ISOLATION AND CHARACTERIZATION OF RHIZOBIA FROM LEGUMINOUS PLANTS AND ASSESSING THEIR PLANT GROWTH PROMOTING PROPERTIES

MSc THESIS

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AUGUST 2018
HARAMAYA UNIVERSITY, HARAMAYA

Isolation and Characterization of Rhizobia from Leguminous Plants and Assessing their Plant Growth Promoting Properties

A Thesis Submitted to the School of Biological Sciences and Biotechnology, Post Graduate Program Directorate
HARAMAYA UNIVERSITY

In Partial Fulfillment of the Requirements for the Degree of
MASTER OF SCIENCE IN BIOTECHNOLOGY

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August 2018
Haramaya University, Haramaya

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BIOGRAPHICAL SKETCH

The author was born on December 1990 G.C from his father Mesele Ejigu and his mother Lezebu Kahsay in Korem, southern Tigray. He attended his education at Kola-Stihdi Elementary School, Korem Secondary School and Biruh-Tesfa Preparatory School. After completing his preparatory school, he joined Mekelle University in September 2012 G.C and graduated with BSc in Biotechnology (regular program) in July 2015 G.C. Immediately, he joined Mekelle University, Department of Biotechnology and served as a graduate assistant (GA) for a year. Then after, he joined the Post Graduate Program Directorate in 2016 G.C to pursue his M.Sc study in Biotechnology at the School of Biological Sciences and Biotechnology, Haramaya University.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my major advisor Dr. Ameha Kebede, School of Biological Sciences and Biotechnology, Haramaya University, for his excellent advice, valuable comments and consistent encouragement throughout my thesis work.

I would also like to thank to my co-advisor Yisehak Tsegaye (Associate Professor), Department of Veterinary Science, Mekelle University, for his fruitful comments, encouragement and inspiring guidance during the research process. Indeed, I am thankful to him for coordinating microbiology and molecular biology laboratories, providing expensive chemicals and reagents, assisting and directing in operating laboratory equipments etc.

I am highly grateful for Tigray Agricultural Research for giving me faba bean samples from their research field and to farmer Assefa Tareke for his honest cooperation to collect sample from his farm land.

I am greatly indebted to different departments of Mekelle University such as Biology Department and Chemistry Department for allowing me to access chemicals.

I am also highly indebted to my friends Hailelul Eyasu, Ashenafi Teklay, Melaku Mekonen and Samson Zemikael who supported me ideally and practically during the course of work.

Finally, I am very happy to acknowledge for Mekelle University that fully sponsored me to engage in this academic development.

ACRONYMS AND ABBREVIATIONS

CR	Congo Red
EPS	Exopolysaccharide
GPA	Glucose Peptone Agar
HCN	Hydrogen Cyanide
IAA	Indol-3- acetic acid
KL	Ketolactose
LB	Luria-Bertani
Nif	Nitrogen fixation
Nod	Nodulation
PCI	Phenol- chloroform-isoamyl alcohol
PCR	Polymerase Chain Reaction
PGPP	Plant Growth-Promoting Properties
PGPR	Plant-growth-promoting rhizobacteria
SE	Solubilization Efficiency
SI	Solubilization Index
TE	Tris-EDTA
UV	Ultraviolet
YEMA	Yeast Extract Mannitol Agar

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ABSTRACT

The increasing world population led to mass application of chemicals in agriculture to increase food production and meet the demand. Today, attentions are diverted into biofertilizers than the chemical fertilizers. This study was initiated to isolate and characterize rhizobia from Faba bean (Vicia faba L.) and assessment for plant growth prompting substance. Faba bean samples were collected by systematic random sampling from farmers' fields of three geographically varied locations of Tigray region (Korem, mekelle and Tanqa abergele). A total of 90 isolates were subjected to biochemical tests viz. catalase, oxidase, congo red, glucose peptone agar and ketolactose. Isolates were assessed for production of indole acetic acid, ammonia, hydrogen cyanide, exopolysaccharide and for phosphate solubilization. Finally molecular test was carried out for nitrogen fixation and nodulation using nifH and nodA genes. Data on exopolysaccharide production and phosphate solubilization were subjected to statistical analysis. Based on biochemical tests, all the isolates were gram negative, both catalase and oxidase positive. 71 (78.9%) of the isolates didn't absorb Congo red, 52 (57.8%) showed poor or no growth on GPA and 79 (87.8%) didn't produce 3- ketolactose. In total, 27 isolates were selected for plant growth promoting properties test. The results showed that all isolates were capable of producing indole acetic acid and ammonia. Whereas, only 3 isolates found to produce hydrogen cyanide. Then 21 isolates were exopolysaccharide producers and the max value (1900 µg ml^{-1}) was produced by isolate Ko1b followed by Ko10c (1850 µg ml^{-1}) and Ta8c (1750 µg ml^{-1}). Similarly, 19 isolates were able to solubilize phosphate with the highest solubilization index (3.33) by the isolate Ko1c. Bands from the gel showed that about 781bp nifH gene and about 850bp nodA gene fragments were found in some isolates. Generally, the potential isolates throughout the tests are promising and important to be used as bioinoculant production. But large scale bioinoculant production must be after field trial of isolates because in vitro results may not be the same as in vivo based experiments.

Key words: Biofertilizers, Faba bean, nifH gene, Nodules, Rhizobia

1. INTRODUCTION

The need for increased food production due to the rapid growth of the world population led to indiscriminate application of nitrogenous chemicals, pesticides and fertilizers for agricultural intensification to meet the demand of the population.

Legumes grown in many African soils without addition of chemical fertilizers are characterized by low levels of production (Mnalku *et al*., 2009). However, excessive use of agricultural chemicals is known to cause undesirable effects on agriculture, food, biodiversity and environment (Namkeleja *et al*. 2016). Some specific problems include pesticide resistance in pests, resurgence of target and non-target organisms, destruction of beneficial organisms like honey bees, and accumulation of chemical residues in food, feed and fodder, pollution of ground water, acidification of soil, enhancement of denitrification that results in higher emission of N_2O to the atmosphere, and on top of these, the burning of fossil fuel for manufacturing N-fertilizers produces hazardous byproducts that pose a threat to human health and the environment (Mia *et al*., 2010). Not only it causes concern on the environment, but also its price has increased and reached to the level that subsistence farmers often face difficulty to purchase and utilize it (Mnalku *et al*., 2009; Mia *et al*., 2010). The most common chemical N fertilizer source that has been used for decades in Ethiopia is urea (Mnalku *et al*., 2009).

Awareness about the health hazards and environmental problems of inorganic fertilizers created an intention to find other best alternatives for resolving the problem of poor soil fertility and to ensure environmental safety. Among the alternative mechanisms is the use of living microbes like rhizobia as bioinoculants (Raffal, 2013). On the other hand, the application of rhizobia in legume crop production is underlined as ecologically safe and relatively low-cost source of nutrient to improve crop yield than chemical fertilizers (Namkeleja *et al.*, 2016). This is important biotechnological method for subsistence and small-holder farmers in developing countries like Ethiopia (Jida and Assefa, 2011).

The symbiotic relationship between the nodule forming bacteria called rhizobia and leguminous plants such as alfalfa, clover, peas, beans, soybeans, peanuts etc results in the enzymatic fixation of atmospheric nitrogen in root nodules and secretion of other plant growth

promoting chemicals. This symbiosis interaction offers special advantage to legumes as seed inoculation with effective strains of rhizobia can meet the appropriate nutrient requirement of the legume to achieve growth and development (Shoukry *et al*., 2013; Tariq *et al*., 2014). Plant growth promoting activities are due to both bacterial and plant interaction in the production of phytohormones, solubilization of phosphate and or acting as disease suppressive through direct inhibition of pathogens' development (through competition or antibiosis) or indirect inhibition by stimulating plant defense systems (Pashapour *et al.* 2016).

Rhizobium species are usually defined as gram negative and nitrogen-fixing soil bacteria capable of inducing the formation of root or stem nodules on leguminous plants in which they reduce atmospheric nitrogen to ammonia for the benefit of the plant. Due to their considerable agricultural and environmental significance, extensive researches have been carried out regarding these legume symbionts (Agrawal *et al*., 2013, Jida and Assefa 2011, Beyene *et al*. 2004, Simon *et al*., 2014). The rhizobia are represented by at least 14 genera containing more than 98 species. The majority of them belong to α- *Proteobacteria* class, in the genera of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium, Allorhizobium* and *Ensifer* (formerly *Sinorhizobium*), *Methylobacterium*, *Devosia*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium* and *Shinella.* Two genera (*Burkholderia* and *Cupriavidus*) belong to beta proteobacteria and one genus (*Pseudomonas*) to gamma proteobacteria (Berrada and Fikri-Benbrahim, 2014; Weir, 2016).

Several phenotypic, biochemical and molecular mechanisms have been readily adopted and are being used by many researchers worldwide for effective identification, characterization and analyzing genetic variability of agronomically useful rhizobial species. Jida and Assefa (2011) and Beyene *et al*. (2004) reported the results of organized works on isolation, selection and characterization of rhizobial species from leguminous plants in some areas of Ethiopia.

Ethiopia is among the countries endowed with mega-biodiversity of several biosphere reserves including the nitrogen fixing rhizobial biodiversity. Although Ethiopia is rich in rhizobial biodiversity, the existence of ineffective species in the natural habitat leads to failure of symbiosis and nodulation with host plants and this resulted in poor crop yields (Ahmed, 2010; Abere, 2010). The inefficiency of indigenous rhizobia for nodulation and nitrogen fixation can

be solved by isolation and inoculation of effective rhizobia (Tsegaye *et al.* 2015). Hence scientific researches regarding rhizobia-host crop interactions and the *in vitro* assessment for plant growth promoting properties are needed to obtain effective isolates. In order to establish an environmental friendly biological nitrogen fixation technology and bioinoculant production, successful study on the nature of rhizobia is preliminary action (Namkeleja *et al*., 2016). Therefore, isolation, characterization, screening and *in vitro* testing of the rhizobial species for their effectiveness as biofertilizer using conventional and molecular methods is necessary to create a platform for expanded studies in field experimentation and large scale bioinoculant production (Simon *et al*., 2014). This will be beneficial especially for the infertile soils and chemical fertilizer allergic soils of Ethiopia to improve crop productivity, via legume-cereal intercropping, and chemical fertilizer reduction.

General Objective

The main objective of this study is to isolate rhizobia from root nodules of Faba bean; characterize them using biochemical and molecular methods; and evaluate their plant growth-promoting properties (PGPPs).

Specific Objectives

- To isolate rhizobia from root nodules of Faba bean (V*icia faba* L.).
- To characterize the isolates biochemically using catalase, oxidase, congo red, ketolactose and glucose peptone agar tests.
- To assess the plant growth-promoting properties (indole acetic acid, ammonia, hydrogen cyanide, exopolysaccharide and phosphate solubilization) of the rhizobia.
- To characterize the isolates at a molecular level by the amplification of *nifH* and *nodA* genes.

2. LITERATURE REVIEW

2.1. Legumes

Legumes are flowering plants categorized in the Fabaceae (Leguminosae) family that contain about 690 genera and 18,000 species. The word legume is derived from the Latin word *legere* which means 'to gather'. The Leguminosae family is classified into three sub-families the so called Papilionoideae, Caesalpinioideae, and Mimosoideae. Each sub-family is identified by its flowers. Papilionoideae consists of edible legume crops including soybean, chickpea, bean, pea etc. The seeds of many legumes are important for food consumption worldwide because they are rich in both oils and proteins. They have higher protein content than any other food plant which is close to animal meat in quality. In fact, they are often called "poor man's meat" because they are inexpensive sources of high-quality protein. The high protein content of legumes is correlated with the presence of nitrogen-fixing bacteria in the root nodules that help the plant to synthesize proteins and other nitrogen-containing compounds (Levetin and McMahon, 2008).

Table 1. List of some common leguminous plant species (Al-Mujahidy *et al*. 2013)

Scientific name	**Common name**	**Local name**
Cicer arietinum	Chick pea	Senebera
Glycine max	Soybean	Akuri
Pisum sativum	Pea	Atare
Trifolium	Clover	Wudma
Lens culinaris	Lentil	Mesere
Vicia faba	Broad bean	Duke baqela
Vigna unguiculata	Cowpea	Boleke
Arachis hypogaea	Peanut	Lewuzi
Phaseolus vulgaris	Common bean	Yegara bakela

2.2. Rhizobia as Legume Inoculants

Rhizobia are a form of soil bacteria that fix nitrogen (diazotrophs) after they are established inside the root nodules of legumes (Fabaciae). Rhizobia require a plant host as they cannot fix nitrogen independently. In general, they are gram-negative, aerobic, motile, non-spore forming rods.

There are several genera of rhizobia which all belong to the Rhizobiales, a monophyletic group of proteobacteria. They are able to colonize the 18,000 species of the *Leguminosae* family and infect their root hairs to produce effective N_2-fixing nodules in which they reduce atmospheric nitrogen to ammonia to the benefit of the plant and their benefit (Sy *et al*., 2001; Mia and Shamsuddin, 2010).

Application of *Rhizobium* inoculants in legume seeds is the oldest agro-biotechnological practice in which legume seeds are purposefully inoculated with rhizobia to promote Biological Nitrogen Fixation by providing nitrogenous compounds to the crop. If this is effectively carried out, it increases crop productivity and decreases application of chemical fertilizers with the consequent reduction of environmental pollution (Kaur, 2014). Rhizobia inoculants can be purchased commercially in the form of solid inoculants, liquid inoculants or freeze-dried forms. However, solid inoculants are commonly used.

Lupwayi (2006) explained that the genetic traits of the *Rhizobium* strains, legume genotypes and inoculant delivery methods are factors that affect nitrogen fixation and nodulation efficiency. Soil inoculation seems to be often better and never worse than seed inoculation for initiating nodulation and N_2 fixation because the number of rhizobia in pre-treated seeds tends to be minimized.

2.3. Biological Nitrogen Fixation

Nitrogen is the most essential nutrient for growth and development of all living organisms. Nitrogen plays role in plant cells for synthesis of enzymes, proteins, chlorophyll, DNA and RNA. Thus, it is essential for plant growth and production of food and feed. Nitrogen deficiency in plants causes reduced growth, leaf yellowing, reduced branching and small trifoliate leaves in legumes. Two nitrogen pools are found on the planet earth. One pool

accounts about 99% of the total amount of nitrogen and it is the gaseous dinitrogen (N_2) form of the atmosphere. The other pool is found with other elements such as carbon (C), hydrogen (H) or oxygen (O) bounded by chemical reactions. It accounts 1% of total nitrogen and has been described as “reactive nitrogen” (Torres, 2008).

Biological nitrogen fixation is a process of converting atmospheric gaseous N into plant assimilating form such as ammonia through a cascade of reactions between symbiotic nitrogen fixing prokaryotes and symbiont plants with the use of complex enzyme systems (Gopalakrishnan *et al*,. 2015). This process is essentially undertaken due to the fact that the atmospheric dinitrogen (N_2), which makes up 80% of the air we breathe, is inert as a result of its strong triple bond (N≡N). Therefore, the nitrogen, to be used for growth of living things, must be fixed (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions in the presence of substantial amounts of energy input. Although the majority of nitrogen which is about 60% world's supply of fixed N is through biological nitrogen fixation processes, other processes such as atmospheric lighting and chemical fertilizer industries are also responsible for nitrogen fixation in the biosphere. Lightning probably accounts for about 10% of the world's supply of fixed nitrogen. The industry also provides about 25% of chemically fixed Nitrogen in the form of urea and ammonium nitrate (NH_4NO_3) (Torres, 2008).

An important requirement for efficient Biological Nitrogen Fixation is to have diazotrophic bacteria growing endophytically within plants. Examples of organisms that do this are rhizobia and *Frankia* (in symbiosis) and *Azospirillum* (Mia and Shamsuddin, 2010).

Namkeleja *et al*. (2016) reported that some species of bacteria that belong to the genus *Bacillus* like *B. cereus*, *B. fusiformis*, *B. marisflavi* and *B. alkalidiazotrophicus* are involved in nitrogen fixation even though rhizobia species remain as the most effective biological nitrogen fixers with a mean annual fixation rate of 55,140 kg N per hectare, compared with 0.330 kg N per hectare for other biological systems. In addition to this, they underlined their estimations that symbiotic interaction between microbes and legumes worldwide reduce about 100 million metric tons of atmospheric nitrogen per year hence saving about US$ 8 billion per year in fertilizer and making biofertilizers inexpensive compared to the chemical nitrogen fertilizers. Accordingly, there is currently an increasing effort on the clarification of the significance of

BNF, enhancing its availability and applying inoculants in agriculture through isolation and characterization of rhizobia in various legumes. If biological nitrogen fixation is efficiently utilized, it will enhance legume production as well as reduce cost of production.

2.4. Principles of Biofertilizers

Biofertilizers, also termed as microbial inoculants, can be generally defined as preparations containing live or latent cells of efficient strains of nitrogen fixing, phosphate solubilizing, phytohormone producing or cellulolytic microorganisms used for application on seeds, soils or composting areas with the objective of increasing the numbers of such microorganisms and accelerating certain microbial processes to supplement the availability of nutrients in a form which can be easily incorporated into the plant bodies. The term may be used to include all organic resources, in broader sense, needed for plant growth and which are rendered in an available form for plant absorption by the action of microorganisms or plant-microbe interactions (Mohammadi and Sohrabi, 2012).

Certain soil microorganisms can be artificially multiplied in culture to produce microbial inoculants (biofertilizers), which are eco-friendly, low cost and renewable source of plant nutrients, used as to replace chemical fertilizers for improving soil fertility and crop productivity (Poonia, 2011). Mahdi *et al*. (2010) reported that the nitrogen fixers like *Rhizobium*, *Azospirillum*, *Azotobacter*, cyanobacteria and phosphate solubilizing bacteria like *Bacillus magaterium*, *Pseudomonas striata*, and phosphate mobilizing mycorrhiza have been widely accepted as bio-fertilizers. Also they stated that large scale production and long term use of biofertilizers will solve the near future threats of the world such as maximum food grain production demand, increasing cost of chemical fertilizers, depletion of soil fertility, increasing environmental hazards and threats to sustainable agriculture.

2.5. Plant Growth Promoting Traits of Rhizobia

Rhizobia are important members of plant-growth-promoting rhizobacteria (PGPR) that exert the positive effects on plant growth via direct and indirect mechanisms. Plant-growth-promoting (PGP) activities include biological nitrogen fixation (ammonium production), the production of phytohormones (eg. IAA production), siderophores production,

exopolysaccharide production and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase synthesis as well as the solubilization of inorganic phosphate (Pongsilp, 2012; Islam *et al*., 2015; Mahbouba *et al*., 2013; Kaur, 2014). Plant growth promoting traits like nitrogen fixation, phosphate solubilization, siderophore formation and phytohormone production are direct promotions and biocontrol abilities of the microbe and induction of plant resistance against biotic and abiotic stresses are indirect promotion traits (Gopalakrishnan *et al*., 2015). The most important plant growth promoting traits tested in this investigation, either direct or indirect mechanisms are reviewed below.

2.5.1. Phytohormone production

Plant hormones, also termed as *phytohormones,* are chemicals involving in control and coordination of plant growth and functioning. Phytohormones regulate cellular activities (division, elongation and differentiation), organogenesis, reproduction, sex determination, and responses to abiotic and biotic stresses. These include auxins, cytokinins, gibberellins, ethylene and Abscisic Acid (ABA). Indole-3-acetic acid is the foremost auxin which enhances plant growth and development by controlling cell enlargement, cell division, root initiation, growth rate, phototropism and apical dominance. Rhizobia have been investigated for production of Indole-3-acetic acid by many researchers (Joseph *et al*., 2007; Saengsanga, 2018; Saharan and Verma, 2017; Pashapour *et al.,* 2016).

Shoukry *et al*. (2018) were successfully screened 21 IAA producing isolates by growing on yeast mannitol medium. Datta *et al*. (2015) also reported that *Rhizobium* strains isolated from the root nodules of *Trifolium* (clover), *Vigna radiata* (mung bean), *Glycine max* (soybean) and *Lens culinaris* (lentil) showed maximum IAA production.

2.5.2. Solubilization of phosphates

Phosphate solubilization process by bacteria like rhizobia is very important next to nitrogen fixation that solves the problem of the low availability of phosphorus to plants in the soil. The low availability of P in the soil is because the majority of soil P is found in the form of insoluble phosphates, while the plants can only absorb it after it converted in to soluble forms known as monobasic ($H_2PO_4^-$) and the diabasic (HPO_4^{-2}) ions (Vessey, 2003). Phosphatic

fertilizers are frequently applied in agriculture to overcome P deficiency in the soil. However, regular use of these chemicals are costly and environmental unfriendly. This problem leads to search ecologically safe and economically reasonable biological substitute for chemical phosphate fertilizers.

Phosphate solubilizing microbes are promising biofertilizers that can enhance plant growth by supplying phosphorus in P deficient soils (Ahemad and Kibret, 2014). From the soil bacterial communities, rhizobia have been identified as effective phosphate solublizers and promising bioinoculants (Goswami *et al.* 2016). Jadhav (2013) reported all his rhizobial isolates (20) from soybean were efficient phosphate solublizers after longer time incubation.

2.5.3. Hydrogen cyanide (HCN) production

Apart from growth regulation, rhizobia are able to induce plant resistant against disease by attacking pathogens, insects and weeds (Namkeleja *et al.,* 2016). They secrete defensive enzymes and chemicals that suppress growth of phytopathogens. Production of the toxic gas, hydrocyanic acid (HCN) is one of the biocontrol mechanisms. HCN inhibits growth of pathogens by influencing the electron transport system, specifically the cytochrome- C oxidase and other metalloenzymes, of the pathogenic cells (Ahemad and Kibret, 2014; Gopalakrishnan *et al*., 2014; Datta *et al*., 2014). Pashapour *et al*. (2016) isolated potential HCN producing *Sinorhizobium* species from root nodules of alfalfa.

2.5.4. Production of ammonia

Nitrogen and nitrogen-based compounds are essential elements for all forms of life and used as a precursor for nucleic acid and protein synthesis. Ammonia released by the rhizobacterial community plays as signaling role in the interaction between rhizobacteria and plants (Ahemad and Khan, 2012). It is also used as a nitrogen source to the host plant and fulfills nutritional need of the plant (Goswami *et al.,* 2013). Ammonia is reported to indirectly influence the plant growth (Joseph *et al*., 2007; Geetha *et al.,* 2014). Joseph *et al.* (2007) reported about 74.2% of their total isolates from chickpea (*Cicer arietinum* L.) were ammonia producers. Karthik *et al.* (2017) also characterized multi-potential Rhizobium strain ND2 as potent ammonia producer.

2.5.5. Exo-polysaccharide production

Rhizobia produce extracellular polysaccharides for their own protection and infection thread formation during nodulation. After rhizobia cells stimulated by flavonoids produced by the roots of legumes in the soil, they started to secret exopolysaccharides called lipochitooligosaccharides. These signaling molecules are called Nod factor which are used for root hair curling, infection thread formation and finally formation of the nodules (Bomfeti *et al.*, 2011; Janczarek *et al.*, 2015). *Rhizobium* spp., *Mesorhizobium* spp., *Agrobacterium and Bradyrhizobium* spp. were reported as EPS producers (Ribeiro and Burkert, 2016). Rhizobia also produce EPS for their cellular protection. It thought to play a role in protection against desiccation, toxic compounds, bacteriophages, osmotic stress and to permit adhesion to solid surfaces and biofilm formation. Castellane *et al*. (2014) evaluated exopolysaccharide production from *Rhizobium tropici*.

2.6. Isolation of Rhizobial Strains

The isolation mechanism for rhizobia strains is carried out by collecting nodule samples from the host legume or directly from soil sample, sterilizing them by using ethanol and sodium hypochlorite, followed by crushing it in sterile petri dish by using blunt tipped sterilized forceps and lastly streaking drop of the nodule suspension on the media (Namkeleja *et al*., 2016; Kaur, 2014). Potato Dextrose Agar (PDA), (Islam *et al*., 2015), or Yeast Extract Mannitol Agar (YEMA) and / or peptone glucose agar are used as growth media while bromo thymol blue (BTB) or Congo Red (CR) can be used as indicators to increase evidence (Namkeleja *et al*., 2016; Singh *et al*., 2013). However, yeast extract mannitol agar (YEM) medium is the major selective medium for isolation and identification of rhizobium species. Isolation of rhizobia is a very important process in many rhizobia related researches that helps to get strong strain for nitrogen fixation from existing ones and discovering new effective strains in terms of fixing N_2.

2.7. Phenotypic Characterization of Rhizobia

It is based on morphological and biochemical properties of the microorganism. Morphological study includes size, shape, margin, color, growth pattern and texture of colonies of cultured

rhizobia *(*Datta *et al*., 2015; Singh *et al*., 2013; Islam *et al*., 2015; Kaur, 2014*).* Likewise, biochemical characterization includes oxidase test, catalase test, citrate utilization, methyl red test, glucose peptone agar assay (GPA), nitrate reduction, detection of Ketolactose production, bromo thymol blue test (BTB), lactose assay (LA assay), KOH solubility test, BIOLOG test etc. (Singh *et al*., 2013; Islam *et al*., 2015; Kaur, 2014). A study conducted by Agrawal *et al.* (2012) indicated that Rhizobia grown on YEMA plates which are isolated from Soya bean and French bean both were found to be slow growers and larger colonies were observed in case of soya bean but medium sized colonies were found in French bean.

2.8. Molecular Characterization of Rhizobia

Since rhizobia are taxonomically very diverse, morphological and physiological characters did not reflect true evolutionary relationship. Efficient strain classification methods are needed to identify genotypes displaying superior nitrogen-fixation capacity and for discovery of several new bacterial phylogenetics that were previously unknown. Robust, accurate and highly specific molecular characterization tools are invented to identify and distinguish between bacterial genera, species and even strains (Ogutcu *et al*., 2009). Among the molecular techniques used in characterization of the bacteria includes; Multi Locus Sequence Analysis (MLSA), sequence analysis of 16S rDNA, 16S rRNA gene sequence analysis, DNA-DNA hybridization, Phylogenetic Analyses, DNA Sequencing, polymerase chain reaction for box elements (Box-PCR), amplified ribosomal DNA-restriction analysis (ARDRA) techniques, Random Amplified Polymorphic DNA (RAPD), PCR-RFLP analysis, SDS-PAGE analysis of the whole-cell proteins and so on (Namkeleja *et al*., 2016; Tan, 2014; Maatallah *et al*., 2002; Costa *et al*., 2014). Detection of nodulation (nod) genes and nitrogen fixation (nif) genes are also important to identify potential rhizobial isolates.

2.8.1. Nitrogen fixing genes

It's known that nitrogen fixing organisms use nitrogenase enzyme encoded by *nif* genes to fix atmospheric nitrogen to ammonia. About 20 nitrogen fixing (*nif*) genes work coordinately in the assembly of nitrogenase components (Poza-Carrión *et al.,* 2013). Nitrogenase consists of two protein components, the homodimeric Fe-protein, the structural subunits encoded by the

nifH gene, and the heterotetrameric MoFe-protein encoded by the structural genes nifD and nifK. It has been described that the majority of the *nif* genes are plasmid born in the rhizobia, but are also located on chromosome in some rhizobia such as *Bradyrhizobium* (Shamseldin, 2013). The *nifH* gene is selected as a nitrogen fixation marker.

2.8.2. Nodulation genes

The *nod* genes are unique to rhizobia and define the central functions required for plant invasion and host plant recognition. *nodA, nodB, nodC, and nod*D are the genes commonly found in all rhizobia and cooperatively work to be nodulation process effective. nodD is the sensor gene which recognizes flavonoids produced by plant roots and controls the expression of the other nod genes. Then the common *nodABC* genes synthesize lipo-chitooligosaccharides (sulfated and acylated beta-1,4-oligosaccharides of glucosamine) called Nod factors, involving in nodule formation (Cardinale *et al.,* 2008). *nodA* encodes an acyltransferase responsible for the attachment of a fatty acid group to the oligosaccharide backbone, while *nodC* encode an N-acetylglucosaminyl transferase (chitin synthase*).* The *nod* and *nif* genes can be located on transmissible elements such as plasmids in many rhizobial species or transposon-like elements in *Mesorhizobium* that makes DNA extraction challenging next to exopolysaccharides (Kaur, 2014). Several molecular works on nitrogen fixation and nodulation genes reported different amplified products of *nifH* and *nodA* genes containg different molecular weights. Some of the experimental results are depicted on the Table 2 below.

Table 2. Different sizes of nitrogen fixation and nodulation gene amplicons identified by different researchers.

Gene	*NifH*	*nodA*	*nodC*	Reference
Molecular weight of the amplicon	600 bp		800 bp	Laranjo *et al.* (2008)
	350 bp	—	—	McDonald *et al.* (2015)
	780bp	—	—	Singh *et al.* (2013)
	300-bp	—	—	Liu *et al.* (2017)
	300bp	200bp	—	Wedhastri *et al.* (2013)
	600-bp	650-bp	900 bp	Diouf *et al.* (2010)
	781 bp		500bp	Nahar *et al.* (2017)
	601 bp	666 bp	—	Haukka *et al.* (1998)
	—	645 bp	—	Ardley *et al.* (2013)

2.9. Polymerase Chain Reaction

The polymerase chain reaction, PCR, is a molecular biology technique to amplify a single or a few copies of a piece of DNA up to several orders of magnitude ($10^{11\text{-}12}$ copies) of a particular DNA sequence. This automated process bypasses the need to use bacteria for amplifying DNA. Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis who received the Nobel Prize and the Japan Prize for developing PCR. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications such as diagnosis of a growing number of diseases, forensics laboratories, in molecular cloning and to detect human genes, bacteria and viruses (Joshi and Deshpande, 2011). PCR procedure consists of a series of 20-40 repeated temperature changes called cycles. Thus steps are denaturation at 94 - 96 ^{o}C, primer annealing at 45 -60 ^{o}C (depending on the primer) and primer extension, usually, at 72 ^{o}C.

2.9.1. Degenerate primers and touch down (Step-down) PCR

A variant of PCR developed to increase specificity of the PCR reaction by avoiding mispriming and the production of nonspecific products is the touch down PCR (TD-PCR). The exact melting temperature (Tm) calculation is the main factor in PCR amplification as an incorrect estimation leads to inappropriate product formation (Mattick and Korbie, 2008). Tm is the temperature at which half of the DNA double strand is dissociated to become single stranded. The annealing temperature at the initial cycles is usually set 3-10°C above the theoretical Tm of the primers, while at the later cycles; it is a few degrees 3-5°C below the primer T_m. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles. Touchdown (TD) PCR is essential when the sequence of the primer might not match the target, if the sequence of the primer has been deduced from amino acid sequences (i.e. degenerate primers), when template DNA contains several closely related targets, or when the target DNA, used to design the primers, is of a different species (Green and Sambrook, 2018).

A degenerate primer is an oligonucleotide sequence produced by combing more unique sequences coding for a specific protein and after combination the degenerate primer contains

all the possible bases on parts of its sequence (Linhart and Shamir, 2005). Such primers are used to amplify when only the protein sequence of a gene is known and to isolate similar genes from a variety of species. Let say, these three sequences “TATCATCCC” “TACCATCCA” or “TACCACCCG” could able to code for one amino acid sequence. All can be combined to form one degenerate primer sequence (TAYCARCCN) based on IUPAC code (Iserte *et al.*, 2013). Increase in degeneracy causes unspecific annealing of the primers. However, degenerate primers increase chance of finding unknown divergent variants of a sequence.

3. MATERIALS AND METHODS

3.1 Description of the Study Area

The selected study sites were Mekelle, Korem (Ofla) and Tanqa Abergele which are found in the northern part of Ethiopia in Tigray Regional State. Fig. 1 shows the three selected sampling sites of the study area where the legumes were grown.

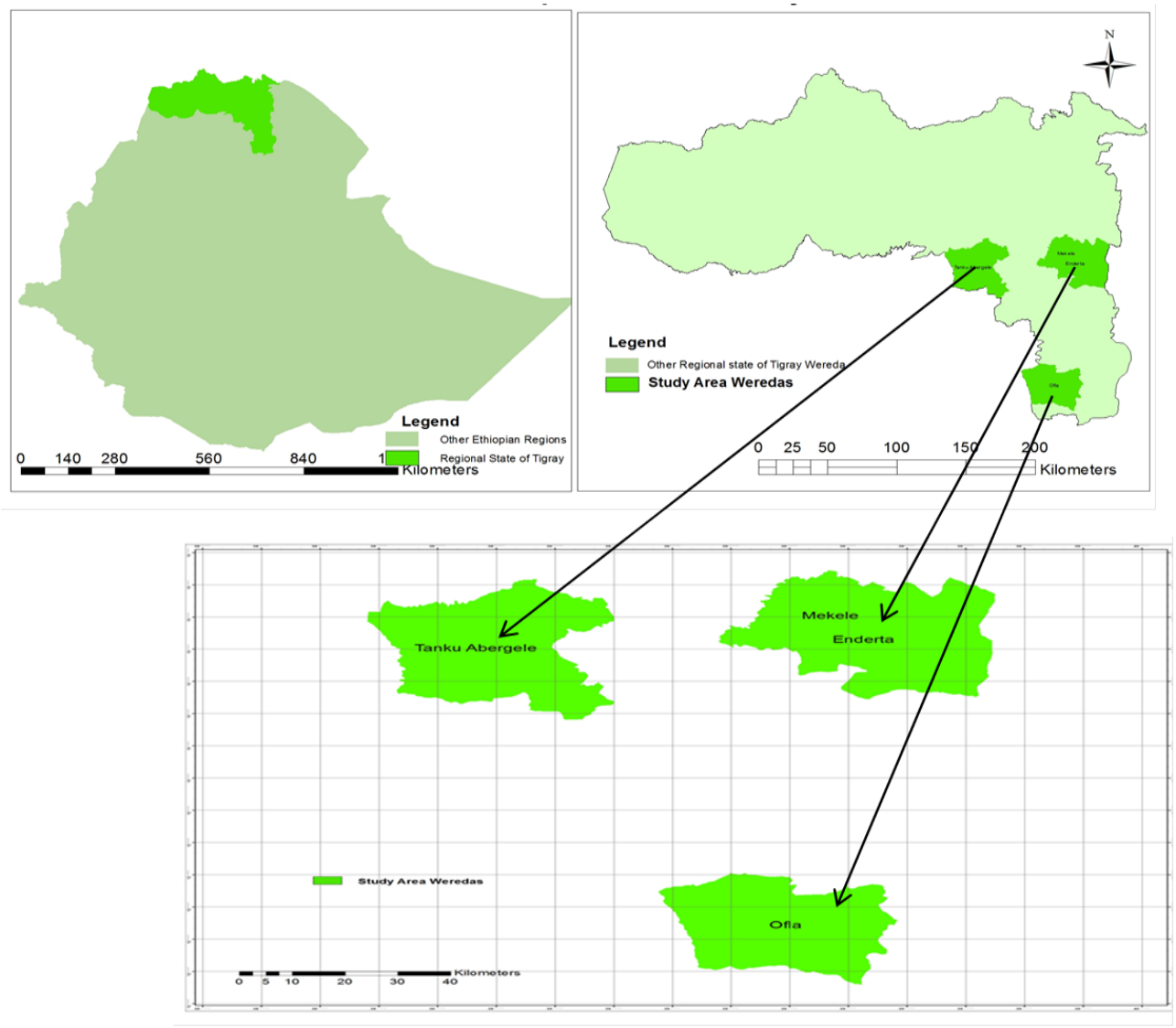


Figure 1. Map of Ethiopia and Tigray regional state showing sampling sites of Mekelle, Korem and Tanqua Abergele.

3.2. Research Design

The research was carried out using laboratory based experimental design. Faba bean samples were collected by systematic random sampling from Faba bean growing fields in three selected sites namely: Korem (Ofla), Mekelle and Tanqua abergele, of Tigray region which are representative of different geographical locations. The root nodules were collected on October, 2017. Tests were replicated at least 3 times to increase reliability.

3.3. Sample Collection

Faba bean (V*icia faba* L.) samples were collected from farmers' fields of three different geographical locations of Tigray regional state (Table 3). Those sampling sites were Mekelle, Korem (Ofla) and Tanqa abergele which were believed to be representatives of midland, highland and lowland respectively. Healthy representative samples from each area were randomly selected and carefully uprooted by excavating the surrounding soil, 15 centimeters (diameter) by 20 centimeters (depth). The clump of soil was carefully removed by hand and by dipping in running water. The shoots of the samples were removed and the roots were placed in alcohol sterilized plastic bags and then transported to biotechnology laboratory, Mekelle University. The samples were then kept at room temperature until isolation of rhizobia was performed.

Table 3. Total number of Faba bean samples and their corresponding sites.

No.	Sampling site	Isolate code	Total number of samples
1	Korem (Ofla)	Ko1- Ko10	10
2	Mekelle	Me1-Me10	10
3	Tanqua abergele	Ta1- Ta10	10
Grand total			30

Where, Ko= Korem, Me= Mekelle, Ta= Tanqua abergele

3.4. Isolation of Rhizobia

Five reddish and comparatively larger Faba bean (*Vicia faba* L.) nodules were removed from each plant using sterilized forceps. The nodules were thoroughly washed with water to remove adhering soil particles and rinsed in 95% alcohol for 30 sec. Then, nodules were immersed in H_2O_2 for 4 minutes and immediately washed five times with sterile water so as to remove traces of the chemicals. The nodules were transferred to glass Petri plates containing 0.5 ml of water and crushed with a glass rod until a milky bacterial suspension was obtained. A loop-full of the resulting suspension were then streaked on yeast extract mannitol agar (YEMA) (Yeast extract 1g/L, Mannitol 10g/L, Dipotassium phosphate 0.5g/L, Magnesium sulphate 0.2g/L, Sodium chloride 0.1g/L, Agar 20g/L, 6.8±0.2) and aerobically incubated for 24hrs at 28^{o}C. After 24 hrs of aerobic incubation, different morphotypes were selected visually and sub-cultured until the purity of the cultures was confirmed. The resulting pure cultures were kept in a refrigerator at +4°C for the next subsequent experiments (Kaur, 2014; Nigam and Xaxlo, 2016; Tan, 2014; Shoukry *et al*., 2013; Tariq *et al*., 2013; Tsegaye *et al*., 2015). Gram staining was performed according to standard Gram's procedure by preparing a thin smear using a loopful of the pure culture maintained on YEMA media to confirm that the rhizobial cells are pure and gram negative.

3.5. Biochemical Characterization of Rhizobia

3.5.1. Catalase test

For the ability of the isolates to produce catalase enzyme, catalase test was performed based on standard procedures (Kaur, 2014). A smear of the bacterial pure culture was prepared on a clean and dry micrcoscope slide. Then a drop of 3% H_2O_2 was added and mixed with the smear. Productions of gas bubbles, due to effervescence of oxygen on the bacterial smear, were considered as indicators of catalase positive isolates.

3.5.2. Oxidase test

For the ability of the isolates to produce oxidase enzyme, oxidase test was carried out using Kovac's oxidase reagent (1% dimethyl-p-phenylenediamine dihydrochloride). Aliquots of the

reagent were dropped on a filter paper and fresh bacterial cells from YMA agar plates were streaked up on the filter paper. The oxidase enzyme producing rhizobial isolates turned into lavender color which became dark purple to black within 1-3 minutes (Kaur, 2014).

3.5.3. Congo red dye absorption test

This test is used to distinguish Rhizobia from other contaminants like *Agrobacterium.* Yeast extract mannitol agar (YEMA) supplemented with Congo red (0.025 g/L) was prepared and inoculated with rhizobial cultures. CRYEMA plates were incubated at 28^{o}C for 48 hours. Generally, rhizobium strains do not show chromogenesis or they absorb the dye very weakly which is distinguishing character from other bacteria which absorb the dye strongly (Singha and Mazumder, 2013).

3.5.4. Ketolactose test

This test is used to differentiate rhizobia from highly contaminating *Agrobacterium* based on the ability of *Agrobacterium* to produce Ketolactase enzyme which converts Lactose to 3-ketolactose. Lactose medium was prepared and rhizobium cultures were streaked on the Petri plates. Plates were incubated for 48 hrs at 28 ^{0}C. After incubation, 5 ml of Benedict's reagent was poured into each petri plate and incubated at room temperature for one and half hrs. *Agrobacterium* colonies were surrounded by yellowish zone of copper di oxide, whereas no such yellow zone was developed around the rhizobial colonies (Kaur *et al*., 2012; Singh *et al*., 2013).

3.5.5. Glucose peptone agar assay (GPA)

Glucose peptone agar medium is widely used for isolating pure Rhizobial colonies and distinguishing them from other contaminants. Most rhizobia show no growth or very poor growth on glucose peptone agar medium. Observation of heavy bacterial growth is indicative of contamination. Glucose peptone agar (40g/L glucose, 5g/L peptone, 15g/L agar) medium was streaked with rhizobial isolates and incubated at 28^{0}C for 48 hrs and the growth of the bacteria was observed after 24 hrs (Nigam and Xaxlo, 2016; Singh *et al.,* 2013).

3.6. *In vitro* Characterization of Rhizobia as Plant Growth Promoters

The rhizobial isolates were also studied *in vitro* for plant growth promoting properties including phytohormones (indole-3-acetic acid) production, production of ammonia, solubilization of phosphate, hydrogen cyanide production, and exopolysaccharide (EPS) production. For each experiment, at least three replications and controls were used.

3.6.1. Production of indole-3- acetic acid (IAA)

Detection of IAA production by bacterial strains was carried out by growing cultures in liquid Luria-Bertani (LB) medium in a shaker incubator (140 rpm) at 30°C for 5 days (Mahbouba *et al*., 2013). Bacterial cells were recovered by centrifugation at 15000 rpm for 1 min. One ml of the supernatant was mixed carefully with 2 ml of Salkowski's reagent (1 ml of 0.5 M $FeCL_3$, 50 ml distilled water and 30 ml concentrated H_2SO_4) and incubated at room temperature in the dark for 30 min. Development of pink to red color is an indication of IAA production (Islam *et al.,* 2015).

3.6.2. Production of ammonia

The isolated rhizobial cells were colorimetrically tested for their capacity to produce ammonia in peptone water. Fresh culture of bacteria were inoculated in 10 ml of peptone water in test tubes and incubated for 72 hrs at 30^{o}C (Saengsanga, 2017). After 72 hours incubation, Nessler's reagent (0.5 ml) was added to the bacterial suspension to detect accumulation of ammonia. Development of faint yellow to brownish color indicated a positive test for ammonia production (Joseph *et al*., 2007; Mahbouba *et al*., 2013).

3.6.3. Hydrogen cyanide (HCN) production

Rhizobial isolates were tested qualitatively for hydrogen cyanide production using the method described by Pashapour *et al*. (2016) with some modifications. Wattman filter paper No.1 was sterilized by autoclaving and then soaked in sodium picrate solution (2% Na_2CO_3 in 0.5% picric acid solution) and dried in the safety cabinet. The bacterial isolates were streaked on Luria Bertani (LB) agar medium (Dürr, 2014) supplemented with 4.4 g/L glycine (Joseph *et al*., 2007) as a precursor of hydrogen cyanide biosynthesis in bacteria. The filter papers were

aseptically placed under the petriplate covers and plates were sealed with parafilim and incubated at 28^{o}C for 5 days. Uninoculated petriplate was incubated together with samples to serve as a negative control. Gaseous hydrogen cyanide (HCN) released by the bacteria was observed by color change on the filter paper. A color change of the filter paper from yellow color to orange, dark orange or reddish brown is indicator of hydrogen cyanide production.

3.6.4. Exopolysaccharide (EPS) production

Exopolysaccharide production was estimated according to Al-Mujahidy *et al*. (2013), who described the method, with minor modifications. Fresh bacterial cultures were inoculated into test tubes containing 10 ml of yeast extract mannitol (YEM) broth supplemented with 1% of glucose as a carbon source. The inoculated tubes were incubated at 30°C in a shaker incubator for 72 hrs. After incubation, the culture broth was centrifuged at 3500 × g for 30 min and the cell free supernatant was mixed with 96% cold ethanol at 1:3 (V/V) ratio of ethanol-supernatant to precipitate the exopolysaccharide (Castellane *et al*., 2014). The crude exopolysaccharide precipitated were collected by centrifugation at 3500 × g for 30 min. Then each sample was weighed after half an hour of drying at 50^{o}C to estimate the polysaccharide produced (Sayyed *et al*., 2011). The result was reported as micrograms of polysaccharide per milliliter of the medium.

3.6.5. Phosphate solubilization ability

Phosphate solubilization ability of the isolates was assessed on Basal Sperber Agar medium containing insoluble tri-calcium phosphate (yeast extract 0.5 g/L, glucose 10 g/L, magnesium sulphate heptahydrated 0.25 g/L, calcium chloride 0.1 g/L, calcium phosphate 2.5 g/L, agar 10 g/L) (Hajjam *et al*., 2016). Rhizobial colonies were spot inoculated on the medium and incubated at 30 ^{o}C for five days. The tri-calcium phosphate solubilization was determined by the formation of clear halo zone around the bacterial colonies. Phosphate solubilization index and solubilization efficiency of the isolates were quantified according to Singh *et al*. (2014) using the following formulas.

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

$$\text{Solubilization Efficiency (\%SE)} = \left[\frac{\text{Solubilization diameter}}{\text{Colony diameter}}\right] \times 100$$

3.7. Molecular Characterization of Rhizobia

The biochemically confirmed rhizobial isolates were then selected and subjected to molecular techniques to characterize at genetic level.

3.7.1. Isolation and purification of genomic DNA

Genomic DNA of the isolates was extracted by chemical method of DNA extraction known as phenol-chloroform method as described by Dash (2016) with major modifications. The bacterial isolates were cultivated in YEM broth medium for 24hrs at 28^0C.

One mL of bacterial culture was pipetted into 1.5 ml microcentrifuge tube and pelleted by centrifugation at 10,000 rpm for 5 minutes and then the supernatant was discarded. The pellet was washed with normal saline twice with centrifugation (10,000 rpm, 5 min) in between. 200µl of lysis buffer was added to the cell pellet and vortexed for complete resuspension. Then 10 µl of each of the RNAse A and proteinase-K were added followed by incubation at 50 °C for 3 hrs in water bath. Equal volume of PCI (phenol: chloroform: isoamyl alcohol, 25:24:1) was added and gently vortexed for 30 sec and mixed by inverting tubes for 2-3 minutes. Then the samples were centrifuged at 12,000 rpm at 4°C for 15 minutes and the upper aqueous phase was carefully transferred to a new tube. Two volumes of 97% chilled ethanol was added and stored at -20°C up to one day for complete precipitation of the genetic material. The samples were then centrifuged at 12,000 rpm for 15min at 4°C to collect the DNA pellet. The supernatant was decanted and the pellets were washed with 70% ethanol. The supernatant was discarded and the DNA pellet was air dried at room temperature. The pellet was finally resuspended in 100 µl TE buffer and was allowed to dissolve and later stored at -20˚C for the next experimentation.

3.7.2. Amplification of *nifH* gene

The amplification of the *nifH* gene was completed using the degenerate primers (Table 4).The amplification was performed as stated by previous reports with minor modifications (Liu *et al*., 2017). Amplification was carried out in 25 µL reaction volume for each sample containg 5 µL of template DNA and the following ingredients of reaction mixture: 0.25 µL FIREPol ® DNA Polymerase, 2.5 µL 10x Buffer B or BD, 2.5 µL $MgCl_2$ (all from Solis BioDyne), 0.25 µL dNTP mix, 0.75 µL of each nifH primers and 13 µL of nuclease free water. After 20 µL of the master mix was transferred to each PCR tube, the corresponding DNA template (5 µL) was added to each tube. To amplify the gene, PCR contents were subjected to initial denaturation of (94°C, 5min), 40 cycles of denaturation (94°C, 1min), annealing (50°C, 1min), extension (72°C, 2min), and finally, stable extension for 10 min at 72°C. PCR products were stored at 4°C until separated by agarose gel electrophoresis.

Table 4. Primers for DNA amplification and respective target genes used in this study

Primer	Sequence 5'-3'	Target gene	Source
nifH f	TGYGAYCCNAARGCNGA	Nitrogen fixation (*nifH*)	Eurofins Genomics India Pvt. Ltd
nifH r	ADNGCCATCATYTCNCC		
NodA-F	TGCRGTGGAARNTRNNCTGGGAAA	Nodulation gene (*nodA*)	
NodA-R	GGNCCGTCRTCRAAWGTCARGTA		

Where, Y= (C or T); N= (any base); R= (A or G); D= (A or G or T); W= (A or T)

3.7.3. Amplification of *nodA* gene

The majority of nitrogen fixing rhizobia can also form nodules on their hosts (Liu *et al*., 2017). To identify the isolates whether they were potent in nodule formation, the *nodA* gene responsible for encoding Nod factors (used for nodule organogenesis) was amplified using NodA primers listed on Table 4. The formulation of the PCR components was similar to that used in *nifH* gene amplification. However, touch down PCR protocol was used for the

amplification of the *nod*A gene (Cardinale *et al*., 2008). The PCR programme was as follows: an initial denaturation step at 94°C for 5 min; 16 cycles at 94°C for 1 min, annealing temperatures decreasing from 60 to 49°C for 1 min (with 1°C decremental step), extension at 72°C for 1 min followed by 24 cycles of denaturation at 95°C for 1 min; annealing at 49°C for 1 min; extension at 72°C for 1 min and final extension at 72°C for 7 min.

3.7.4. Agarose gel electrophoresis

About 20 µl of PCR amplified products were mixed with 4 µl of 6x DNA Loading Dye Buffer Double Blue (from Solis BioDyne) and loaded into ethidium bromide stained 2% molten agarose gel. Amplicons were subjected to electrophoresis and visualized under UV illumination after separation. 100 bp DNA Ladder (from Solis BioDyne) was used as a marker.

3.8. Data Analysis

All experiments were done in triplicates and the data represent average values of three. Data from phosphate solubilization and exopolysaccharide production was analyzed using descriptive statistics.

4. RESULTS AND DISCUSSION

4.1. Isolation and Morphological Characteristics of Rhizobia

In this study, a total of ninety isolates were obtained from thirty samples of faba bean *(Vicia faba* L.) , collected from farmers' fields of three different geographical locations of Tigray regional state, during the early pod setting stage on October, 2017. During the culture process, colony morphology of the isolates was observed. Most of the isolates were found to have white, circular, translucent, raised, single and mucilaginous colony characteristics (Appendix 7). All the isolates were found to be gram negative and rod shaped under the light microscope having 1000X magnification (Data not shown). Researches viz. Abere (2010), Kaur *et al*. (2012), Singha and Mazumder (2013), Hamza and Alebejo,(2017), who isolate rhizobia from Faba bean *(Vicia faba* L.), soybean rhizosphere, root nodules of *Crotolaria junceae L*. and cowpea, Elephant and Lablab plants, respectively, reported similar observations of colony features of rhizobia species.

4.2. Biochemical Characterization of Rhizobia

This was the preliminary characterization of isolates to evidently isolate and purify them for the coming major tests of plant growth promoting substances and molecular characterizations.
In the catalase and oxidase tests, all the isolates were found to be positive as observed by the effervescence of the gas around the colonies and coloration of the filter paper strip respectively (Table 5). These results are in line with results of Kaur (2014) and Kaur *et al*. (2012) who found 100% catalase and oxidase positive rhizobial isolates.

Congo red dye absorption test is one of the biochemical methods used for the differentiation of rhizobia from its major contaminants, i.e. *Agrobacterium* species. This is because *Agrobacterium* species absorb the dye strongly and produce stained reddish colonies in contrast to the rhizobial colonies which do not absorb the dye and stand out white and translucent or poorly absorb it. Most of the isolates failed to absorb the Congo red dye when grown on YEMA medium supplemented with 0.025 g/L Congo red after incubation period of 48 hrs at 28°C. Deshwal and Chaubey (2014) also reported similar results by growing rhizobia on YEMA medium containg Congo red. However, in this study, some isolates19 (21.1%) were

observed which absorb the Congo red dye poorly and appeared as pale red colonies. In this regard, Abere (2010) found five faba bean isolates that were pigmented with the dye after incubation on CR-YEMA. In addition, Tsegaye *et al*. (2015) reported on his paper that three isolates from sixteen isolates showed slight Congo red absorption and resulted in light red colony appearance.

Glucose peptone agar (GPA) medium was also used to examine the growth of the rhizobia by using the glucose as an exclusive carbon source (Rai and Sen, 2015). This medium was used to purify rhizobial cultures from other contaminants so that rhizobia could not show growth or show very poor growth in contrast to the contaminants which show massive growth on this medium. The results generally showed that out of the total isolates, 52 (57.8%) showed either no growth or poor growth on GPA medium which meets the characteristics of rhizobia. However, the remaining 38 (42.2%) isolates showed massive growth on the medium (Table 5). This result, fortunately, is in agreement with the report of Singha and Mazumde (2013) who found 12 rhizobial isolates that indicated features of rhizobia and 2 other isolates that showed well growth on the same medium as described above. Similar results were also reported by Abera *et al*. (2016), Rai and Sen, (2015) and Tsegaye *et al*. (2015).

Keto-lactose test is also widely used by soil microbiologists to differentiate and purify rhizobia isolates from other contaminants especially *Agrobacterium* species. This happens because *Agrobacterium* cells produce ketolactase enzyme when grown on lactose medium and convert lactose to 3-ketolactose which in turn results in the formation of yellowish zone of Cu_2O around the colony after flooded with Benedict's reagent. In contrast, rhizobial colonies are not able to do so. Accordingly, the majority of the isolates 79 (87.8%) did not show yellow zone around the colonies and such negative ketolactose test confirmed the identity of the isolates as rhizobia. However, 11 (12.2%) of the tested isolates gave positive result for ketolactose test which showed coloration around the colonies after 30 minutes reaction with Benedict's reagent and this was not the characteristics of rhizobia. Singh *et al.* (2013) and Rai and Sen (2015) reported ketolactose negative isolates while working with rhizobium isolates from *Sesbania sesban* and French bean, respectively.

In general, the result was in close agreement with the earlier works carried out by Kaur *et al.* (2012) in rhizobia isolates from soybean rhizosphere and Sharma *et al.* (2010) from soybean nodules.

Table 5. Biochemical characterization of rhizobial isolates

Biochemical test	Reaction	% of isolates showing reaction
Grams reaction	Positive	0%
	Negative	100%
Catalase	Positive	100%
	Negative	0%
Oxidase	Positive	100%
	Negative	0%
CR test	Positive	21.1%
	Negative	78.9%
GPA test	Positive	42.2%
	Negative	57.8%
KL test	Positive	12.2%
	Negative	87.8%

CR= Congo red; GPA= glucose peptone agar; KL= ketolactose

4.3. *In vitro* Characterization of Rhizobia for Plant Growth Promoting Properties

A total of twenty seven isolates were selected based on their morphological and biochemical characteristics and were subjected to the *in vitro* determination of plant growth promoting properties such as production of IAA, ammonia production, hydrogen cyanide production, phosphate solubilization and exopolysaccharide production. The percentage of isolates producing such PGPPs is shown in (Figure 2).

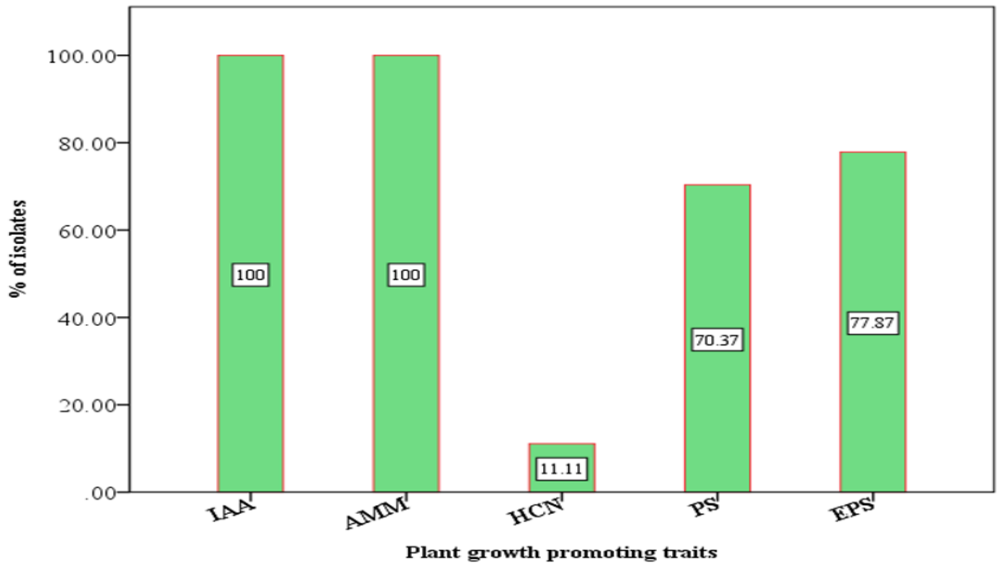


Figure 2. Percent distribution pattern of different plant growth promoting traits across tested rhizobial isolates

4.3.1. Indole-3- acetic acid (IAA), ammonia and hydrogen cyanide (HCN) production

Indole-3-acetic acid (*IAA*) is the main *auxin class in plants which plays important role in* cell elongation, cell division and differentiation, root initiation, phototropism as well as it serving as a signaling molecule necessary for growth and development of plant organs (Elzanaty *et al*., 2015). Rhizobia have the ability to produce high amount of IAA when free living and in symbiosis with their host plants. Although different colors were observed, all tested isolates showed positive reaction after treatment with Salkowiski reagent (Table 6). The color variation (from deep pink to pale pink) was an indicator of different isolates of rhizobia with different potentials to produce indole acetic acid. The results of this study were in agreement with previous studies that were reported by Elzanaty *et al*. (2015) and Shoukry *et al*. (2018). These researchers found that production of IAA by rhizobia varies among various species and even strains and depends on the nature and availability of substrates and culture conditions. Jida and Assefa (2012) also found auxin producing *Mesorhizobium* species isolated from chickpea (*Cicer arietinum* L.) and indicated that the bacteria's capability of releasing growth hormones and other plant growth promoting substances made them important to be used as bioinoculants.

Rhizobia also have been well studied not only for production of plant growth regulators but also ammonia and hydrogen cyanide production. Rhizobia are able to convert atmospheric nitrogen into ammonia and then taken by plants (Saengsanga, 2017). They can also produce low molecular weight molecules viz. HCN that can be used for antagonism against phytopathogenic microorganisms to protect their host plants. Hydrogen cyanide has the ability to inhibit electron transport of the pathogens and halt their energy supply so that growth inhibition may result (Gopalakrishnan *et al.,* 2015; Datta *et al.,* 2014). Development of yellow to brown color after addition of Nessler's reagent to the fully grown bacterial suspension in peptone water was considered as an indicator of ammonia production.

The results showed that almost all the tested isolates had great potential to produce ammonia (Table 6). Like phytohormone production, ammonia production was the most common characteristic of the isolates. This result was in line with results of Manasa *et al*. (2017) who tested 15 isolates of rhizobia and observed all were positive for ammonia production. Another work by Saharan and Verma (2015) also reported similar results during their work on rhizobia with other rhizospheric bacteria.

In contrast to the production of IAA and ammonia, hydrogen cyanide production was a very rare characteristic of the rhizobial isolates. Out of 27 isolates only 3 isolates (Table 6) were found to give strong positive reaction that changed the yellow color of the filter paper to reddish brown color indicating higher level of hydrogen cyanide gas production. Manasa *et al*. (2017) reported that more than half of the isolates they tested were found to be HCN producers with records of strong to weak HCN production. However, various studies reported that HCN production is not widely spread in rhizobia species when compared to other rhizobacteria like *Pseudomonas* spp. and *Bacillus* spp (Datta *et al.,* 2014; Verma *et al.,* 2010; Ahmad *et al.,* 2008). For instance, Ahmad *et al.* (2008) reported that *Mesorhizobium ciceri* was active for the production of direct plant growth promoting traits such as IAA and NH_3 production, phosphates solubilization rather than for the indirect plant growth promoting traits viz. siderophore production, antifungal activity and HCN production when compared with *Azotobacter* spp., *Fluorescent Pseudomonas* and *Bacillus* spp.

Table 6. Production of plant growth promoting substances

	Plant Growth Promoting Substances		
Isolate code	**IAA**	**AMM**	**HCN**
Ko1a	++	++	–
Ko1b	++	++	–
Ko1c	++	++	–
Ko3b	+	++	–
Ko5a	++	++	–
Ko5b	++	++	–
Ko6a	++	++	–
Ko6b	++	++	–
Ko10c	+	++	–
Me1a	++	++	–
Me2a	+	++	–
Me4b	+	++	–
Me6b	++	++	–
Me7b	+	++	–
Me10b	+	++	–
Ta4b	+	++	++
Ta4c	+	++	++
Ta5a	++	+	–
Ta5b	++	+	–
Ta7a	++	+	–
Ta7b	++	++	–
Ta8a	++	+	–
Ta8b	++	+	–
Ta8c	++	+	–
Ta9b	+	++	++
Ta9c	++	++	–
Ta10c	++	++	–

(++), strong reaction (+), weak reaction (–), negative reaction

4.3.5. Exopolysaccharide (EPS) production

Rhizobia species are known EPS producers to establish effective symbiosis with legumes because these EPS molecules, also called Nod factors, are important for initiating root hair curling and infection thread formation resulting in the formation of the nodules where nitrogen fixation takes place (Bomfet *et al*., 2011). It is also documented for other functions such as nutrient gathering, biofilm formation, protection against desiccation and environmental stresses (Janczarek *et al.*, 2015).

In the present study, of the twenty seven isolates tested, twenty one were exopolysaccharide producers as elucidated in Fig. 3. The other six isolates did not show EPS production. The existence of EPS-deficient strains of rhizobia was previously described by Janczarek *et al.* (2015). In the present study, in addition to the presence of such deficient isolates, significant variation was observed among the producers in extracellular polysaccharide production. Maximum amount of polysaccharide (1900 µg ml^{-1}) was produced by the isolate Ko1b followed by Ko10c (1850 µg ml^{-1}) and Ta8c (1750 µg ml^{-1}) when compared the mean value of the EPS produced in the medium. Whereas, up to 233 µg ml^{-1} (minimum value) of extracellular polysaccharide production was recorded for isolate Ta4b.

Similar observations were recorded by Nirmala *et al*. (2011) and Kucuk and Kivanc (2009) who reported that rhizobia species produced EPS of up to 1680 µg ml^{-1} and 1182 µg ml^{-1} respectively by growing the isolates on media supplemented with additional carbon sources. The findings of the present investigation are also in close agreement with the observations of Castellane *et al*. (2015) who concluded that the genus *Rhizobium* is a non-pathogenic potential source of polysaccharides.

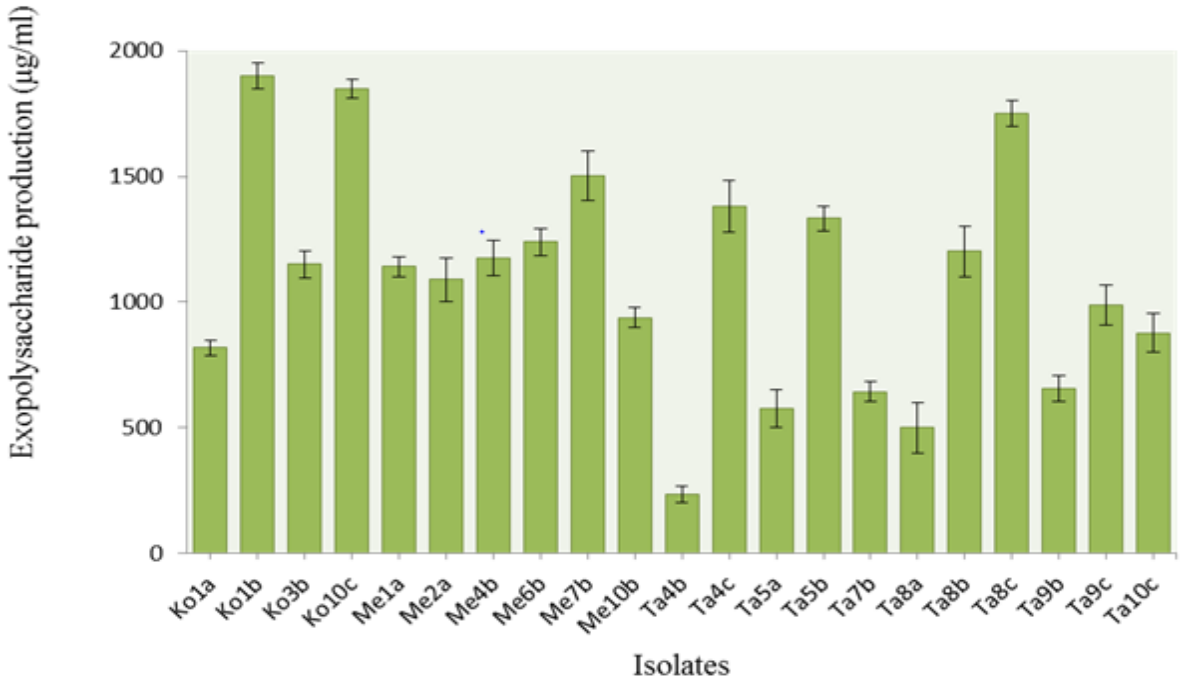


Figure 3. Exopolysaccharide production by different rhizobial isolates. Error bars are standard errors from three replicates per treatment.

4.3.4. Phosphate solubilization ability

Phosphorus (P), which is found in the form of insoluble organic and inorganic phosphates in the soil, is among the most essential macronutrients used for plant growth and development (Datta *et al.*, 2014). This, inaccessible form of phosphate by plants, must be converted into soluble forms like monobasic (H_2PO4^-) and dibasic (H_2PO4^{2-}) phosphates to be used by plants and this process is carried out by powerful phosphate solubilizing microorganisms like the rhizobia (Namkeleja *et al*., 2016; Gopalakrishnan *et al.*, 2015).

Out of the twenty seven rhizobial isolates tested for phosphate solubilization in this study, nineteen isolates produced clear halo zone surrounding their colonies after incubation on Basal Sperber Agar medium for five days indicated that they were inorganic phosphate solublizers. However, solubilization variation was observed among the nineteen isolates which showed solubilization index (SI) in the range of 1.13 to 2.33 as depicted in Table 7. These isolates might be effective phosphate solubilizes in the soil helping their host plants to grow well. Tan *et al.* (2014) reported almost similar results of rhizobial isolates with phosphate solubilization index values ranging from 1.31 to 2.51 upon incubation of the isolates from day two (D2) up to day ten (D10). A similar result was also recorded by Singh *et al.* (2014) while working on Chickpea rhizobia. The causes of the solubilization variation in rhizobia species and other microorganisms have been well investigated by other studies and found to be the result of differences is the amount of carbon sources in the medium or due to different diffusion rates of organic acids secreted by the organism when grown on agar plates (Hajjam *et al*., 2016).

Table **7**. Phosphate solubilizing ability of rhizobial isolates

Isolate code	Zone of Solubilization (mm)	Colony Diameter (mm)	Solubilization Index (SI)	Solubilization Efficiency (%)
Ko1a	9	4	2.25	125
Ko1c	10	3	3.33	233
Ko5a	9	4	2.25	125
Ko6a	10	5	2	100
Ko6b	11	7	1.57	57
Me1a	12	9	1.33	33
Me2a	14	12	1.17	17
Ta4b	13	5	2.6	160
Ta4c	9	5	1.80	85
Ta5a	6	5	1.20	20
Ta5b	5	3	1.66	66
Ta7a	9	8	1.13	13
Ta7b	5	3	1.66	66
Ta8a	6	4	1.50	50
Ta8b	11	4	2.75	175
Ta8c	10	4	2.50	150
Ta9b	12	6	2	100
Ta9c	7	3	2.33	133
Ta10c	7	3	2.33	133

4.4. Molecular Identification of Rhizobia

Molecular characterization of the rhizobial isolates collected from different faba bean growing areas of Tigray Region was done using primers (*nifH* and *nodA*) of specific nitrogen fixation and nodulation genes.

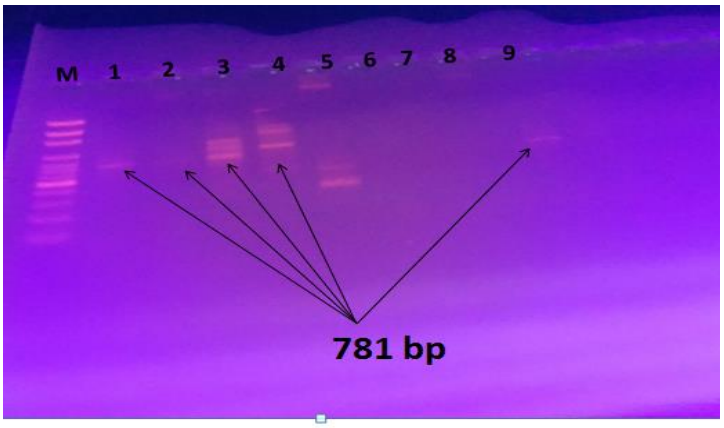
4.4.1. Amplification of *nifH* and *nodA* genes

The nitrogenase enzyme plays an essential role in the reduction of atmospheric dinitrogen (N_2) to ammonia (NH_3). This enzyme complex is encoded by various *nif* genes in rhizobia and other prokaryotes but these genes are absent in plant genomes. The *nifH* gene is one of the important genes in biological nitrogen fixation which encodes the nitrogenase reductase portion of the nitrogenase enzyme complex in nitrogen fixing organisms (Liu *et al.*, 2017; Singh *et al.*, 2013). In the present investigation, five isolates showed specific replicon with a molecular weight of approximately 781 bp confirming the presence of *nifH* gene in their

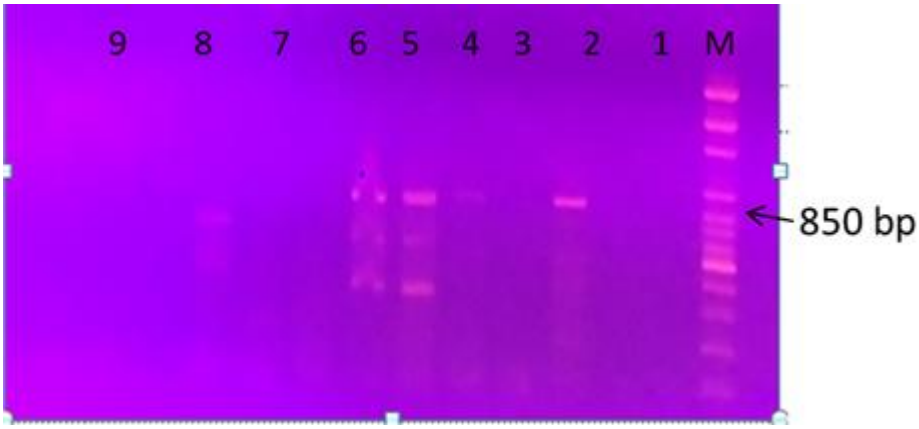
genome. These rhizobial isolates appeared the most important *nifH* gene containing genera with a potential of atmospheric nitrogen fixation (Fig 4).

These potential isolates of *rhizobial* spp. also showed positive amplification for *nodA* gene (Fig 4). But only two isolates (2 and 4 from the Fig. 4 which represent for isolates Ko1b and Ko3b) carry both nitrogen fixation and nodulation genes. For *nodA* gene amplification, an amplified product with a molecular weight of approximately 850bp was obtained. The results of *nifH* gene amplification were in agreement with the works of Singh *et al.* (2013) who reported similar *nifH* gene product of six *Rhizobium* strains from the root nodules of *Sesbania sesban* and confirmed that they had nitrogen fixing capability. In addition, Kaur (2014) reported 850bp approximate molecular weight of *nodA* gene fragment when he amplified the gene from *Rhizobium* genome using the same primers used in this study. *Rhizobium* nodulation gene NodA plays an essential role in the biosynthesis of Nod factors viz. lipochitin oligosaccharides.

When we observe the gel output, additional bands besides the expected bands were formed (Fig 4). This phenomenon was described to be appeared due to the use of degenerate primers, lower annealing temperatures and the use of many PCR cycles (>35). However, the presence of such extra bands never prevent from predicting the previously expected band (Wedhastri *et al.*, 2013).



(a)



(b)

Figure 4. Gene fragment amplification (a) nifH (b) nodA

5. SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary

Faba bean (*Vicia faba* L.) samples were collected from farmers' fields of three different geographical locations of Tigray Regional State. Rhizobia were isolated following standard experimental procedures. Isolates were biochemically characterized using catalase test, oxidase test, congo red test, GPA test and ketolactose test to purify and confirm the rhizobia. Isolates which scored positive in biochemical test were further tested for plant growth promoting traits like IAA production, ammonia production, EPS production, HCN production and phosphate solubilization. Finally, potential isolates were molecularly characterized for nitrogen fixation and nodulation.

White, circular, translucent, raised and mucilaginous colony characteristics were observed when rhizobial isolates grown on yeast extract mannitol agar (YEMA) plates aerobically. Although all isolates were positive for grams reaction, catalase and oxidase tests, they were effectively screened by CR test, GPA test and KLT test. Isolates that highly absorbed the congo red dye, isolates that showed massive growth on GPA medium and isolates that produced yellowish zone around the colonies were automatically discarded because such behaviors of the cells are considered as contaminants rather than rhizobia.

Twenty seven isolates, out of 90 primary isolates, were selected for PGPR characterization. The data on Indole-3- Acetic Acid (IAA) and Ammonia production showed that all isolates were IAA and ammonia producers. The different colors of the reaction showed the nature of the isolates that some isolates produce too high amount of IAA or ammonia and others less amount. Unlike IAA and ammonia, HCN production was not common characteristic among the rhizobial isolates. Only 3 isolates were able to generate HCN gas.

According to the data from exopolysaccharide production, the majority of the isolates were extra cellular polysaccharide producers and it is important for symbiosis and self-protection for rhizobia cells. Production variation among the isolates still exists and this indicates the presence of different species. Most of the isolates also showed phosphate solubilization ability when grown on Basal Sperber Agar medium for five days.

The *nifH* and *nodA* genes were amplified using respective primers to test isolates for nitrogen fixation and nodule formation. Bands from the gel revealed that *nifH* and *nodA* genes were clearly present in isolates Ko1b and Ko3b (2&4 from the Fig 4) together. Whereas, the other positive isolates carry either *nifH* gene or *nodA* gene. Both genes are not always present together in all rhizobial species. The results showed other extra bands besides the expected band but this cannot prevent from predicting the expected target band. Most experiments involving degenerate primers, lower annealing temperatures and the use of many PCR cycles (>35) usually lead to the production of other additional bands.

5.2. Conclusion

Generally, the *in vitro* assessment of plant growth promoting properties proved that the isolates are promising for plant growth and development. As a result, they can be used as a biofertilizer that makes them more important than chemical fertilizers in environmental protection and sufficient nutrient supply to the plants.

5.3. Recommendations

Isolation and characterization of promising rhizobial isolates *in vitro* condition may not effectively show the same result as *in vivo* condition. Therefore, this laboratory work must be supported by field trial on corresponding plants for large scale bioinoculant production and application.

Isolates must be studied for different environmental factors such as temperature, pH and salinity to develop inoculants as rhizobia are ecologically competitive.

Extensive research must be carried out in the genetics of rhizobia especially on the nitrogen fixation and nodulation genes as the genes have not commonly designed and highly specific primers.

Finally, the potential isolates should be identified at species level and beyond.

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7. APPENDICES

Appendix 1: Preparation of Media and Reagents

Isolation of rhizobia

Yeast Extract Mannitol Agar (YEMA) medium

Yeast extract (1g)
Mannitol (10 g)
Dipotassium phosphate (0.5 g)
Magnesium sulphate heptahydrated (0.2 g)
Sodium chloride (0.1 g)
Agar (20 g)
Distilled water (1000 ml)
pH (6.5 - 7.0).

Assessment of Plant Growth Promoting Properties of rhizobial isolates

Indole acetic acid (IAA) production test

Luria-Bertani medium

Casein enzymic hydrolysate (10 g)
Yeast extract (5 g)
Sodium chloride (10 g)
Distilled water (1000 ml)
pH (7.5)

Preparation of Salkowiski Reagent to test indole acetic acid

Developed by Sakowiski, 1885.
Used for colorimetric determination of of Indole-3- Acetic acid.
Contains 0.5 molar ferric chloride (0.5M $FeCl_3$), oxidizing acid (in this case I used 98% sulphuric acid, H_2SO_4) and distilled water in the proportion of 1:30:50 (V: V: V).
0.5M $FeCl_3$ was calculated as follows using molarity formula.

$$Molarity\ (M)\ of\ FeCl_3 = \frac{Number\ of\ mole\ of\ \mathrm{FeCl3}\ (\mathrm{n})}{volume\ of\ \mathrm{FeCl3}\ (\mathrm{L})}$$

$$\mathrm{n} = \frac{grams\ (g)\ of\ \mathrm{FeCl3}}{molecular\ weight\ (MW)\ of\ \mathrm{FeCl3}}$$

Grams of $FeCl_3$ = MW x M x V

Grams of interest were dissolved in required amount of distilled water and combined with concentrated sulphuric acid to obtain Salkowiski reagent.

Ammonium Production test

Peptone water

Peptic digest of animal tissue (10 g)
Sodium chloride (5 g)
Distilled water (1000 ml)
pH (7.2)

Nessler's reagent

For qualitative detection of ammonia production by rhizobia.

Hydrogen cyanide (HCN) production test

Luria-Bertani agar medium

Casein enzymic hydrolysate (10 g)
Yeast extract (5 g)
Sodium chloride (10 g)
Agar (15 g)
Distilled water (1000 ml)
pH (7.5)

L-glycine as supplementary

4.4 g/L
Used as precursor for HCN biosynthesis in bacteria.

Filter paper

Wattman filter paper number one (90 cm diameter).

Preparation of sodium picrate (2% Na_2CO_3 in 0.5% picric acid solution)

Used for colorimetric detection of HCN production.
2% Na_2CO_3 means 2 grams of sodium carbonate dissolved in 100 ml of solvent.
0.5% picric acid using dilution formula, $C_1V_1 = C_2V_2$, from the original solution (1.3% picric acid).
To prepare 100 ml of sodium picrate, 35.8 ml picric acid is required. 2 grams of Na_2CO_3 was weighed and dissolved in 35.8 ml picric acid and the beaker was filled with dH_2O until it reaches 100 ml.

Exopolysaccharide Production test

Yeast Extract Mannitol (YEM) broth

Yeast extract (1 g)
Mannitol (10 g)
Dipotassium phosphate (0.5 g)
Magnesium sulphate heptahydrated (0.2 g)
Sodium chloride (0.1 g)
Distilled water (1000 ml)
pH (6.5 - 7.0)

Chilled acetone

For precipitation of exopolysaccharide from the supernatant.

Phosphate solubilization test

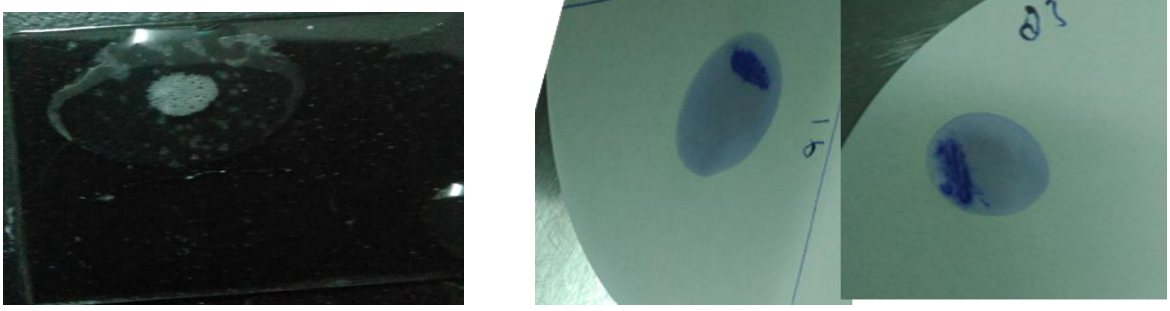
Basal Sperber Agar Medium

Yeast extract (0.5g)
Calcium chloride (0.1g)
Magnesium sulphate heptahydrated(0.25g)
Tri-calcium ortho- phosphate (2.5g)
Glucose (10 g)
Agar (10g)
Distilled water (1000ml)

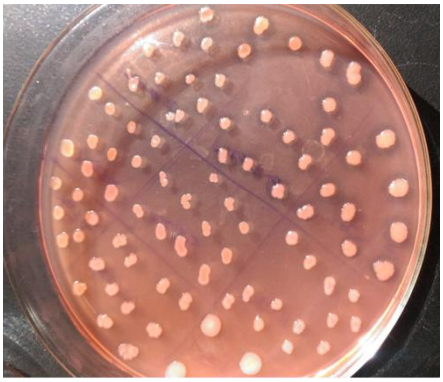
Appendix Figure 1. Sample collection from Faba bean (V*icia faba* L.)

Appendix Figure 2. Rhizobia growth on YEMA medium

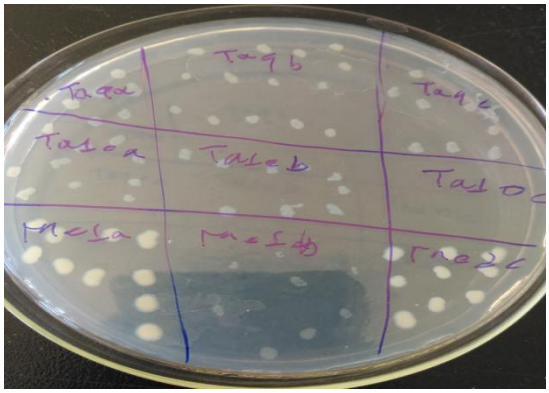
Appendix Figure 3. Biochemical Tests



Catalase

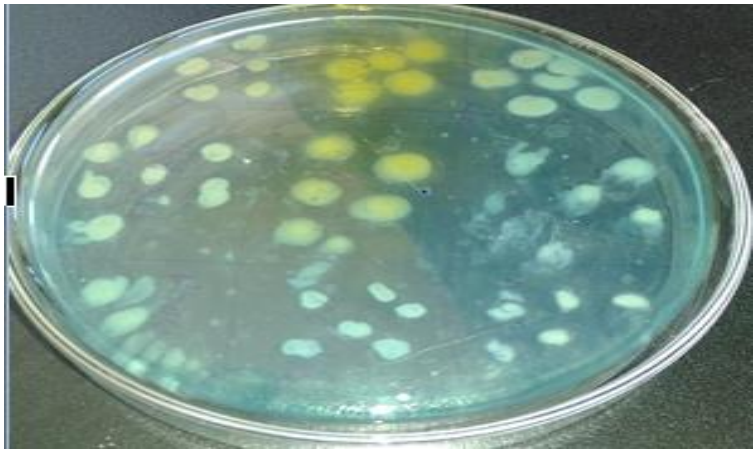


Oxidase



Congo red

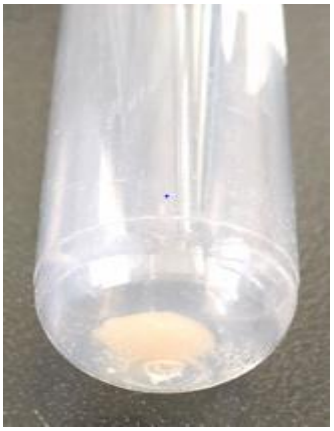
GPA



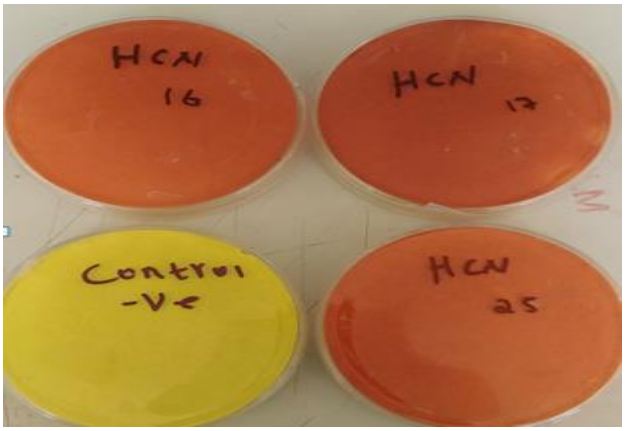
Ketolactose

Appendix Figure 4. Plant Growth Promoting Properties of rhizobial isolates

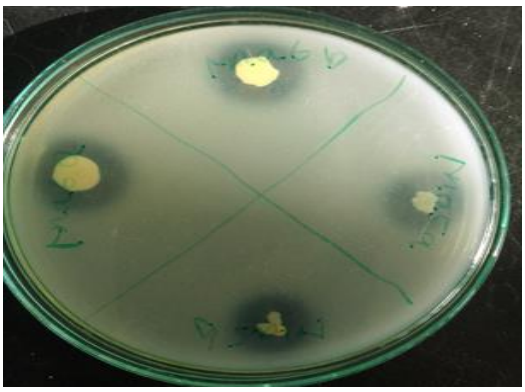
Indole acetic acid



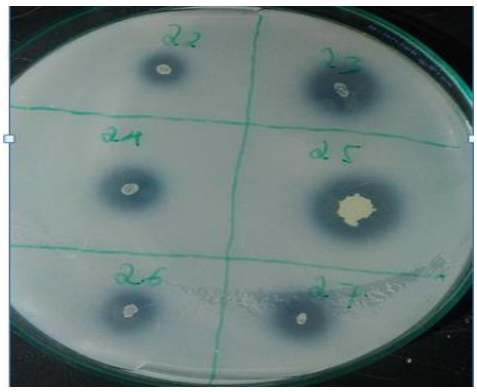
Ammonia



EPS



Hydrogen cyanide



Phosphate solubilization