct production of pathogenesis related proteins in one pathway. The production of pathogenesis related proteins is generally the result of attack by pathogenic microorganisms. Pathogenesis related proteins are generally produced as a result of wounding or necrosis inducing plant pathogens. Both pathways are alternate mechanisms for induction. The pathogen induced pathway relies on salicylic acid (SA) that is produced by the plant as a signalling molecule, whereas the wounding pathway relies on jasmonic acid (JA) as the signalling molecule (Devendra *et al.*, 2007). Rhizobacterial isolates can elicit ISR through production of volatile compounds such as: 2,3-butanediol. The bacterial species of *Bacillus amyloliquefaciens* and *Bacillus subtilis* reported to trigger ISR in *Arabidopsis* by producing this compound (Van Loon and Bakker, 2005).

2.4. Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) is one of the most important symbiotic fungi found in association with plant roots. They play important role in plant growth promotion by increasing nutrient uptake capacity specially phosphorus. Reduce plant attack by soil pathogenic microorganisms and also interact with soil microorganisms such as PGPR to benefit the host plant (Gosling *et al.*, 2010).

Mycorrhizal symbiosis is important for growth of coffee plant (Lebro *et al.*, 2012). The role of AMF has been described as a fundamental link between plant and soil. They have ability to protect host plants from root pathogens and mitigate the effects of extreme variations in temperature, pH and water stress. It is take part in the cycles and transfer of

mineral elements in the soil and into the roots (AL-Areqi *et al.*, 2013). And also, it functions as an extension of the plant root system and increasing absorptive area.

The most important effect of AMF is improving phosphorus nutrition of the host plant in soils with low phosphorus levels. This is due to the large surface area of their hyphae and high affinity phosphorus uptake mechanisms. They can also produce organic acids that solubilise the insoluble mineral phosphates. It was reported that, AMF mycelia have capability to increase uptake of many nutrients such as N, S, B, Cu, K, Zn, Ca, Mg, Na, Mn, Fe, Al and Si (Diriba, 2007).

According to Douira *et al.* (2014) study on rhizosphere soil of the carob tree, the existence of a community of mycorrhizal fungi were very abundant and diverse. Al-Areqi *et al.* (2013) detected diversified AMF such as *Glomus proliferum, Glomus etunicatum, Acaulospora sporocarpia, Acaulospora and Glomus* species in the rhizosphere of *Coffea arabica* in Republic of Yemen. They reported that, frequency of *Glomus* species in the coffee rhizosphere of study sites was 93%.

Zerihun *et al.* (2013) studied AMF colonization potential of root samples and rhizosphere soil of nine acacia species (*Acacia abyssinica*, *Faidherbia albida*, *A. nilotica*, *A. senegal*, *A. seyal*, *A. sieberiana*, *A. saligna*, *A. tortilis* and *A. robusta*) from Bishoftu, Zeway and Addis Ababa sites with different land use types to assess their AMF diversity, spore density and root colonization. They reported that, roots of all acacia species were colonized from low to moderate or relatively high levels by AMF with the occurrence of arbuscules, vesicles and hyphae. The highest AM fungal colonization was found in *A. seyal* (67.3%) from open grazing field at Zeway followed by *A. nilotica* (44%), whereas the lowest AMF colonization of 12% was recorded in *A. saligna* at Bishoftu.

Rhizosphere soils of acacia species harboured AMF fungal spores ranging from 3.7 spores g^{-1} soil in *A. nilotica* to 15.0 spores g^{-1} in *A. seyal* from open grazing field at Zeway. A total of 41 AMF species in 14 genera and 7 families of the Glomeromycota were identified. Nine species belonged to *Acaulospora*, 6 to *Funneliformis*, 4 each to *Gigaspora*, *Glomus*, and *Rhizophagus*, 3 each to *Claroideoglomus*, and *Scutellospora*, 2 each to *Racocetra* and *Diversispora*, and 1 each to *Entrophospora*, *Sclerocystis*, *Paraglomus* and *Pacispora.* Based on relative abundance and isolation frequency of spores, *C. claroideum*, *C. etunicatum*, *C. luteum*, *F. geosporus* and *G. aggregatum* were the dominant species of AMF in the study.

3. MATERIALS AND METHODS

3.1. Description of the study area

The study site was Gawo Kebe district, Qellem Wallaga Zone, Oromia Regional State, Ethiopia (Figure 3). The district is located at 594 km away from the capital city and 86 km away from Dambi Dollo toward the west. It is found between the altitudinal range of 1500 to 3303 metre above sea level (Zelalem, 2015). Two types of soils identified in the study were loam soil and sand soil. The major rainy seasons in the area are spring (April to May), summer (June to August) and autumn (September to October). The local farmers commonly practise agroforestry and monoculture cultivation systems. The main crops cultivated in this area are maize, *tef*, coffee, barley, bean and pea. Based on the GPS data recorded during sample collection, sites of sample collection were between the elevation of N $8^0$59’3”; E 34^0 58’16.5” and N $8^0$59’21.7”; E $34^0$59’18.3”.

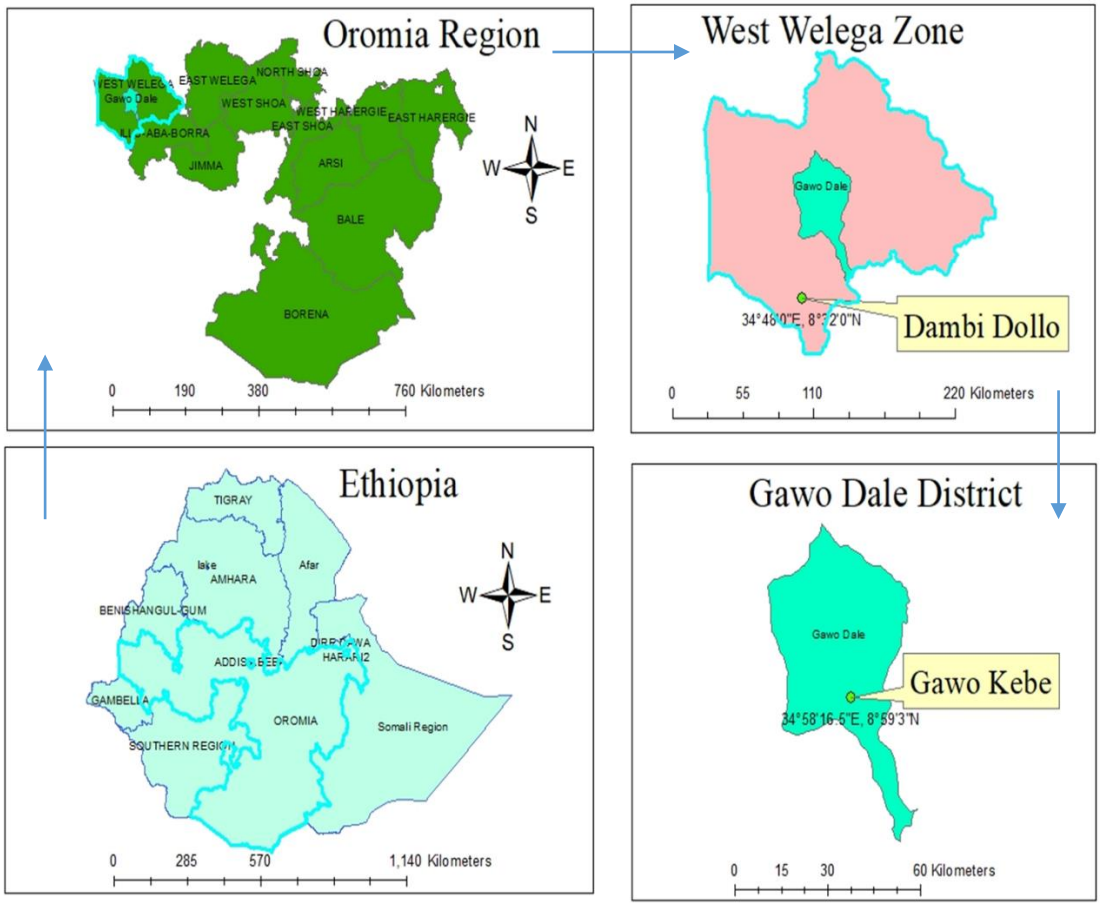


Figure 3: Map of the coffee trees rhizosphere soils and roots sample collection site from Gawo Kebe district.

3.2. Sample Collection

Fifteen samples of rhizosphere soil and fine roots, nine from monocultures and six from agroforestry coffee production system were collected during February to March, 2018. Three coffee trees from each field was systematically selected. One kilogram of coffee rhizosphere soil at the depth of 30 cm from five fields, two from agroforestry system and three from monoculture (in triplicate from each field) and fine fresh fibrous coffee roots were collected from each site. Four coffee varieties were selected for the study considering the presence of the varieties in the area. The coffee varieties included in this study were the variety of *Coffea arabica* species: 74110, Black coffee (Buna Gurraacha), Mana sibuu and Buna aduu (Sun coffee or non-shading coffee). The samples were pooled into one composite sample depending on coffee variety and farming system. Soil samples were collected in sterilized polyethylene plastic bag, air dried and stored at room temperature for further analysis. Fine coffee roots sample were collected and stored in 50% of alcohol and stored at 4°C for determination of root colonization by AMF (Brundrett *et al.*, 1995).

3.3. Soil Physicochemical Analysis

3.3.1. Soil Samples pH Analysis

For the determination of soil pH each soil sample was added into separate beaker and dissolved in distilled water by one to two point five (1:2.5) ratios of soil to water. The content was stirred for thirty minutes and the residue was allowed to settle for two minutes. Then the pH meter was calibrated with pH buffers. A pH meter wire was inserted into the beaker that contains soil suspension and pH reading was recorded for each sample.

3.3.2. Soil Chemical Analysis

Soil chemical analysis was carried out at Ethiopian Institute of Agricultural Research, Agricultural and Nutritional Research Laboratory, Bishoftu. Soil phosphorus was extracted from the soil by using Bray II solution as extractant. The extracted phosphorus was measured colourimetrically, based on the reaction with ammonium molybdate and development of the molybdenum blue colour. Then, the absorbance of the compound was measured at 882 nm in a spectrophotometer. This is directly proportional to the amount of phosphorus extracted from the soil (Bray and Kurtz, 1945).

Soil nitrogen content was analysed by using Kjeldahl method. The soil sample was digested in strong sulphuric acid in the presence of a catalyst, which helps in the

conversion of the amine nitrogen to ammonium ions. The ammonium ions were then converted into ammonia gas. The ammonia gas was led into a trapping solution where it dissolves and becomes an ammonium ion. Finally, the amount of ammonia trapped was determined by titration with a standard solution (Baethgen and Alley, 1989).

Soil organic carbon content was analysed by using Walkley-Black chromic acid wet oxidation method. Oxidisable matter in the soil was oxidised by $K_2Cr_2O_7$ solution. The reaction was assisted by the heat generated when two volumes of H_2SO_4 were mixed with one volume of the dichromate. The remaining dichromate was titrated with ferrous sulphate. The titre is inversely related to the amount of carbon present in the soil sample (Walkley and Black, 1934).

3.4. Isolation of Bacteria from Coffee Rhizosphere

Isolation of bacteria were performed by serial dilution of the soil suspension in tenfold dilution series. One gram of soil sample was diluted in 9 ml of sterilized distilled water. All treated samples were further diluted up to 10^{-10} and 0.1ml of the diluent was spread plated over nutrient agar media. Then the plates were incubated at 28^0C for 48 hours and number of colonies was determined. Representative bacterial colonies were selected depending on the cultural characteristics and they were further streaked on nutrient agar for purification. After isolation of pure colony, the isolates were transferred into nutrient agar slant for preservation and stored at 4ºC until further analysis.

3.4.1. Colony Characterization of Bacterial Isolates

Colony shape and colour were recorded after 48 hours of growth on nutrient agar. Shape of bacterial isolate and Gram reaction was determined by Gram staining method.

3.4.1.1. Gram Staining Procedure

Bacterial colony was smeared on clean glass slide and allowed to air dry. The smear was heat fixed with Bunsen burner flame and flooded with crystal violet for one minute followed by washing with water. Next, it was flooded with iodine for one minute and after one minute washed with water. Thirdly, it decolorized with 95% of ethanol for fifteen seconds after rinsing with distilled water. Finally, the slide was stained with Gram's safranin for one minute and washed with water. Then, the slide was air dried and observed under compound microscope by using oil immersion to identify shape (coccus, rod and spiral) and Gram's reaction of bacterial isolates.

3.5. Biochemical Characterization of Bacterial Isolates

Different biochemical tests were carried out to identify the bacterial isolates. For this identification, catalase, citrate test, methyl red, urease test, indole test, triple sugar iron agar and lysine iron agar tests were carried out (Elmanama, 2009). Depending on the tests result bacterial isolates were grouped under different genera.

3.5.1. Catalase Test

Pure culture of bacterial colony was placed on clean glass slide and one drop of 3% of H_2O_2 was added to it and checked for bubble formation. This catalase enzyme can be produced by bacterial isolates that can convert hydrogen peroxide to water and oxygen (Woodland, 2004).

3.5.2. Citrate Test

A 24 hours of bacterial culture was streaked on prepared Simon's citrate agar slant and incubated at room temperature (25^0C) for four days. The isolates that have capability to utilize citrate as carbon source by producing citrate permease enzyme had changed the colour of slant from green to blue (Woodland, 2004).

3.5.3. Methyl Red Test

MR-VP broth medium was prepared and inoculated with bacterial isolates. The inoculated broths were incubated at 37^0C for 48 hours. Then, one millilitre of each aliquot was transferred into separate clean test tubes and two drops of methyl red indicator was added to it. Isolates that had capability of retaining acidic end product by fermenting glucose and converting yellow to red on the top of MR-VP broth (Elmanama, 2009).

3.5.4. Urease Test

For the urease test, slant was prepared from urea agar base and urea. Bacterial isolate was streaked on the slant and incubated at 35^0 C to allow the bacteria for growth. The bacteria that could converted urea to carbon dioxide, water and ammonia convert yellow slant to bright pink (Elmanama, 2009).

3.5.5. Indole Test

Pure culture of bacterial isolate was inoculated into prepared tryptone broth and incubated at 37^0C for 48 hours. After bacterial growth turbidity was observed, five drops of kovac's

reagent was added to it. Isolates that had tryptophanase enzyme had convert yellow broth to top red circled (Elmanama, 2009).

3.5.6. Endospore Stain

The pure bacterial culture was smeared on the slide, air dried and heat fixed. Then, the smear was covered with malachite green and placed on warmed hot plate for three minutes without evaporating the stain. The slide was removed from hot plate and allowed to cool. The slide was rinsed with water and safranin was applied to it. After thirty seconds, the smear was rinsed with water and air dried. Finally, the slide was examined under oil immersion. Endospore former bacteria should appear pink and the spores should appear green (Elmanama, 2009).

3.5.7. Triple Sugar Iron Agar (TSIA) Test

After TSIA (glucose, lactose and sucrose) slant was prepared, each bacterial culture was first stubbed in the medium and then streaked on the slant. The medium was incubated at 35^0C for 48 hours to allow the growth of inoculated bacteria. From this test, glucose, sucrose and lactose fermentation and H_2S production were analysed (Woodland, 2004).

3.5.8. Lysine Iron Agar (LIA) Test

Pure bacterial culture was inoculated into prepared LIA slant first by stabbing and streaking on the slant. The slant was incubated at 35^0C for 48 hours and then after glucose fermentation, H_2S production, lysine decarboxylation and deamination were analysed (Elmanama, 2009).

3.5.9. Oxidation-Fermentation (OF) Test

Two tubes containing sterilized Hugh and Leifson's medium was inoculated with test organisms by stabbing half way of the bottom of the tubes. One tube was covered with sterilized liquid paraffin to create anaerobic condition in the tube by preventing diffusion of oxygen. The other tube was left open to create aerobic condition. Finally, the tubes were incubated at 35^0C for 48 hours. Aerobic microbes convert colour of indicator bromothymol blue from green to yellow in open tubes while anaerobic microbes convert green to yellow in closed tubes. Components of Hugh and Leifson's medium per 1000ml of distilled water (5 gram of sodium chloride, 0.3 gram of di potassium phosphate, 2 gram of peptone, 0.03 gram of bromothymol blue, 3 gram of agar and 10 gram of glucose) (Pradhan, 2015).

3.6. *In-Vitro* Screening of Isolates for Multiples PGP Activities

3.6.1. Phosphate Solubilisation Test of Bacterial Isolates

Phosphate solubilisation test of bacterial isolate was performed on Pikovskaya's medium according to Pikovskaya (1948). The pH of prepared Pikovskaya's agar medium was adjusted at seven before autoclaving. Pikovskaya's agar components in g/l were 0.5g of $(NH_4)_2SO_4$, 0.1g of $MgSO_4.7H_2O$, 0.02g of KCl, 0.003g of $FeSO_4.7H_2O$, 0.003g of $MnSO_4.H_2O$, 5g of $Ca_3(PO_4)_2$, 10g of glucose, 0.5g of yeast extract and 15g of agar (Firew *et al.,* 2016). Each bacterial culture was inoculated on the centre of Pikovskaya's agar medium plates containing tricalcium phosphate ($Ca_3(PO_4)_2$) as insoluble phosphate source. Then, the plates were incubated at 30^0C to observe clear zone around the colony (Majeed *et al.,* 2015). Bacterial isolates those have potential to solubilise phosphorus were identified by clear halo zone produced around the isolated bacterial colonies. Phosphate solubilisation index was determined according to Premono *et al.* (1996).

$$\text{Solubilisation Index(SI)} = \frac{\text{Colony diameter} + \text{holo zone diameter}}{\text{Colony diameter}}$$

3.6.2. Production of Indole Acetic Acid (IAA)

Indole acetic acid production of bacterial isolates was performed according to Tekalign *et al.*(2016) and Shakeela *et al.*(2017). Pure culture of bacterial isolate was grown in Luria Bertani (LB) broth at 30^0C overnight. Overnight grown cultures were centrifuged at 10,000 rpm for fifteen minutes and the supernatants were collected. And then, two millilitres of supernatant were added into tubes and two drops of orthophosphoric acid (H_3PO_4) was added to it followed by four millilitres of Salkowski reagent (7.5ml of 0.5M $FeCl_3.6H_2O$ + 150ml of H_2SO_4 and 250ml of distilled water were mixed together). Indole acetic acid producers bacterial isolate should manifest pink colour. Components of Luria Bertani broth per 1000ml of distilled water (10 g of Tryptone, 5 g of yeast extract and 5 g of NaCl).

3.6.3. *In-Vitro* Anti-Fungal Activity Test of Bacterial Isolates Against Fungal Plant Pathogen (*Gibberella Xylarioides*)

Antagonism of bacterial isolate against coffee fungal pathogen (*Gibberella xylarioides)* was performed by using dual plate method. Each 24 hours old culture of rhizosphere bacterial isolate was spot inoculated at the distance of three centimetres from the centre of potato dextrose agar medium containing sucrose at four points. A four millimetre diameter

of five days old culture of *Gibberella xylarioides* grown on potato dextrose agar was cut by using cork borer and placed at the centre of each petri plate. Then, the plates were incubated at temperature of 30^0C. And the other petri plate with four millimetre diameter of *Gibberella xylarioides* without bacterial culture was placed at the centre of the potato dextrose agar medium was also incubated at the same temperature and used as a control. Colony diameter of the fungal pathogen was measured and compared with that of control. After five days incubation the percent of inhibition in growth of pathogen was calculated according to method suggested by Toppo and Naik (2015).

$$\%\ \text{of inhibition} = \frac{R1 - R2}{R1} \times 100$$

where, R_1 = radial growth of fungus in control;

R_2 = radial growth of fungus in dual inoculation.

3.7. Determination of AMF Spore Density and Root Colonization

3.7.1. Determination of AMF Spore Density

AMF spore was extracted from one hundred grams of coffee rhizosphere soil by wet sieving and decanting method (Gerdemann and Nicolson, 1963). In a two-litter beaker, one hundred grams of each composite soil sample was submerged in 0.5 L of tap water and stirred for one minute with spatula. After it settled, the supernatant was passed through a series sieve with decreasing size (710, 125 and 63 µm) and it was repeated two times. Contents retained by the sieves of 710 to 63 microns was divided into four tubes and centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded and fifty percent (50%) of sucrose solution (w/v) was added in to each centrifuge tube. The mixture was stirred and the tubes were again centrifuged for one minute at 2000 rpm. The supernatant was carefully poured through a 63µm sieve and carefully washed with tap water to remove the sucrose. Finally, spores, spore clusters and sporocarp were carefully washed and transferred to a Petri dish to prepare for spore counting under the dissecting microscope (ISO 1006) at ×4. Enumeration of spore numbers per gram of dry soil was performed according to: (https://invam.wvu.edu/methods/spores/enumeration-of-spores, n.d.).

3.7.2. Staining and Quantification of AMF Root Colonization

Coffee roots were first washed with water, cut into a length of one centimetre and then they were immersed in 10% potassium hydroxide (KOH) solution. They were heated in autoclave at 121^0C for five minutes to remove intracellular components and washed with tap water three times. Then after, the roots were rinsed and whitened in a solution of 10% hydrogen peroxide (H_2O_2) for five minutes at room temperature and washed with tap water. The roots were acidified with 10% HCl for 15 minutes and finally stained with a solution of 0.05% trypan blue in autoclave at 121^0C for ten minutes. Then, the stained roots were finally rinsed repeatedly with tap water and stored in distaining solution of lactoglycerol (lactic acid, glycerol and distilled water 1:1:1) ratio for three days in dark place. Finally, roots were mounted in polyvinyl-lacto-glycerol (PVLG) mountant on microscopic slides and covered with 40×22 mm coverslips. The slides were then observed under a compound microscope and each fragment was checked along its entire length to record the mycorrhizal structures: arbuscules, vesicles, and hyphae (intra cellular and intercellular, hyphae and extra-matrix) (Brundrett *et al.*, 1996).

The percent of root length colonized by any AMF structures were quantified by the ratio between number of root bits colonized to total number of root bits observed by using the magnified intersection method of (McGonigle *et al.*, 1990).

$$\text{RLC }\% = \frac{\text{Total Root Colonization (G)} - \text{Negative Colonization (N)}}{\text{Total Root Colonization (G)}} \times 100$$

$$\text{AC }\% = \frac{\text{Number of Arbuscle}}{\text{Total Root Colonization (G)}} \times 100$$

$$\text{VC }\% = \frac{\text{Number of Vesicle}}{\text{Total Root Colonization (G)}} \times 100$$

where, $\text{RLC}\% = \text{Percentage of Root Length Colonnization}$

$\text{AC}\% = \text{Percentage of Arbuscle Colonization}$

$\text{VC}\% = \text{Percentage of Vesicle Colonization.}$

3.8. Statistical analysis

The data obtained in this study was subjected to analysis of variance (ANOVA), Excel and Minitab version 17 statistical software. Mean and correlation between the recorded data were done. The value of $p < 0.05$ is used as statistically significant (Rahman *et al.*, 2017).

4. RESULTS

4.1. Soil Physicochemical Analysis

The physico-chemical properties of coffee rhizosphere soil sample in the study area is shown in table 1. The pH of all soil samples was in the range from acidic to near acid. Available P was ranged from the minimum (13.25 ppm) from monoculture to the maximum (44.6 ppm) from Mana sibu agroforestry. Nitrogen availability was ranged from 0.19% to 0.27% while organic carbon was ranged from 2.48% to 3.41% in monoculture and agroforestry respectively.

Table 1: The physico-chemical properties of the soil samples from rhizosphere of coffee trees.

Soil Sample	Coffee variety	Cultivation system	pH	P (ppm)	TN%	OC%
1	74110	Monoculture	4.625	39.58	0.19	2.57
2	Black Coffee	Monoculture	4.5	27.59	0.25	2.74
3	74110	Agroforestry	5.5	16.54	0.27	2.53
4	Mana Sibu	Agroforestry	5.75	44.60	0.27	3.41
5	Buna Aduu (Non-shading Coffee)	Monoculture	5.6	13.25	0.23	2.48

P: phosphorus; TN: total nitrogen; OC: organic carbon.

4.2. Total Bacterial Count of the Soil Samples

In this study, the bacterial colony counts were between 3.4 x 10^4 and 5.0x10^7. The colony counts from monoculture coffee cultivation system were with highest number of colony (5x10^7 CFU) with a few distinction of colony shape and colour. However, in agroforestry coffee cultivation system least number of bacterial colonies were counted (3.4x10^4 CFU). The samples from monoculture were found with highest abundance of bacterial number and those from agroforestry were found with less bacterial abundance (Table 2). However, the isolates from agroforestry cultivation system found with higher diversity than monoculture (Table 4).

Table 2: Number of culturable bacterial colonies isolated from coffee rhizosphere soil on plate count agar medium.

Soil sample	Coffee variety	Cultivation system	Total bacterial colony count g^{-1} of soil (CFU g^{-1})
1	74110	Monoculture	6.5×10^{6}
2	Black Coffee	Monoculture	5.0×10^{7}
3	74110	Agroforestry	3.3×10^{5}
4	Mana Sibu	Agroforestry	3.7×10^{6}
5	Buna Aduu (Non-shading Coffee)	Monoculture	3.4×10^{4}

4.3. Colony Characterization of Rhizobacteria

The colony characteristics were recorded for each rhizobacterial isolates. The colony morphology such as shape, elevation, margin and colour was recorded for each isolates. From the total of thirty-five coffee rhizosphere bacterial isolates 42.9% were Gram positive while 57.1% of them were Gram negative isolates. This showed that most of the bacterial isolates from this study were Gram negative bacteria (Table 3).

Table 3: Cultural characteristics of bacterial isolates.

Coffee variety	Cultivation System	Soil sample	Isolate code	Colony morphology				Gram's stain	Shape of bacterial isolate
				Shape	Elevation	margin	Colour		
74110	Monoculture	1	$RIMCS_1$	Irregular	Crateriform	Undulate	White	-	Rod
			$RIMCS_2$	Circular	Umbonate	Undulate	White	+	Rod
			$RIMCS_3$	Circular	Convex	Entire	Grey	-	Rod
			$RIMCS_4$	Circular	Raised	Entire	White	-	Rod
Black Coffee	Monoculture	2	$RIMCSBC_1$	Irregular	Crateriform	Undulate	Yellow	+	Rod
			$RIMCSBC_2$	Circular	Convex	Entire	White	-	Rod
			$RIMCSBC_3$	Circular	Convex	Entire	Yellow	-	Rod
74110	Agroforestry	3	$RIACS_1$	Circular	Convex	Entire	White	-	Rod
			$RIACS_2$	Circular	Umbonate	Undulate	Yellow	+	Rod
			$RIACS_3$	Circular	Convex	Entire	Pink	+	Cocci
			$RIACS_4$	Circular	Convex	Entire	Yellow	-	Rod
			$RIACS_5$	Irregular	Umbonate	Undulate	White	+	Rods
			$RIACS_6$	Circular	Convex	Entire	White	-	Rod
			$RIACS_7$	Irregular	Pulvinate	Undulate	White	+	long rod
			$RIACS_8$	Irregular	Crateriform	Undulate	White	+	short rod

			$RIACS_9$	Rhizoid	Umbonate	Undulate	White	-	Rod
			$RIACS_{10}$	Circular	Convex	Entire	Grey	+	Rod
			$RIACS_{11}$	Irregular	Pulvinate	Lobate	Grey	+	Coccus
Mana Sibu	Agroforestry	4	$RIACSMS_1$	Circular	Raised	Entire	Red	-	long rod
			$RIACSMS_2$	Circular	Convex	Entire	Yellow	-	Rod
			$RIACSMS_3$	Circular	Convex	Entire	Grey	+	Thin rods
			$RIACSMS_4$	Circular	Convex	Entire	Pink	-	Rod
			$RIACSMS_5$	Circular	Convex	Undulate	White	-	Rod
			$RIACSMS_6$	Rhizoid	Umbonate	Lobate	White	-	Rod
			$RIACSMS_7$	Rhizoid	Umbonate	Filiform	Grey	-	Rod
			$RIACSMS_8$	Irregular	Crater form	Undulate	Yellow	-	Rod
Buna aduu (Non-shading Coffee)	Monoculture	5	$RIMCSNS_1$	Circular	Convex	Entire	Grey, gum	-	Short rod
			$RIMCSNS_2$	Circular	Convex	Entire	White, gum	+	Rod
			$RIMCSNS_3$	Rhizoid	Raised	Entire	White	+	Streptobacilli
			$RIMCSNS_4$	Irregular	Crateriform	Undulate	Grey, dry	+	Rod
			$RIMCSNS_5$	Rhizoid	Umbonate	Undulate	Yellow	-	Rod
			$RIMCSNS_6$	Rhizoid	Umbonate	Undulate	White	+	Short rod
			$RIMCSNS_7$	Irregular	Raised	Lobate	Red	-	Rod
			$RIMCSNS_8$	Filamentous	Pulvinate	Filiform	Red	-	Rod
			$RIMCSNS_9$	Irregular	Umbonate	Entire	White	+	Short rod

(+) = Gram positive; (-) = Gram negative

RIMCS: Rhizobacteria isolated from monoculture cultivation system of 74110;

RIMCSBC: Rhizobacteria isolated from monoculture cultivation system of black coffee;

RIACS: Rhizobacteria isolated from agroforestry cultivation system of 74110;

RIACSMS: Rhizobacteria isolated from agroforestry cultivation system of mana sibu coffee variety;

RIMCSNS: Rhizobacteria isolated from monoculture cultivation system of non-shading coffee.

4.4. Biochemical Characterization of Rhizobacteria

In this study, twelve different bacterial isolates were identified from five composite coffee rhizosphere samples (three from monoculture and two from agroforestry cultivation system). The bacterial isolates from agroforestry showed high diversity while those from monoculture cultivation system showed low diversity of genera. According to Bergey's Manual of Determinative Bacteriology, the isolates were identified to the genus *Bacillus, Pseudomonas, Enterobacter, Corynebacterium, Citrobacter, Serratia, Brevibacterium, Micrococcus, Erwinia, Gordonia, Klebsiella and Burkholderia.* The *Bacillus* genera were found with highest frequency (17.1%) followed by *Pseudomonas* (14.9%), *Enterobacter* (14.9%), *Corynebacterium* (8.6%), *Citrobacter* (8.6%), *Serratia* (8.6%), *Brevibacterium* (5.7%), *Micrococcus* (5.7%), *Erwinia* (5.7%), *Gordonia* (5.7%), *Klebsiella* (2.9%) *and Burkholderia* (2.9%) (Table 4).

Table 4: Biochemical tests of bacterial isolates.

Coffee Variety	Cultivation system	Soil sample	Bacterial isolates code	Biochemical tests															
										TSIA				LIA					
				Catalase	Citrate	MR	Urease	Indole	Endospore stain	H_2S production	Glucose	Lactose	Sucrose	H_2S production	Decarboxylation	Glucose	Deaminase	Oxidation-fermentation (OF)	Recommended Genera of bacterial isolates
74110	Monoculture	1	$RIMCS_1$	+	-	+	+	+	-	+	+	+	-	+	-	+	-	Fermentative	*Enterobacter*
			$RIMCS_2$	+	-	+	-	-	+	-	+	-	-	-	-	+	-	Oxidative	*Bacillus*
			$RIMCS_3$	+	+	-	-	-	-	-	+	+	+	-	+	+	-	Fermentative	*Klebsiella*
			$RIMCS_4$	+	+	-	-	-	-	-	+	-	-	-	-	+	-	Oxidative	*Pseudomonas*
Black Coffee	Monoculture	2	$RIMCSBC_1$	+	-	+	-	-	-	+	+	-	-	+	-	+	-	Oxidative	*Corynebacterium*
			$RIMCSBC_2$	+	-	-	+	+	-	+	+	+	+	+	-	+	-	Fermentative	*Enterobacter*
			$RIMCSBC_3$	+	-	+	+	+	-	+	+	+	-	+	-	+	-	Fermentative	*Enterobacter*
74110	Agroforestry		$RIACS_1$	+	+	+	-	-	-	+	+	+	-	+	-	+	-	Fermentative	*Enterobacter*
			$RIACS_2$	+	+	+	-	-	-	-	+	-	-	-	+	-	-	Oxidative	*Brevibacterium*

			$RIACS_3$	+	-	-	-	-	-	-	+	-	-	-	+	-	-	Oxidative	*Micrococcus*
			$RIACS_4$	+	+	+	-	-	-	+	-	+	+	-	+	-	-	Fermentative	*Citrobacter*
			$RIACS_5$	+	+	-	-	-	+	-	+	-	-	-	+	-	-	Oxidative	*Bacillus*
		3	$RIACS_6$	+	+	-	-	-	-	-	+	+	-	+	-	+	-	Oxidative	*Pseudomonas*
			$RIACS_7$	+	+	+	-	-	+	-	+	-	-	-	+	-	-	Oxidative	*Bacillus*
			$RIACS_8$	+	-	-	-	-	-	+	+	-	-	+	-	+	-	Oxidative	*Corynebacterium*
			$RIACS_9$	+	+	+	-	-	-	+	+	+	+	-	+	-	-	Oxidative	*Pseudomonas*
			$RIACS_{10}$	+	-	-	-	-	-	-	+	-	-	-	-	+	-	Oxidative	*Brevibacterium*
			$RIACS_{11}$	+	-	-	-	-	-	-	+	-	-	-	-	+	-	Oxidative	*Micrococcus*
Mana Sibuu	Agroforestry		$RIACSMS_1$	+	+	-	-	-	-	+	+	-	-	+	+	+	-	Fermentative	*Citrobacter*
			$RIACSMS_2$	+	-	+	-	-	-	-	+	-	-	-	-	+	-	Oxidative	*Pseudomonas*
			$RIACSMS_3$	+	-	+	-	-	+	-	+	-	-	-	+	-	-	Oxidative	*Bacillus*
		4	$RIACSMS_4$	+	+	-	-	-	-	+	+	-	-	+	-	+	-	Fermentative	*Citrobacter*
			$RIACSMS_5$	-	-	-	+	-	-	-	-	-	-	-	+	-	-	Fermentative	*Erwinia*
			$RIACSMS_6$	+	+	-	-	-	-	+	+	-	-	-	-	+	-	Oxidative	*Pseudomonas*
			$RIACSMS_7$	+	+	-	-	-	-	+	+	-	-	+	+	-	-	Oxidative	*Burkholderia*

			RIACSMS$_8$	+	+	+	-	-	-	+	+	-	-	+	+	+	-	Fermentative	*Serratia*
Non-shading Coffee	Monoculture	5	RIMCSNS$_1$	-	-	-	+	-	-	+	+	-	-	+	-	+	-	Fermentative	*Erwinia*
			RIMCSNS$_2$	+	-	-	-	-	-	-	-	-	-	-	+	-	-	Oxidative	*Corynebacterium*
			RIMCSNS$_3$	+	+	-	-	-	+	-	+	-	-	-	+	-	-	Oxidative	*Bacillus*
			RIMCSNS$_4$	+	-	-	+	-	+	-	+	-	-	-	+	-	-	Oxidative	*Bacillus*
			RIMCSNS$_5$	+	-	-	+	-	-	+	+	+	+	+	-	+	-	Fermentative	*Enterobacter*
			RIMCSNS$_6$	-	-	-	+	-	-	+	+	-	-	+	-	+	-	Oxidative	*Gordonia*
			RIMCSNS$_7$	+	+	-	-	-	-	-	+	+	-	-	+	-	-	Fermentative	*Serratia*
			RIMCSNS$_8$	+	+	+	-	-	-	-	+	-	-	-	+	-	-	Fermentative	*Serratia*
			RIMCSNS$_9$	-	-	+	-	-	-	-	+	-	-	-	-	+	-	Oxidative	*Gordonia*

(+) = positive for the test; (-) = negative for the test

RIMCS: Rhizobacteria isolated from monoculture cultivation system of 74110;

RIMCSBC: Rhizobacteria isolated from monoculture cultivation system of black coffee;

RIACS: Rhizobacteria isolated from agroforestry cultivation system 74110;

RIACSMS: Rhizobacteria isolated from agroforestry cultivation system of mana sibu coffee variety;

RIMCSNS: Rhizobacteria isolated from monoculture cultivation system of non-shading coffee.

Oxidative= Bacteria those utilize carbohydrates aerobically

Fermentative = Bacteria those utilize carbohydrates anaerobically.

4.5. *In-Vitro* Screening of Isolates for Multiples PGP Activities

4.5.1. Phosphate Solubilisation

The result obtained from this study showed that out of the total thirty-five bacterial isolates fourteen of them (40%) exhibited phosphate solubilisation ability with solubilisation index ranging from 1.25 to 3.0 (Table 5). Both Gram negative and Gram positive bacteria were fifty-fifty on phosphate solubilisation. The isolate $RIACS_8$ (*Corynebacterium* sp.) showed highest holo zone (8 mm) with phosphate solubilisation index of (3) while isolate $RIMCS_1$ (*Enterobacter* sp.) exhibited least halo zone (1 mm) on Pikovskaya's agar with phosphate solubilisation index of (1.25). The bacterial isolates those solubilise and non-solubilise inorganic phosphate were shown in Figure 4.

Table 5: Phosphate solubilisation ability of bacterial isolates.

Phosphate solubilisers	Bacterial Genera	Colony size (mm)	Clear zone (mm)	Phosphate solubilisation index
$RIMCS_1$	*Enterobacter*	4	1	1.25
$RIMCS_2$	*Bacillus*	4	2	1.5
$RIMCSBC_1$	*Corynebacterium*	4	2	1.5
$RIACS_5$	*Bacillus*	4	5	2.25
$RIACS_6$	*Pseudomonas*	4	5	2.25
$RIACS_7$	*Bacillus*	4	5	2.25
$RIACS_8$	*Corynebacterium*	4	8	3
$RIACSMS_6$	*Pseudomonas*	4	6	2.5
$RIACSMS_8$	*Serratia*	4	3	1.75
$RIMCSNS_1$	*Erwinia*	4	3	1.75
$RIMCSNS_4$	*Bacillus*	4	5	2.25
$RIMCSNS_7$	*Serratia*	4	6	2.5
$RIMCSNS_8$	*Serratia*	4	7	2.75
$RIMCSNS_9$	*Gordonia*	4	4	2

(+) = Gram positive; (-) = Gram negative

RIMCS: Rhizobacteria isolated from monoculture cultivation system of 74110;

RIMCSBC: Rhizobacteria isolated from monoculture cultivation system of black coffee;

RIACS: Rhizobacteria isolated from agroforestry cultivation system 74110;

RIACSMS: Rhizobacteria isolated from agroforestry cultivation system of mana sibu coffee variety;

RIMCSNS: Rhizobacteria isolated from monoculture cultivation system of non-shading coffee.

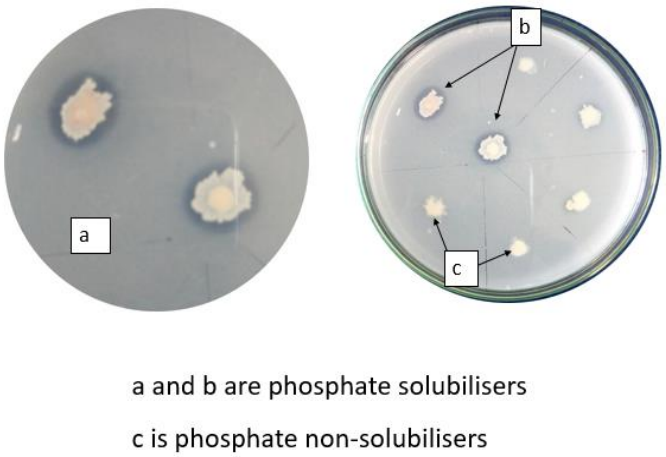


Figure 4: Phosphate solubilisers and non-solubilisers bacterial isolates.

4.5.2. Indole Acetic Acid Production

In this study, all the thirty-five rhizobacterial isolates were tested for the production of indole acetic acid production under *in-vitro* condition. However, all isolates did not show indole acetic acid characteristics.

4.5.3. Antagonism of Bacterial Isolates Against Fungal Coffee Plant Pathogen (*Gibberella Xylarioides*)

In the present study, seventeen out of the total of thirty-five bacterial isolates (48.6%) showed inhibition against the fungal mycelial growth. The bacterial genera of *Enterobacter, Pseudomonas, Citrobacter, Bacillus, Corynebacterium, Gordonia, Erwinia and Serratia* were showed antagonism against *Gibberella xylarioides* under *in-vitro* condition. They showed variable percent of fungal mycelial growth inhibition ranging from 7.7 to 46.2%. The highest percent (46.2%) of growth inhibition was exhibited by isolate RIACSMS$_{6}$ (*Pseudomonas* sp.) from agroforestry and the lowest (7.7%) growth inhibition was formed by isolates RIMCS$_{1}$ (*Enterobacter* sp.) and RIMCS$_{4}$ (*Pseudomonas* sp.) from monoculture, and RIACS$_{1}$ (*Enterobacter* sp.) from agroforestry (Table 6). Rhizobacterial isolates that showed antagonism against Gibberella xylarioides was shown in figure 5.

Table 6: Antagonism of bacterial isolates against coffee wilt disease (*Gibberella xylarioides*).

Antagonist isolates	**Bacterial Genera**	***Gibberella Xylarioides* colony size by (mm)**	**% of inhibition**
Control		2.6	
$RIMCS_1$	*Enterobacter*	2.4	7.7
$RIMCS_4$	*Pseudomonas*	2.4	7.7
$RIMCSBC_3$	*Enterobacter*	2.2	15.4
$RIACS_1$	*Enterobacter*	2.4	7.7
$RIACS_5$	*Bacillus*	1.7	34.6
$RIACS_6$	*Pseudomonas*	2.0	23.1
$RIACS_7$	*Bacillus*	1.6	38.5
$RIACS_8$	*Corynebacterium*	2.0	23.1
$RIACS_9$	*Pseudomonas*	1.9	26.9
$RIACSMS_3$	*Bacillus*	2.2	15.4
$RIACSMS_5$	*Erwinia*	1.8	30.8
$RIACSMS_6$	*Pseudomonas*	1.4	46.2
$RIACSMS_8$	*Serratia*	2.0	23.1
$RIMCSNS_4$	*Bacillus*	1.8	30.8
$RIMCSNS_6$	*Gordonia*	2.3	11.5
$RIMCSNS_7$	*Serratia*	1.8	30.8
$RIMCSNS_9$	*Gordonia*	2.0	23.1

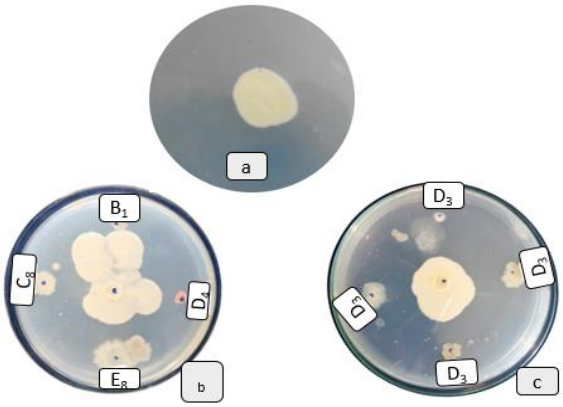
RIMCS: Rhizobacteria isolated from monoculture cultivation system of 74110;

RIMCSBC: Rhizobacteria isolated from monoculture cultivation system of black coffee;

RIACS: Rhizobacteria isolated from agroforestry cultivation system 74110;

RIACSMS: Rhizobacteria isolated from agroforestry cultivation system of mana sibu coffee variety;

RIMCSNS: Rhizobacteria isolated from monoculture cultivation system of non-shading coffee.



a. Control (*Gibberella xylarioides* colony)

b. Antagonism test with different bacterial isolates

c. Antagonism test with the same bacterial isolates

Figure 5: Antagonism of bacterial isolates against *Gibberella xylarioides.*

4.5.4. Multiple Plant Growth Promotion Activity

From the total of thirty-five rhizobacterial isolates ten of them showed both phosphate solubilisation and antagonism against *Gibberella xylarioides* growth inhibition ability under *in-vitro* condition. Both Gram negative and Gram positive bacteria were fifty-fifty. The rhizobacterial isolate $RIACSMS_6$ (*Pseudomonas* sp.) showed highest while $RIMCS_1$ (*Enterobacter* sp.) showed the least plant growth promotion activity (Table 7).

Table 7: Rhizobacterial isolates that showed both phosphate solubilisation and antagonism against *Gibberella xylarioides*.

Isolates	**Bacterial Genera**	**PS clear holo zone by millimeter**	**PSI**	**% of *G. xylarioides* growth inhibition**
$RIMCS_1$	*Enterobacter*	1	1.25	7.7
$RIACS_5$	*Bacillus*	5	2.25	34.6
$RIACS_6$	*Pseudomonas*	5	2.25	23.1
$RIACS_7$	*Bacillus*	5	2.25	38.5
$RIACS_8$	*Corynebacterium*	8	3.0	23.1
$RIACSMS_6$	*Pseudomonas*	6	2.5	46.2
$RIACSMS_8$	*Serratia*	3	1.75	23.1
$RIMCSNS_4$	*Bacillus*	5	2.25	30.8
$RIMCSNS_7$	*Serratia*	6	2.5	30.8
$RIMCSNS_9$	*Gordonia*	4	2.0	23.1

G. xylarioides; PS = Phosphate solubilisation; PSI = Phosphate solubilisation index

RIMCS: Rhizobacteria isolated from monoculture cultivation system of 74110;

RIMCSBC: Rhizobacteria isolated from monoculture cultivation system of black coffee;

RIACS: Rhizobacteria isolated from agroforestry cultivation system 74110;

RIACSMS: Rhizobacteria isolated from agroforestry cultivation system of mana sibu coffee variety;

RIMCSNS: Rhizobacteria isolated from monoculture cultivation system of non-shading coffee.

4.6. AMF Spore Density and Root Colonization

4.6.1. Determination of AMF Spore Density

In this study, the AMF spore abundance showed variation between the soil samples ranging from 1.66 to 3.68 g^{-1} of soil. Rhizosphere soil of coffee variety from agroforestry showed highest number of spore density (3.68 g^{-1}) compared to the soil sample from the monoculture (1.66 g^{-1}) (Table 8). The AMF spore was present in all rhizosphere soil of coffee tree with variable density ranging from 1.87 to 3.68 g^{-1} of coffee rhizosphere soil sample for agroforestry while that of monoculture was ranging from 1.66 to 2.5 g^{-1}. The highest AMF spore abundance was recorded from Mana Sibu coffee variety (sample 4) 3.68 g^{-1} while the lowest spore density was recorded from 74110 coffee variety (sample 1) 1.66 g^{-1} of soil sample (Table 8).

Table 8: AMF spore density per one hundred gram of coffee rhizosphere soil sample.

Soil sample	Coffee variety	Cultivation system	AMF spore $g\ 100^{-1}$ of soil	AMF spore g^{-1} of soil
1	74110	Monoculture	166	1.66 ± 0.21
2	Black Coffee	Monoculture	211	2.11 ± 0.44
3	74110	Agroforestry	187	1.87 ± 0.17
4	Mana Sibu	Agroforestry	368	3.68 ± 0.60
5	Buna Aduu (Non-shading Coffee)	Monoculture	250	2.5 ± 1.56

4.6.2. Quantification of AMF Root Colonization

The microscopic observations of AMF root colonization of coffee trees showed all coffee varieties were colonized with AMF structures hyphae, Vesicle and arbuscule (Table 9). The colonization percentage was varied from sample to sample. The coffee roots of agroforestry cultivation system showed highest colonization (95%) while the coffee roots from monoculture showed the lowest colonization (61.9%). The maximum average percentage of arbuscular colonization was observed in coffee root from agroforestry (7.5%) while that of monocultures system showed the minimum (3.8%). The highest percentage of vesicle colonization was recorded by agroforestry of Mana sibu coffee variety (sample 4) which was 5% while the lowest percentage of vesicle colonization was recorded by monoculture 74110 coffee variety (sample 1) which was 1%. The root colonization by hyphae, vesicle and arbuscule where shown in figure (6 a, b and c).

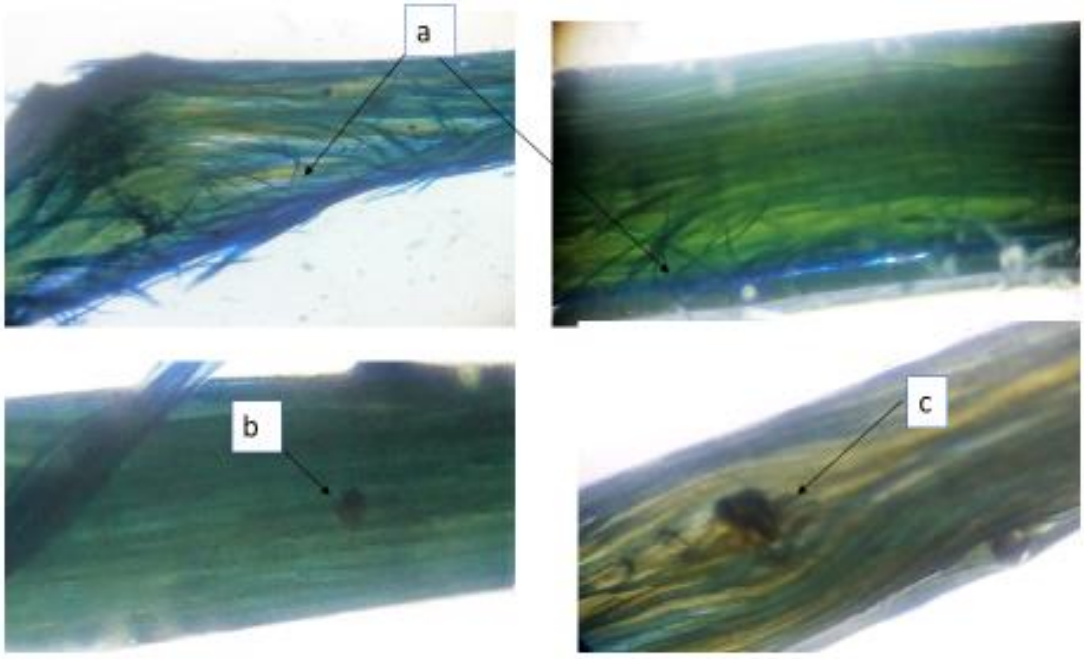
Table 9: Percentage of AM fungal colonization in five samples of coffee trees.

Sample	Negative	Arbuscule	Vesicle	Hyphae	RLC%	AC%	VC%
1	7	2.3	0.3	21.5	77.5	7.4	1
2	11.7	1.3	0.7	17	61.9	4.2	2.3
3	4.3	1.3	0.7	28.3	87.6	3.8	2
4	2	3	2	33	95	7.5	5
5	4.7	1.7	0.3	18.7	81.5	6.7	1.2

Where: RLC% = Percent of Root Length Colonization

AC% = Percent of Arbuscule Colonization

VC% = Percent of Vesicle Colonization



a. AMF hyphae
b. AMF vesicle
c. AMF arbuscule

Figure 6: Arbuscular mycorrhizal fungi colonization in roots of coffee.

5. DISCUSSION

PGPR are soil inhabiting group of bacteria that stimulate plant growth by secretion of plant growth substances. In this study, most of the rhizobacterial isolates (57.1%)were Gram negative while others (42.9%) were Gram positive (Table 3). Similarly, Befekadu *et al.* (2017) isolated a wide array of Gram negative and Gram positive bacteria associated with rhizosphere of coffee plants from south-western Ethiopia. They reported that, the isolates were dominated with Gram negative bacteria (73.2%) and the Gram positives were (26.8%).

In this study, most of the rhizobacteria were isolated from rhizosphere of coffee trees of agroforestry cultivation system and few of them were from rhizosphere of coffee tree of monoculture cultivation system (Table 4). Yang *et al.* (2016) showed the bacterial community diversity in rhizosphere soil samples were more diverse under intercropping system than under sole-cropping. Intercropping increased the numbers of beneficial bacteria in the soil and improved the diversity of the bacterial community, which was conducive to improving soil nutrient (N and P) supply capacity and soil micro-ecosystem stability (Li *et al.,* 2018).

The bacterial isolates were tested for their ability to solubilise insoluble tri-calcium phosphate ($Ca_3(PO_4)_2$) under *in-vitro* condition. Both Gram positive (50%) and Gram negative bacteria (50%) showed phosphate solubilisation ability. The isolates showed phosphate solubilisation ability were identified to the genus *Bacillus, Serratia, Corynebacterium, Pseudomonas, Enterobacter, Gordonia and Erwinia*. From the total of thirty-five rhizobacterial isolates, 40% of them were found to be phosphate solubilisers. The solubilisation index of the isolates was ranged from 1.25 to 3 (Table 5). In consistent with this study, Befekadu *et al.* (2017) reported that, 32.5% (55/169) of the rhizobacterial isolates screened from south-western Ethiopia showed clearly visible haloes (>0.50 cm) around their colonies on Pikovskaya's agar. They recorded phosphate solubilisation index ranging from 0.53 to 6.1.

The largest phosphate solubilisation index (3) was produced by Gram positive bacterial isolate $RIACS_8$ (*Corynebacterium* sp.) from coffee trees of agroforestry cultivation system and the least (1.25) was produced by $RIMCS_1$ (*Enterobacter* sp.) from monoculture cultivation system. About 28.6% of phosphate solubilisers in this study was *Bacillus* sp.

Diriba *et al.* (2013) reported phosphate solubilising bacteria associated with *Coffea arabica* from natural coffee forests of south-western Ethiopia. Members of the phosphate solubilising bacteria were dominated by the genus *Pseudomonas* (33.9%) followed by *Burkholderia* (12.9%), and *Bacillus*. The solubilisation index was ranging from 2.05 to 5.82.

In this study, seventeen out of thirty-five (48.6%) of rhizobacterial isolates from coffee rhizosphere soil showed mycelial growth inhibition *of Gibberella xylarioides*. The highest fungal mycelial growth inhibition was produced by isolate $RIACSMS_6$ (46.2%) which was identified as *Pseudomonas* sp. and least percentage of fungal mycelial inhibition (7.7%) was produced by isolate $RIMCS_1$ and $RIMCS_4$ (*Enterobacter* sp. and *Pseudomonas* sp.) respectively and $RIACS_1$ (*Enterobacter* sp.) (Table 6). Tekalign *et al.* (2016) isolated *Bacillus* sp. from rhizosphere of *Coffea arabica* and studied their antagonism against two fungal pathogens *Colletotrichum gloeosporioides* and *Fusarium oxysporum* for radial mycelial growth inhibition. They reported that, sixteen isolates of *Bacillus* sp. showed greater than 40% mycelial growth inhibition. Among these isolates, BT_{42} showed maximum mycelial growth inhibition against *Colletotrichum gloeosporioides* (78%) and *Fusarium oxysporum* (86%).

In another study, Melkamu *et al.* (2013) isolated rhizobacteria from rhizosphere of *Coffea arabica* and *Pisum sativum*, and tested their antagonism against coffee wilt disease (*Gibberella xylarioides*). Out of the eighty-one rhizobacterial isolates eleven of them (13.6%) showed inhibition of mycelial growth of *Gibberella xylarioides* in dual culture plate method. The percent of mycelial growth was ranging from 18% to 71.5%. The highest mycelial growth inhibition was exhibited by *Bacillus* and *Pseudomonas* sp. under *in-vitro* condition. They reported that, coffee seedling treated with *Bacillus* sp. were effectively reduced incidence and severity of coffee wilt disease under *in-vivo* condition in greenhouse.

The coffee rhizosphere soils and roots from agroforestry cultivation system were also colonized by AMF than those from monoculture cultivation system (Table 8 and Table 9). All coffee roots were colonized with AMF structures (arbuscule, hyphae and vesicle) with various colonization potential (Table 9; Figure 6). There was positive correlation between average spore density per one gram of coffee rhizosphere soil sample and number of arbuscule, vesicle and hyphae (r= 0.68, 0.87 and 0.58) respectively (data not shown).

However, it did not show statistically significant correlation ($p>0.05$). Zerihun *et al.* (2013) also reported that, the percentage of root colonization and the number of AMF spores observed in the acacia trees did not correlate significantly ($r = 0.48$, $p>0.05$).

In this study, the correlation between percent of available phosphorus in coffee rhizosphere and spore density showed weak positive correlation ($r= 0.393$, $p= 0.513$) (data not shown). This means there was no statistically significant correlation since $p>0.05$. There was positive correlation between percent of nitrogen ($r= 0.523$, $p= 0.366$) and spore density; between percent of organic carbon ($r= 0.870$, $p= 0.055$) and spore density. However, no statistically significant correlation between the two parameters. Lebron *et al.* (2012) studied AMF root colonization of different coffee cultivars and showed that the concentrations of phosphorus in the soil can affect the interaction between AMF and plants. High phosphate concentration showed to inhibit AMF colonization of roots. The high levels of soil phosphorus contributed to the low extra-radical hyphal lengths of AM fungi. However, in this study, no such effect was observed.

6. CONCLUSIONS

In this study, the rhizobacteria isolated from rhizosphere of coffee plant showed various plant growth promoting activities, including phosphate solubilisation and antagonism against coffee wilt disease (*Gibberella xylarioides*). Based on the morphological and biochemical characteristics, the bacterial genera showed plant growth promoting activity under *in-vitro* condition were *Bacillus, Pseudomonas, Corynebacterium, Serratia, Gordonia, Enterobacter* and *Erwinia*. The $RIACS_8$ (*Corynebacterium* sp.) showed highest phosphate solubilisation by forming holo zone (8 mm) with PSI of (3). The highest percent (46.2%) of *Gibberella xylarioides* mycelial growth inhibition was exhibited by isolate RIACSMS6 (*Pseudomonas* sp.). All the coffee root samples were colonized by AMF structures with varied percentage and the rhizosphere soils harboured various number of spores. Maximum spore density (3.68 g^{-1} of soil) and maximum percentage of root colonization (95%) recorded from coffee tree from Mana sibu agroforestry system and minimum spore density (1.66 g^{-1} of soil) and minimum root colonization (61.9%) exhibited in coffee tree from monoculture. This shows the agroforestry cultivation system is more advisable for maintenance of microbial diversity in rhizosphere.

RECOMMENDATIONS

Based on this finding the following recommendations were laid down.

This study depends only on biochemical test for identification of PGPR up to genus level and the species of isolates were not identified. For this reason, molecular characteristics of bacterial isolates should be done to use the isolates as a bio-inoculants.

Some of the rhizobacterial isolates such as *Corynebacterium*, *Bacillus*, *Pseudomonas* and *Serratia* showed plant growth promoting activity under *in-vitro* condition. Therefore, green house test and field test should be done for the isolates to see their competitiveness, tolerance and adaptability to different environments.

The isolates were failed to produce IAA without precursor L-tryptophan. Therefore, further study should be done for IAA production with the supplement of L-tryptophan precursor.

Since AMF is an obligate symbiont of plant roots and improve plant health and productivity by exchanging nutrients with the plant root, further study should be conducted on the diversity and application of AMF as bio-fertilizers.

REFERENCES

Aarab, S., Javier, F., Megias, M., Laglaoui, A., Bakkali, M., and Arakrak, A. (2015). Isolation and screening of bacteria from rhizospheric soils of rice fields in North-western Morocco for different plant growth promotion (PGP) activities: An *in-vitro* study. *International Journal of Current Microbiology and Applied Sciences,* **4**(1), 260-266.

Adesemoye, A.O., and Egamberdieva, D. (2013). Beneficial effects of plant growth-promoting rhizobacteria on improved crop production: prospects for developing economies. *Bacteria in Agrobiology: Crop Productivity* (pp. 45-57).

AL-Areqi, A.H., Chliyeh, M., Sghir, F., Touhami, O.A., Benkirane, R., and Douira, A. (2013). Diversity of arbuscular mycorrhizal fungi in the rhizosphere of *Coffea arabica* in the Republic of Yemen. *Journal of Applied Biosciences,* **64**, 4888-4898.

Alemayehu Asfaw. (2014). Coffee production and marketing in Ethiopia. *European Journal of Business Management,* **6**(37), 109-111.

Ali, S.S., and Vidhale, N.N. (2013). Bacterial siderophore and their application: A review. *International Journal of Current Microbiology and Applied Sciences,* **2(**12), 4-8.

Befekadu Teshome, Misganaw Wassie and Endeshaw Abatneh. (2017). Isolation, screening and biochemical characterization of phosphate solubilizing rhizobacteria associated with *Coffea arabica* L. *Journal of Fertilizers and Pesticides,* **8**(3), 1-5.

Baethgen, W.E., and Alley, M.M. (1989). A natural colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. *Community Soil Science and Plant Analysis,* **20**, 961-969.

Beneduzi, A., Ambrosini, A., and Passaglia, L.M. (2012). Plant growth promoting rhizobacteria (PGPR): Their potential as antagonist's biocontrol: A review article. *Genetics and Molecular Biology*, **35**(4), 1044-1048.

Bhardwaj, D., Ansari, M.M., Sahoo, R.K., and Tuteja, N. (2014). Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microbial Cell Factories*, **13**(66), 1p.

Bray, R.H., and Kurtz, L.T. (1945). Determination of total organic and available forms of phosphorus in soils. *Journal of Soil Science,* **59**, 39-45.

Breed, R.S., Murray, E.G., and Smith, N.R. (1957). *Bergey's Manual of Determinative Bacteriology* (7th ed.). United States of America: Baltimore the Williams and Wilkins Company.

Brundrett, M., Bougher, N., Dell, B., Grove, T., and Malajczuk, N. (1996). Working with mycorrhizas in forestry and agriculture. *ACIAR Monograph* **32**, 374p.

Chauhan, A., Shirko, C.K., Kaushal, R., and Rao, D.L. (2015). Plant growth promoting rhizobacteria of medicinal plants in Northwest Himalayas: Current Status and future Prospects. India: Springer International Publishing Switzerland, 381-407.

Delelegn woyessa and Fassil Assefa. (2011). Diversity and plant growth promoting properties of rhizobacteria isolated from tef (*Eragrostis tef*). *Ethiopian journal of Education and Science,* **6**(2), 5-11.

Devendra, K., Choudhary., Prakash, A., and Johri, B.N. (2007). Induced systemic resistance (ISR) in plants: mechanism of action. *Indian Journal of Microbiology,* **47**, 289-295.

Dey, R., Krishna, K., and Tilak, K.V. (2014). Plant growth promoting rhizobacteria in crop protection and challenges, 31-51.

Diriba Muleta. (2007). *Microbial inputs in coffee (Coffea arabica L.) production systems, South-western Ethiopia*. Doctoral thesis Swedish University of Agricultural Sciences, Uppsala, 9-45.

Diriba Muleta, Fassil Assefa, Borjesson, E., and Granhall, U. (2013). Phosphate solubilising rhizobacteria associated with *Coffea arabica* L. in natural coffee forests of south-western Ethiopia. *Journal of the Saudi Society of Agricultural Sciences*, **12**, 1-10.

Diriba Muleta, Fasssil Assefa, Hjort, K., Roos, S., and Granhall, U. (2009). Characterization of rhizobacteria isolated from wild *Coffea arabica* L. *Journal of Engineering in life Sciences,* **9**(2), 100-108.

Elmanama, A.A. (2009). *General Microbiology Laboratory Manual.* Medical Technology Department, Islamic University, Gaza, 16-73.

Figueiredo, M.V., Seldin, L., Araujo, F., and Mariano, R.L. (2010). Plant growth promoting rhizobacteria: fundamentals and applications. *Microbiology monographs*, 22-35.

Firew Elias, Delelegn Woyessa and Diriba Muleta. (2016). Phosphate solubilization potential of rhizosphere fungi isolated from plants in Jimma zone, Southwest Ethiopia. *International Journal of Microbiology*, 1-10.

Geetanjali and Jain, P. (2016). Antibiotic production by rhizospheric soil microflora: A review. *International Journal of Pharmaceutical Sciences and Research*, **7**(11), 4304-4312.

Geetha, K., Rajithasri, A.B., and Bhadraiah, B. (2014). Isolation of plant growth promoting rhizobacteria from rhizosphere soils of green gram, biochemical characterization and screening for antifungal activity against pathogenic fungi. *International Journal of Pharmaceutical Science Invention*, **3**(9), 47-53.

Gerdemann, J. W., and Nicolson, T. H. (1963). Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Transactions of the British Mycological Society*, **16**(2), 235-244.

Girma Adugna. (2004). *Diversity in pathogenicity and genetics of Gibberella xylarioides (Fusarium xylarioides) populations and resistance of Coffea sp. in Ethiopia*. PhD dissertation, 1-5.

Glick, B. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Hindawi Publishing Corporation*.

Gosling, P., Hodge, A., Goodlass, J., and Bending, G.D. (2005). Arbuscular mycorrhizal fungi and organic farming. *Journal of Agriculture, Ecosystem and Environment*, **113**, 17-28.

Gosling, P., Ozaki, A., Jones, J., Turner, M., Rayns, F., and Bending, G.D. (2010). Organic management of tilled agricultural soils results in a rapid increase in colonisation potential and spore populations of arbuscular mycorrhizal fungi. *Journal of Agricultures, Ecosystems and Environment*, **139**, 273-278.

https://invam.wvu.edu/methods/spores/enumeration-of-spores. (n.d.). (W. V. University, Producer, & West Virginia University) Retrieved 2017, from INVAM, http://invam.caf.wvu.edu.

Islam, S., Akanda, A., Prova, A., Islam, T., and Hossain, M. (2016). Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. *Frontiers in Microbiology*, **6**(1360), 1-9.

Jha, D.K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, **28**, 1328-1343.

Kifle Belachew, Demelash Teferi, Niguse Hundessa and Sisay Tesfaye. (2016). The statue and management of coffee wilt disease (*Gibberella xylarioides*) in Ethiopian coffee production. *Journal of Natural Sciences Research*, **6**(5), 16-20.

Kundan, R., Pant, G., Jadon, R. and Agrawal, P.K., (2015). Plant Growth Promoting Rhizobacteria: Mechanism and Current Prospective. *Journal of Fertilizers and Pesticides*, **6**(2), 1-7.

Lebron, L., Lodge, D.J., and Bayman, P. (2012). Differences in arbuscular mycorrhizal fungi among three coffee cultivars in Puerto Rico. *Journal of International Scholarly Research Network of Agronomy*, 1-6.

Li, Q., Chen, J., Luo, X., Li, N., Arafat, y., and Lin, W. (2018). Belowground interactions impact the soil bacterial community, soil fertility, and crop yield in maize/peanut intercropping systems. *International Journal of Molecular Sciences*, **19**(622), 1-13.

Majeed, A., Kaleem, A.M., Hameed, S., Imran, A., and Rahim, N. (2015). Isolation and characterization of plant growth promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. *Journal of Frontiers in Microbiology*, **6**(198), 1-7.

Martinez-Viveros, M.A., Jorquera, D.E., Crowley, G., Gajardo, P., and Mora, M.L. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of Soil Science and Plant Nutrition*, **10**(3), 293-312.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., and Swan, J.A. (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* (115), 495-501.

Melkamu Tiru, Diriba Muleta, Gezahegn Berecha and Girma Adugna. (2013). Antagonistic effect of rhizobacteria against coffee wilt disease caused by *Gibberella xylarioides*. *Asian Journal of Plant Pathology*, **7**(3), 109-120.

Munees Ahemad and Mulugeta Kibret. (2013). Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University Science,* **26**, 1-16.

Nandal, M., and Hooda, R. (2013). Plant growth promoting rhizobacteria: A review article. *International Journal of Current Research,* **5**(12), 3863-3868.

Nihorimbere, V., Ongena, M., Smargiassi, M., and Thonart, Ph. (2011). Beneficial effect of the rhizosphere microbial community for plant growth and health. *Journal of Biotechnology, Agronomy, social and Environment,* **15**(2), 327-334.

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil connection with the vital activity of some microbial species. In *Microbiology,* (pp. 362-370.).

Pradhan, P. (2015). *http://microbesinfo.com/2015/03/218/.*

Prashar, P., Kapoor, N. and Sachdeva, S. (2013). Isolation and characterization of *Bacillus* sp with i*n-vitro* antagonistic activity against *Fusarium oxysporum* from rhizosphere of tomato. *Journal of Agricultural Science and Technology,* **15**, 1501-1509.

Premono, M.E., Moawad, A.M., and Vlek, P.L. (1996). Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science,* **11**, 13-23.

Rahman, C.H., Ahcene, B., Miloud, B., and Rachid, D. (2017). Screening and characterization of plant growth promoting traits of phosphate solubilizing bacteria isolated from wheat rhizosphere of Algerian saline soil. *Malaysian Journal of Microbiology,* **13**(2), 124-130.

Ramamoorthy, V., Viswanathan, R., Raguchander, T., Prakasam, V., and Samiyappan, R. (2001). Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. *Review Article on Crop Protection,* **20**, 1-8.

Rawat, S., and Mushtaq, A. (2015). Characterization of plant growth promoting rhizobacteria from legume rhizosphere. *Asian Journal of Plant Science and Research,* **5**(4), 38-42.

Rodrigues, A.A., Forzani, M.V., Soares, R.S., Sibov, S.T., and Vieira, J.D. (2016). Isolation and selection of plant growth promoting bacteria associated with sugarcane. **46**(2), 149-155.

Samuel Gebreselassie and Ludi, E. (2006). Agricultural commercialisation in coffee growing areas in Ethiopia. *Ethiopian Journal of Economics,* **15**(1), 87p.

Santhoshkumar, S., and Nagarajan, N. (2017). Arbucular mycorrhizal fungal association in the rhizosphere soil and root colonization of some medicinal plant species in Sirumalai Hills Eastern Ghats of Dindugul District Tamilnadu. *American-Eurasian Journal of Agricultural and Environmental Sciences,* **17**(3), 206-209.

Shahab, S., Ahmed, N., and Khan, N.S. (2009). Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs. *African Journal of Agricultural Research,* **4**(11), 1312-1314.

Shakeela, S., Padder, S.A., and Bhat, Z.A. (2017). Isolation and characterization of plant growth promoting rhizobacteria associated with medicinal plant *Picrorhiza Kurroa*. *Journal of Pharmacognosy and Phytochemistry,* **6**(3), 157-166.

Sihen Getachew, Girma Adugna, Fikre Lemessa and Hindorf, H. (2012). Coffee wilt disease (*Gibberella xylarioides* Heim and Saccas) in forest coffee system of Southwest and Southeast Ethiopia. *Plant Pathology Journal*, 1-6.

Singh, A., Budholiya, N., and Singh, S. (2015). Isolation and identification of phosphate solubilizing bacteria by 16S RRNA sequencing and evaluation of pesticide tolerance capacity. *World Journal of Pharmacy and Pharmaceutical Sciences,* **4**(11), 964-976.

Singh, R.P., and Jha, P.N. (2015). Molecular identification and characterization of rhizospheric bacteria for plant growth promoting ability. *International Journal of Current Biotechnology,* **3**(7), 12-17.

Smith, S., and Read, D. (2008). *Mycorrhizal Symbiosis* (3rd ed.). Australia: Elsevier Ltd.

Tekalign Kajela,Thakkar, R.V., and Thakor, P. (2016). *Bacillus* species (BT42) isolated from *Coffea arabica* L. rhizosphere antagonizes *Colletotrichum gloeosporioides* and *Fusarium oxysporum* and also exhibits multiple plant growth promoting activity. *Journal of Biomedical Central of Microbiology,* **16**(277), 1-11.

Toppo, S.R., and Naik, U.C. (2015). Isolation and characterization of bacterial antagonist to plant pathogenic fungi (*Fusarium* sp.) from agro based area of Bilaspur. *International Journal of Research Studies in Biosciences*, 6-13.

Van Loon, L.C., and Bakker, P.A. (2005). Induced systematic resistance as a mechanism of disease suppression by rhizobacteria. Netherlands, (pp. 35-59).

Walkley, A., and Black, I.A. (1934). An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic soil titration method. *Journal of Soil Science,* **37**, 29-38.

Woodland, J. (2004). Bacteriology. In *USFWS Laboratory Procedures Manual* (2nd ed.). USFWS-Pinetop Fish Health Centre, Arizona, 7-15.

Yang, Z., Yang, W., Li, S., Su, Z., Sun, M., Gao, Z., and Zhang, Ch. (2016). Variation of bacterial community diversity in rhizosphere soil of sole-cropped versus intercropped wheat field after harvest. *PLOS/ONE*, **11**(3), 1-14.

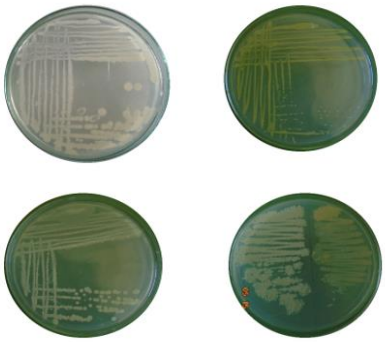
Zelalem Yimer. (2015). *Aspects of land degradation and land manage practices in Gawo Kebe Woreda, Kellem Wellega Zone, Oromia Region*. MSc thesis, Haramaya University, 27-32.

Zephania Simon, Mtei, K., Amare Gessesse and Ndakidemi, P.A. (2014). Isolation and characterization of nitrogen fixing *Rhizobia* from cultivated and uncultivated soils of Northern Tanzania. *American Journal of Plant Sciences,* **5**, 4050-4058.

Zerihun Belay, Fassil Assefa and Vestberg, M. (2013). Diversity and abundance of arbuscular mycorrhizal fungi associated with acacia trees from different land use systems in Ethiopia. *Global Journal of Microbiology Research,* **1**(1), 23-33.

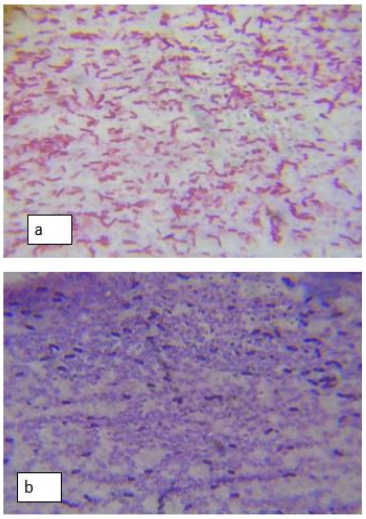
Appendixes

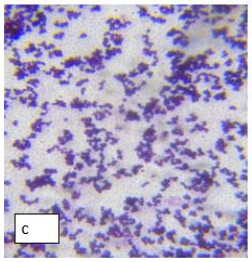
Appendix 1: Isolation of pure culture by streak plate method



Pure culture of bacterial isolates

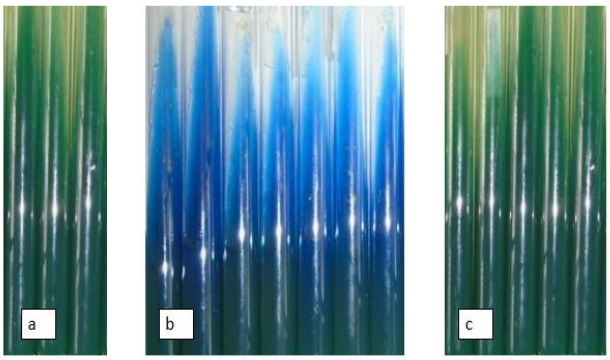
Appendix 2: Representative Gram staining of bacterial isolates





a. Gram negative rods
b. Gram positive rods
c. Gram positive cocci

Appendix 3: Citrate test

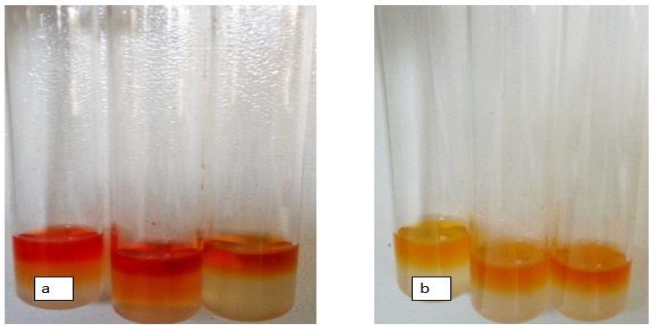


a. Control

b. Citrate test positive isolates

c. Citrate test negative isolates

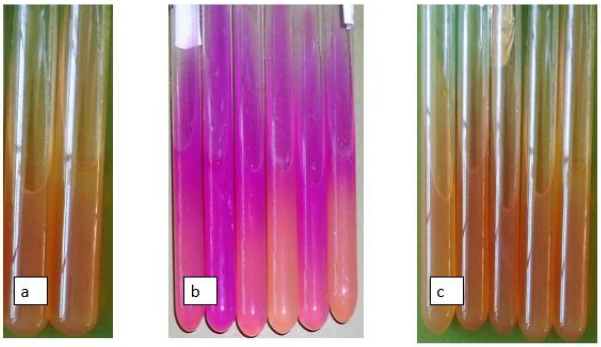
Appendix 4: Methyl red test



a. Methyl red test positive isolates

b. Methyl red test negative isolates

Appendix 5: Urease test

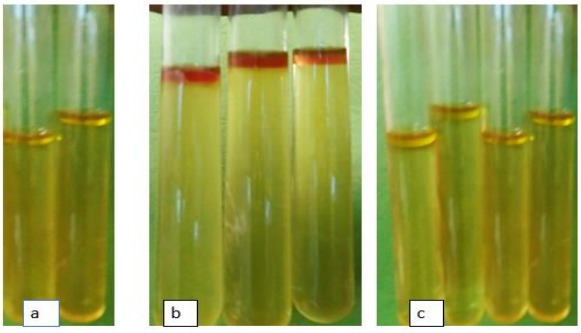


a. Control

b. Urease test positive isolates

c. Urease test negative isolates

Appendix 6: Indole Test

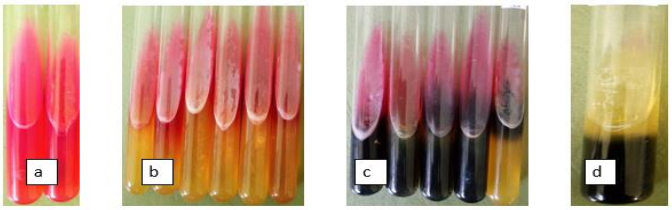


a. Control

b. Indole test positive isolates

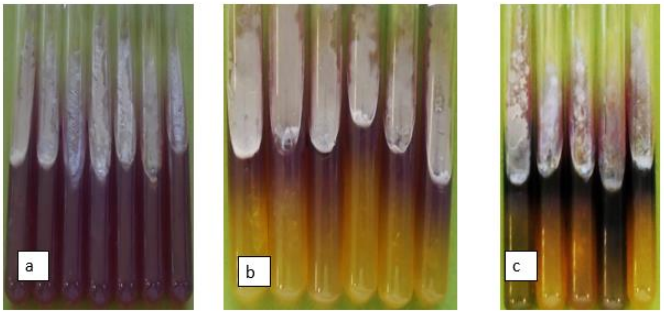
c. Indole test negative isolates

Appendix 7: Triple sugar iron agar (TSIA) test



a. Control
b. Glucose fermentation only
c. Glucose fermenters, H2S producers, Sucrose and lactose non-fermenters
d. Acidic slant/acidic bottom: glucose, lactose and sucrose fermenter, H_2S producer

Appendix 8: Lysine iron agar (LIA) test



a. Lysine decarboxylase producers
b. Non-decarboxylase producers, but glucose fermenters
c. Non-decarboxylase producers, but glucose fermenters and H_2S producers

Appendix 9: Standard correlation coefficient (r) value

r value	**Relationship between two variables**
Exactly -1	Perfect downhill (negative) linear relationship
-0.7	Strong downhill (negative) linear relationship
-0.5	Moderate downhill (negative) linear relationship
-0.3	Weak downhill (negative) linear relationship
0	No linear relationship
+0.3	Weak uphill (positive) linear relationship
+0.5	Moderate uphill (positive) linear relationship
+0.7	Strong uphill (positive) linear relationship
+1	Perfect uphill (positive) linear relationship