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Isolation and Characterization of Rhizobia from Rhizospher and Root Nodule of Cowpea, Elephant and Lab Lab Plants

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Abstract: **Nitrogen is essential element for plant growth and development which is supplied by mutual symbiosis of rhizobia in cultivated legume plants. Biological nitrogen fixation could help to enhance agricultural productivity and ensure food security. This work aimed isolation and characterization of rhizobia from rhizospher and nodule samples collected from the study area. Isolation of rhizobium were undertaken using Yeast Extract Mannitol Agar medium. A total of 120 rhizobium were isolated from four samples. One isolate from each sample was selected for further characterization. Isolate LLsm1, CPsm1, CPnm1 and Esm1 were found to be negative for MR-VP and starch hydrolysis test. All isolates were found to be positive and negative for catalase and citrate utilization test respectively. They are also found to be Gram negative, rod shaped morphology, fast grower and indole producers. All the isolates were found with poor absorption of Congo red dye and no growth on the YEMA with 2% NaCl. Therefore, all isolates were confirmed as Rhizobia and plant growth promoting bacterial strains. These properties suggest that rhizobium isolated in this study could find potential application for development of the sustainable agriculture as to be a good candidate of biofertilizer which help in soil fertilization without applying chemical fertilizers.**

Keywords: **Rhizobium, Nitrogen Fixing, Bacteria, Isolates, Biofertilizer.**

1. INTRODUCTION

Microorganisms are living organisms, they are ubiquitous and live in familiar setting such as soil, water, food, and plant roots. Soil microorganisms constitute the world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. These microbial diversity significantly enhances the rates Carbon and Nitrogen cycle in the ecosystem [1]. They have a profound impact in every facet of human life. The beneficial microbes are fascinating, versatile and carry out extremely useful processes that can't be achieved by other physical and chemical means [2].

Biological nitrogen fixation is carried out by either symbiotic or free living prokaryotic, it is well documented that biological nitrogen fixation mediated by nitrogennase enzymes is a process important to the biological activity of soil. Soil microorganism that have capacity of fixing nitrogen have frequently been reported as plant growth promoters. A number of microorganisms such as Rhizobium , Blue-green algae (cyanobacteria), Azotobacter, Azospirillum and Clostridium can play significant role in agriculture as Nitrogen fixing microorganisms [3, 4].

Rhizobium are soil microorganism that can live on plant residues (saprophytes) or entirely within plants (endophytes) or (rhizo-bacteria) or in close association with the plant roots [5]. They play a central role in the Nitrogen supply of most soil ecosystems through their ability to fix Nitrogen in symbiosis with legumes. Based on ability to fix nitrogen, rhizobia are classified into slow (Bradyrhizobium) and fast growing rhizobia . The process in which the rhizobia colonize the

rhizosphere, infect the roots and fix nitrogen leads to plant development and grain yield improvement [6, 7]. The effectiveness of rhizobia populations in fixing nitrogen is correlated with soil fertility status where acidic soils have been reported to contain less effective rhizobia strains [8, 9, 10]. The following plants are common examples of legumes: clover, alfalfa, soy beans, cowpea, lab lab and chick peas. The breakdown of these legumes by bacteria during ammonification actually returns excess nitrogen not utilized by the plant to the surrounding soil [11, 12].

The major portion of biological nitrogen fixation is carried out by the symbiotic nitrogen fixers such as rhizobium, this have agricultural as well as ecological importance [13, 14]. Much of nitrogen provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health. Biological nitrogen fixation is the cheapest and environment friendly procedure in which rhizobia interacting with leguminous plants and fix aerobic nitrogen into soil [15]. It has been proven that the presence of rhizobia increases plant productivity without any harm to human health and environments. So for maintaining soil fertility, cultivation of leguminous plants is important which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non leguminous plants.

In fact, in Ethiopia, synthetic fertilizers gives short sustain high yield product, but it causes along term negative impact on the farm lands. In addition most farmers in Ethiopia are not aware about the use of native rhizobia inoculants as biofertilizers because the knowledge is not the first priority in agricultural production and even the rhizobia were not explored. Therefore a research project has been initiated with the objectives of isolation and characterization of Rhizobium from rhizospher and root nodules native to Ethiopia.

2. MATERIALS AND METHODS

2.1. Description of the Study Area:

Arba Minch city is found in southern Ethiopia, the first common name for this city was called Ganta Garo. It is located in Gamo Gofa Zone of the Southern Nations, Nationalities, and Peoples Region. About 454 kilometers south of Addis Ababa at an elevation of 1285 meters above sea level. The study will be conduct Arba Minch Agricultural center branch of Sheele Mele Lucy number 9 agricultural farm land ,which is18 Km from Arba Minch city in southern Ethiopia. It is well known for its cultivation of leguminous plants specially Cowpea, Elephant and Lablab plant.

2.2. Sample Collection:

Nodule and soil samples were collected from Arba minch Zuria woreda, at Arba Minch Agricultural center branch of Sheele Mele Lucy number 9 agricultural farm land. Each samples were kept in clean sterile sample bottles sealed and transferred to the microbial and industrial biotechnology laboratory and stored at 4^0C.

2.3. Surface Sterilization of Nodules:

Initially detached nodules were washed under running tap water to remove the adhering soil particles from nodule surface. Nodules were dipped in 0.1% of Mercuric Chloride ($HgCl_2$) solution for 30 seconds and later were washed successively ten times with sterilized distilled water to remove the traces of toxic $HgCl_2$, surface sterilized nodules were transferred in to test tube containing 5ml of sterilized distilled water. These nodules were crushed with the help of sterilized rod to obtain a milky suspension of bacteriods. These were streaked on Yeast Extract Mannitol Agar (YEMA) Media and further identify by gram's staining method [14].

2.4. Isolation of Nitrogen Fixing Bacteria (Rhizobium):

The soil samples were suspended in water by vigorous vortexing and serial dilutions were made up to 10^{-6} in sterile distilled water. 100µl of appropriate dilution were added to petri plate on YEM Agar plate containing Mannitol 10.00 g, $MgSO_47H_2O$ 0.20 g, NaCl 0.10 g, K_2HPO_4 0.50 g, $CaCl_2 \cdot 2 H_2O$ 0.20 g, $FeCl_3 \cdot 6 H_2O$ 0.01 g, yeast extract 1.00 g, agar20.00 g and distilled water 1000 ml) with the right calibration of pH (6.8-7) and incubated for 24 hrs at 32^0C. Bacterial culture was repeated for three times by single colony streaking on YEMA medium [14, 16]. The cultures were subsequently sub-cultured and used regularly. Agar slants were prepared and preserved at 7^0C for further experiments. Identification of the isolates were done by morphological & various biochemical methods.

2.5. Morphological Characterization:

The colony characteristics (i.e. shape, size, color, elevation, margin of the bacterial colony and their growth rate) were determined by observing the colonies on YEMA plates of the overnight grown microorganisms at 32°C. Microscopic observation of the Isolates was done using Gram staining technique.

2.6. Biochemical Characterization:

Isolates were characterized by different biochemical methods; methyl red test, VP test, Catalase test (cover slip method), starch hydrolysis test, citrate utilization test, and indole test.

Catalase activity test, the presence of the enzyme catalase in the rhizobial isolates was examined by suspending one loopful of organism in a drop of 3% Hydrogen peroxide on a glass slide. This test was performed as per standard procedure [14]. Production of bubbles indicates a positive result for catalase test.

Citrate utilization test, citrate utilization by the isolates was observed by the growth on slants of Simmon's Citrate Agar. A distinct change in color from green to blue refers to as a positive result for citrate utilization test.

2.7. Cultural and Metabolic Characterization:

Growth on 2% NaCl: To the basal medium of YEMA, 2% NaCl was added to check the growth of isolates. As 2% NaCl is inhibitory for some rhizobial isolates it may can serve as tools for identification of isolates.

Congo red test: The purity of the rhizobial isolates was detected by adding Congo red in YEMA media. Most rhizobia absorb the dye only weakly whereas contaminants including Agrobacteria, will absorb strongly.

Bromothymol blue media: Yeast Mannitol Agar (YMA) media incorporated with bromothymol blue was used to distinguish fast (acid producing) growing strains from slow (non acid producing or alkali producing) growing rhizobia [14]. In this medium, the fast growers require 48 hours to produce an acidic reaction by turning the color of the media yellow from green, whereas the slow growers take > 96 hours to produce alkaline endpoints with or without changing the color of the media from green to blue.

2.8. Spot Test for Assessment of Indole Production:

Spot test for indole Production was performed on growing Rhizobium strains on Trypton Soya Agar medium amended by adding glucose (10g), K_2HPO_4 (0.5g); $MgSO_4$.$7H_2O$ (0.2gm); NaCl (0.1g); yeast extract (1.0g) for 1L medium [17]. A Whatman Filter paper was placed on the bacterial growth on YEM medium. Then the filter papers were saturated with few drops of Salkawski reagent (1mL 0.5M $FeCL_3$, 50mL H_2SO_4). After two minutes, appearance of pink color was observed which was indicator of IAA production.

2.9. Data Analysis:

The data were analyzed using basic statistical parameters like table, percentage. In addition to this, Microsoft office Excel worksheet 2010 was used to construct tables and data presentation.

3. RESULTS

One hundred twenty Rhizobium were obtained from four sample areas 52, 52, 8 and 8 from Lab lab soil (LLsm), Cowpea soil (CPsm), Cowpea nodule (CPnm) and Elephant soil (Esm) respectively. Hence the strains were identified as Rhizobium (fig 1). Out of 120, 4 Nitrogen fixing bacterial isolates were selected one from each sample for further examination. The present study encompassed the isolation and characterization of nitrogen fixing (Rhizobia) bacterial strain from cowpea nodule and from rhizospher of cowpea, elephant, and lab lab plants. Isolation of nitrogen fixing bacteria was carried out on YEMA selective media and all samples (cowpea soil, lablab soil, and elephant soil and cowpea nodule) were grown well on it.

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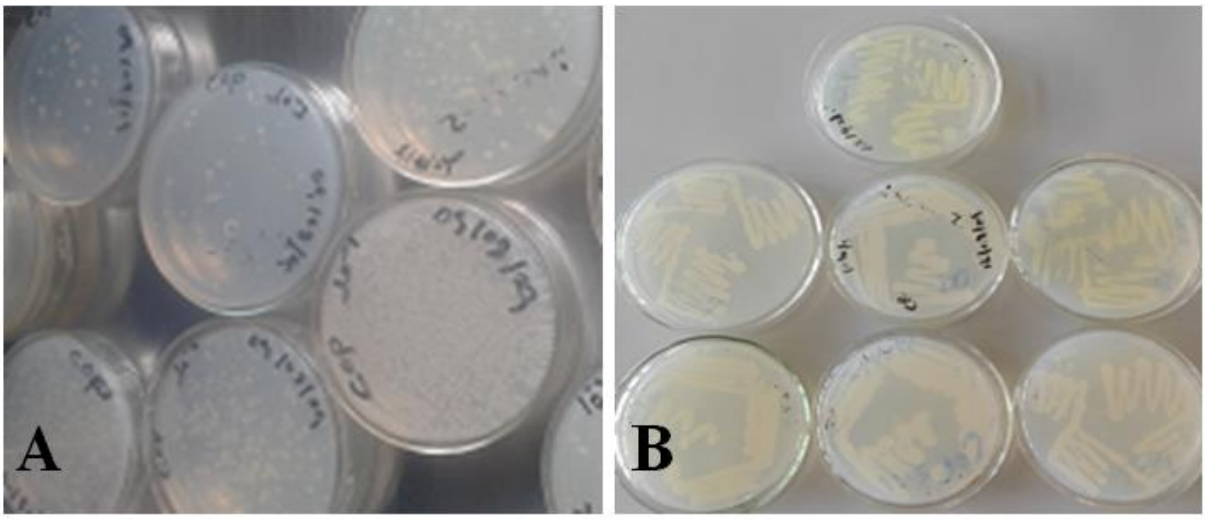

Figure 1.Growth of Rhizobium on YEMA medium; (A) Spread plate and (B) streak plate.

3.1. Morphological Characterization:

Colony morphology of the isolates were observed. Most of the isolates were found to produce, creamy white colony with round shape characteristics. The colonies were characterized as regular and circular configuration and regular margin. Motile and rod shaped cells were observed under light microscope with 1000X magnification. The strains was unable to hydrolyse starch. All the isolates were found to be Gram negative and rod shaped.

3.2. Biochemical Tests:

All isolates were tested for selective biochemical tests which are presented in table 1. In catalase test bubbles were formed and in citrate test no color change observed and it remained green. But all isolates (from nodule and rhizosphere) found to be positive and negative for catalase and citrate utilization test respectively.

Table 1: Results of biochemical tests of potential isolates

Isolates code	Color on Pure YEMA	Methyl Red test	VP test	Catalase test	Starch hydrolysis test	Citrate utilization test	Indole test
LLsm1	White	Negative	Negative	Positive	Negative	Negative	Positive
Esm1	White	Negative	Negative	Positive	Negative	Negative	Positive
CNsm1	White	Negative	Negative	Positive	Negative	Negative	Positive
CSsm1	White	Negative	Negative	Positive	Negative	Negative	Positive

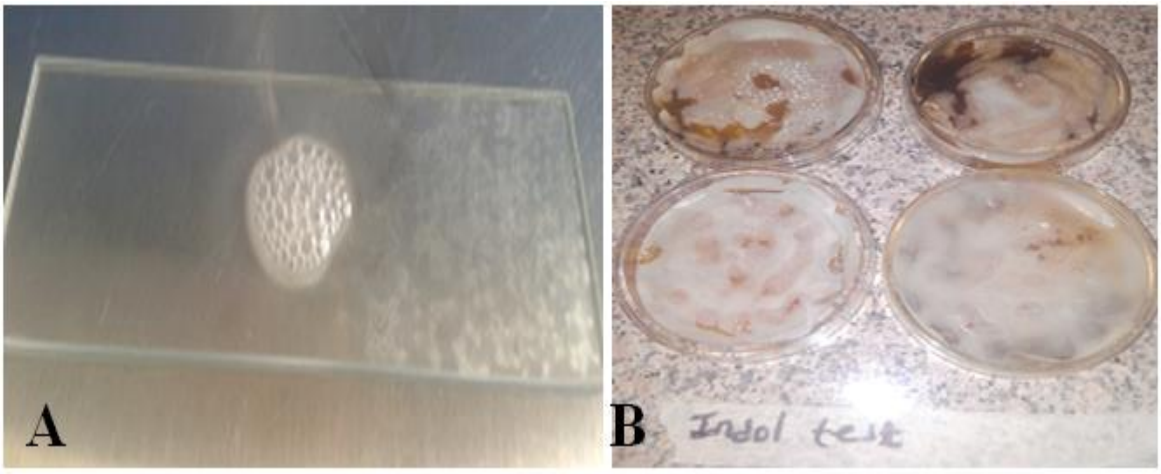

Figure 2. Results of biochemical tests of potential isolate: (A) Catalase test and (B) Indole test

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3.3. Cultural and Metabolic Characterization:

All isolates showed light pink on their growth on YEMA Congo red media since they could not absorbed the red color totally and all isolates grown on YEMA with bromothymol blue showed yellow color (changed the media from green to yellow), this indicate the production of acid by the bacterial isolates. In 2% NaCl no growth of the isolates was also observed. The result of cultural and metabolic characterization are summarized in table 2 and figure 3.

Table 2. Results of cultural and metabolic characterization of selected isolates

Isolate code	Growth on YEMA	Gram Staining	Cell Shape	Growth on 2% NaCl	Congo Red Test	Bromothymol blue Test	Genus of Isolate
LLs1	Yes	Negative	Rod	No Growth	Pink color	Yellow color	*Rhizobium*
Es1	Yes	Negative	Rod	No Growth	Pink color	Yellow color	*Rhizobium*
CPn1	Yes	Negative	Rod	No Growth	Pink color	Yellow color	*Rhizobium*
CPs1	Yes	Negative	Rod	No Growth	Pink color	Yellow color	*Rhizobium*

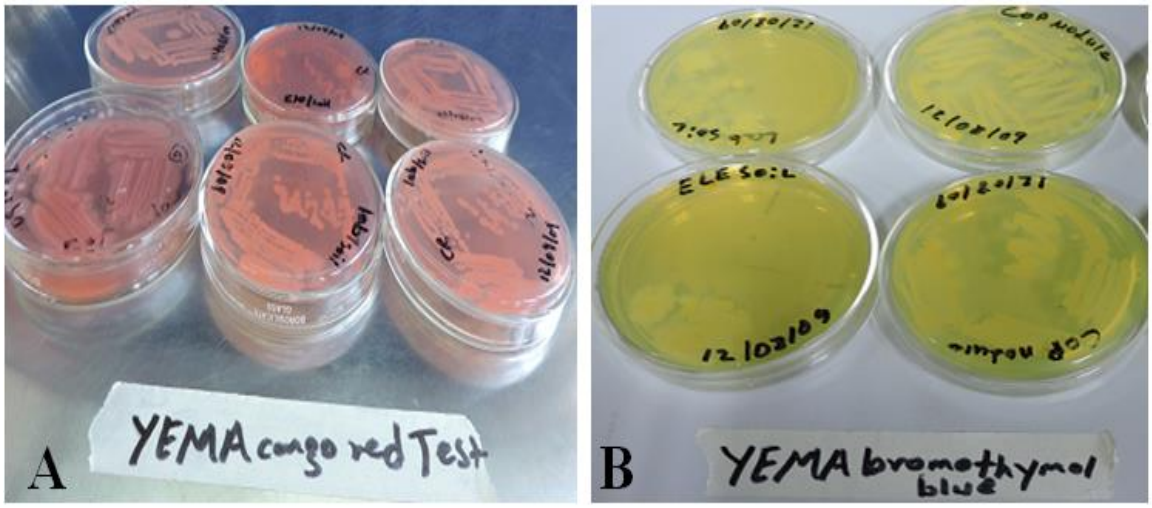

Figure 3. Growth of selected isolates at 32°C (A) on YEMA with Congo red and (B) on YEMA with Bromothymolblue

4. DISCUSSION

Isolation and identification of Nitrogen fixing (Rhizobia) bacteria which have vital ability to fix Nitrogen to the plant and to be used as biofertilizer is one of the main concerns of this study. Accordingly, 147 bacterial strains were isolated from study area. Growth of bacteria on Yeast Extract Mannitol Agar (YEMA) medium indicate their ability to fix atmospheric Nitrogen to the plants (Fig 1). The use of Yeast Extract Mannitol Agar (YEMA) medium for the isolation of Nitrogen fixing (Rhizobia) bacteria has earlier been reported by some workers [14, 17, 18]. As shown in result, soil collected from cowpea rhizosphere is rich in Nitrogen fixing bacteria. This indicates that soil collected from cowpea rhizosphere has potential for screening of Nitrogen fixing bacterial strain which produce industrially important Biofertilizers.

All the isolates (LLsm1, Esm1, CNsm1 and CSsm1) were showed circular, pin head type small sized colonies on Yeast Extract Mannitol Agar (YEMA). Results are almost similar with that investigated by some researchers [19, 20]. Such strains also showed that negative result for methyl red, VP, and starch hydrolysis test. Each isolates were found to be positive and negative for catalase and citrate utilization test respectively. Pervin *et al*, (2017) also reported catalase activity in some isolates of Rhizobium from nodules and rhizosphere were Catalase positive and citrate negative. So in catalase test bubbles were produced and in citrate test no color change observed and it remained green.

All the isolates from rhizosphere and nodules were acted as fast grower and produced acid in Bromothymol blue. In Congo red all the isolates showed pink colour or showed poor absorption of dye Cong red. This fact give further evidence for purity of the Rhizobial isolates [21]. All the isolates were showed no growth on the YEMA with 2% NaCl, thus confirming the rhizobia. Therefore, all isolates were confirmed as Rhizobia.

Minor amount of tryptophan was available in amended trypton soya agar media which is used to test indole production by nitrogen fixing bacteria isolated in this study. The appearance of pink color was the indicator of positive result of indole production. Positive result for isolate LLsm1, Esm1, CNsm1 and CSsm1 were observed in this study (Figure 2B and table 1). These strains may be considered as effective plant growth promoting bacterial strains. Similar observation has been reported by some researchers [17, 18].

Biological Nitrogen fixation play great role by diminishing input of the hazardous chemical fertilizer in the field and contribute to the development of the sustainable agriculture, which is necessary for the agriculture based, under developed country like Ethiopia. The present study is expected to reveal the isolation of rhizobium strains native to Ethiopia. This study showed the presence of nitrogen fixing bacteria on rhizosphere and root nodules of leguminous Plant. These properties suggest that nitrogen fixing bacteria isolated in this study could find potential for development of the sustainable agriculture as to be a good candidate of biofertilizer which help in soil fertilization without applying hazardous chemical fertilizers.

5. CONCLUSIONS

This study showed the presence of nitrogen fixing bacteria on rhizospere and root nodules of leguminous plant. Not only the leguminous plants but also the rhizosphere contains rhizobia which help in soil fertilization. It would have zero impact on the environment as compared to conventional chemical fertilizers. It was found that these nitrogen fixing strains along can substitute the chemical fertilizer. Therefore, isolation of efficient native nitrogen fixing rhizobia for Cow pea, Elephant, Lablab and produce them as inoculants to improve legume production in the country is important. Biological Nitrogen fixation is an important factor for the development of the sustainable agricultural system, especially it is necessary for the agriculture based under developed country like Ethiopia.

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