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Isolation and Characterization of Nitrogen Fixing Bacteria from Rhizospher Soil Collected from Shell Mele Agricultural Center, Southern Ethiopia

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ABSTRACT

The aim of this present study was the isolation and characterization of nitrogen-fixing bacteria from lab rhizospher and root nodule samples collected from Sheele Mele agricultural farmland, southern Ethiopia. Isolation of nitrogen-fixing bacteria was undertaken using Azotobacter and yeast extract mannitol agar medium. Accordingly, a total of 55 nitrogen-fixing microbes were isolated from lab rhizosphere and nodule samples. Further morphological and biochemical characterization was done for the selected isolates. All the selected isolates were found to be motile, gram-negative in spore test, positive in catalase test, negative in citrate utilization test, negative MR test, positive in VP test and positive in indole test. They have also rod-shaped cell. The colonies of LRR29 and LNR16 isolates are a creamy white colony and with poor absorption of Congo red dye when grown in Yeast Extract Mannitol Agar supplemented with Congo red. While the colony of LRA10 has milky white colour, circular configuration and raised elevation. These evidence help to confirm the identity of isolates, LRR29 and LNR16 belong to genus Rhizobium and also LRA10 belongs to genus Azotobacter. These potential isolates have plant growth promoting activity. Treatment of corn seeds with 10 ml microbial biomass produced from isolate LRA10 have enhanced the root length (up to 47%) and the shoot length (up to 42%) over water treated control. Further study is required to examine the impact of these bacterial isolates on the yield and seed quality before application as a biofertilizer.

Keywords: Nitrogen Fixing, Bacteria, Rhizosphere, Root Nodule, and Isolates.

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INTRODUCTION

Microbes can be considered as engineers of soils (Rajendhran and Gunasekaran, 2008) and many ecosystem services that are linked to terrestrial ecosystems, including plant production, safeguarding of drinking water or carbon sequestration, are closely linked to microbial activities and their functional traits (Torsvik and Ovreas, 2002). The soil matrix and physco-chemical properties of soil are the most crucial factor which determines the structure of microbial community (Lombard et al., 2011). In addition to that, the diversity of soil microbes is huge and can still be considered as a black box (Simon and Daniel, 2011). The beneficial microbes are fascinating, versatile and capable of growing on a wide range of substrates and carry out extremely useful processes that cannot be achieved by other physical and chemical means. Moreover, microorganisms are able to degrade many substances to a remarkable spectrum of products that support the growth of living organisms and fulfill numerous human requirements. These all are not a result of a single organism but of microbial communities which closely interact with each other (Aneja et al., 2006). Nitrogen is an essential element for plant growth and development and a key issue of agriculture. Most studies indicate that nitrogen fertilizers contribute to resolving the challenge the world is facing, feeding the human population. High yield production of agriculture was accompanied by an enormous increase in the application of nitrogen fertilizer.

There is, however, a high heterogeneity of its distribution throughout the world: some areas subjected to pollution whereas others to depleted soil, decreased crop production, and other consequences of inadequate supply. Nitrogen (N_2) is the most important element in all form of living things since many organic compounds which have an essential role in the structural and functional processes of life is derived from nitrogen.

Nitrogen enters living organism via nitrogen fixation (Egamberdieva and Kucharova, 2008; Berman-Frank et al., 2003). This is accompanied by microbial processes, these microbes may be symbiotic or free-living in nature (Reghuvaran et al., 2012). The process of biological nitrogen fixation is mediated by nitrogenase enzymes and the process of nitrogen fixation is dependent on certain parameters like moisture conditions, amount of oxygen, the supply of organic substrates, and genotypes of microorganisms and plant (Church et al., 2008). Nitrogen-fixing organisms are generally active in plant root zone soil. Plants that are capable of releasing exudates exhibit higher nitrogen fixation activity in soil (Egamberdieva and Kucharova, 2008). Nitrogen-fixing free-living microorganisms have frequently been reported as plant growth promoters (Requena et al., 1997; González-López et al., 2005). Biological nitrogen fixation can be an important source of nitrogen for supporting aquatic primary productivity (Affourtit et al., 2001). Nitrogen is one of the most essential elements for all forms of life; a basic material for synthesizing proteins, nucleic acids, and other organic nitrogenous compounds. Unfortunately, no plant species are able to reduce atmospheric dinitrogen into ammonia and use it directly for its growth. It appears that only a number of prokaryotic microorganisms including bacteria and cyanobacteria have been shown to possess the ability to fix dinitrogen (Nghia and Gyurján, 1987). Bacteria known collectively as the ‘‘Rhizobia**’’** are famous for their ability to induce nodules on the roots (and occasionally, stems) of legume plants. Within these nodules, the differentiated, ‘‘bacteroid’’ forms fix atmospheric nitrogen and the resultant ammonia being used as a source of fixed nitrogen.

This symbiosis provides the bacteria with an exclusive niche and, in return, the plants obtain a personalized nitrogen source (Johnston et al., 2007). They occur in the so-called free-living forms, for example, aerobic Azotobacter, anaerobic Clostridia or in symbiosis with certain higher plants, for example, Rhizobia with legumes or Azolla Anabaena Azollae with Azolla. The potential for biological nitrogen fixation is increased greatly by the fact that there is a close relationship between plants and nitrogen Prokaryotes. Nitrogen-fixing prokaryotes are able to make ranges of useful associations with plants: from loose associations to intercellular symbioses. There exist associative symbioses in which nitrogen-fixing prokaryotes (for example, *Azospirillum*, *Azotobacter*, *Enterobacter* species) have been found to occur in rhizosphere of different plants such as sugarcane, maize, wheat, rice, grasses and others (Affourtit et al., 2001). The activity of nitrogen-fixing microorganisms depends greatly upon excessive amount of carbon compounds and adequately low level of combined nitrogen (Johnston et al., 2007). Although synthetic fertilizers give short sustain high yield product, it causes long-term negative impact on the farmlands (Nghia and Gyurján, 1987). In Ethiopia, most of the farmers use chemical fertilizers since they have limited knowledge about the role of nitrogen-fixing bacteria. Moreover, these microbes were not explored and their inoculants were not found in the market. Therefore a research project has been initiated with the objectives of isolation and characterization of nitrogen-fixing bacteria native to Ethiopia from lab soil and root nodules.

MATERIALS AND METHODS

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. Soil samples were collected from Arba Minch agricultural center found in Sheele Mele, Lucy number 9 agricultural farmlands, which is 18 km from Arba Minch city in southern Ethiopia. It is well known for its cultivation of leguminous plants especially lab plant and so on.

Materials Used

The materials used during this investigation are conical flasks, pipette, petri dishes, test tubes, filter paper, aluminum foil, glass slides, pH meter, wire loop, incubator, autoclave, electronic weighing balance, microscope, beaker, glass rod, glass rod spreader, hot plate and laminar airflow cabinet (Hood).

Chemical and Reagent Used

Both nitrogen-free and nitrogen-containing media, iodine solution, distilled water, hydrogen peroxide, immersion oil, Salkawski reagent, yeast extract, water, gram staining reagents, glucose and mannitol, sodium hydroxide and hydrochloric acid.

Sample Collection

Rhizosphere soil and root nodule samples were collected from Arba Minch Zuria woreda, at Sheele Mele agricultural farmland. The soil samples were collected by the use of hand glove, spatula, and sterile polythene bag. At the collection sites, spatula was used to remove the

Table 1. Composition of azotobacter medium required for isolation of nitrogen fixing bacteria.

S.No	Name of the Compounds	Formula	Concentration (g /l)
1	Distilled Water	H_20	1000 ml
2	Glucose	$C_6H_{12}O_6$	20.00
3	Calcium carbonate	$CaCO_3$	20.00
4	Anhydrous magnesium sulphate	$MgSO_47H_2O$	0.50
5	Dipotassium phosphate	K_2HPO_4	1.00
6	Agar	$C_{14}H_{24}O_9$	15.0
7	Reference	Rosemary et al. (2013)	

Table 2. Composition of milk agar Medium required for isolation of protease producing bacteria.

S.No	Name of the Compounds	Formula	Concentration (g /l)
1	Distilled water	H_20	1000 ml
2	Mannitol	$C_6H_{14}O_6$	10.00
3	Anhydrous magnesium sulphate	$MgSO_47H_2O$	0.20
4	Sodium chloride	NaCl	0.10
5	Di-potassium hydrogen phosphate	K_2HPO_4	0.50
6	Anhydrous calcium chloride	$CaCl_2$ 2 H_2O	0.20
7	Anhydrous iron chloride	$FeCl_3$ 6 H_2O	0.01
8	Yeast extract	_	1.00
9	Agar	$C_{14}H_{24}O_9$	20
10	References	(Pervin et al., 2017; Gyorgy et al., 2010).	

overlying earth and sample collected from about 3 cm depth. Each sample was kept in clean sterile sample bottles sealed and transferred to the microbial and industrial biotechnology laboratory and stored at 7ºC.

Media Preparation

Both nitrogen-free and nitrogen-containing media were used for the isolation of nitrogen-fixing bacteria.

Azotobacter Medium

The components of the Azotobacter medium are presented in Table 1. Each of the components was measured with the weighing balance and put into a conical flask containing 1000 ml distilled water. The pH was adjusted to 7.4 using pH meter and batches of the medium were autoclaved at 121ºC for 15 min. After autoclaving, the medium was poured into petri dishes for solid agar medium.

Rhizobium Medium (Yeast Extract Mannitol Agar Medium)

The components of Rhizobium medium are described in Table 2. Each of the components was measured with weighing balance and put into a conical flask containing 1000 ml distilled water and then covered with aluminum foil. The pH of the medium was adjusted to 7 using pH meter after this process. The medium was autoclaved at a temperature of 121ºC for 15 min and allowed to cool and was poured into petri dishes. This solidified medium can be used for the enumeration of symbiotic Rhizobium microbes by using spread plate techniques.

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 sec 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 5 ml 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 identified by gram's staining method (Pervin et al., 2017).

Isolation of Nitrogen Fixing Bacteria

First the soil samples were grounded with sterile mortar and pestle to liberate the adhering microorganism before their suspension was prepared. One gram of the soil sample was weighed and dissolved in 9 ml of distilled

water in a test tube and homogenized by vigorous vortexing and subsequently, serial dilutions were made up to 10^{-6} in sterile distilled water. 0.1ml of appropriate dilution was pipetted and added to petri plate containing different media components which are described in Tables 1 and 2. A glass spreader, sterilized with alcohol and flame was used to spread the inoculums evenly on the plates. Then, the plates were incubated for 48 h at 32ºC. Purity was achieved by repeated sub-culturing consecutively by single colony streaking on nutrient agar plates (Pervin et al., 2017; Gyorgy et al., 2010). The purified bacterial cultures were maintained by inoculating into the slant culture medium in test tubes. The cultures were subsequently sub-cultured and used regularly. Agar slants were prepared and preserved at 7ºC for further experiments. Slant culture medium was inoculated with purified bacteria culture obtained by isolation and purification processes. Identification of the isolates was done by morphological and various biochemical methods.

Characterization of Selected Nitrogen Fixing Isolates

Microscopy was done by observing morphology and cultural characteristics of the isolates. Accordingly, the colony characteristics (that is, shape, size, color, elevation, and margin of the bacterial colony) were determined by observing the colonies on nutrient agar plates of the overnight grown microorganisms. Isolates were also characterized by different biochemical methods; methyl red test, VP test, Catalase test (coverslip method), starch hydrolysis test, citrate utilization test and indole test.

Gram Reaction

This was done to differentiate Gram-positive from Gram-negative one. Using a clean slide and sterile wire loop, a loopful of normal saline was dropped at the center of the slide and loop sterilized again, Specimen was collected with the wire loop. A smear was made in a circular manner. After making the smear, it was heat-fixed on the slide by passing it gently over the Bunsen flame. Crystal violet was put on the smear for 30 sec and poured off. Iodine solution was poured on and left for 1 min. It was then washed off with tap water. Then, acetone/ethanol was added for the decolonization purpose and subsequently, the acetone/ethanol was poured off and water poured immediately. Counterstaining using Safranin was done with water within a minute. After drying the slide surface observe through a compound microscope. Finally, immersion oil was added and viewed with x 100 oil immersion objective lens.

Motility Test

This was done to check the presence of locomotors structure. A drop of cultured organisms in a saline suspension was placed on the slide and covered with a coverslip. Then, it was viewed under the microscope. The sharp darting movement in different directions across the field of view of the microscope indicated positive result motility and showed that the organism had locomotive apparatus like flagella.

Spore Staining

This was done to identify the test organisms either spore producer or not. Heat-fixed smear of the isolates was heated to boiling with a mixture of 5% aqueous solution of malachite green and 0.5% safranin. Heat-fixed smear of the sample was made on clean slides. The slide was flooded with malachite green and heated from below with the Bunsen flame. Until when the flame was removed, steam was seen rising from the stain. The dye was allowed to act with the steam rising from it for a minute. The stain was washed off thoroughly with distilled water and the slides drained. The slides were also flooded with safranin and left to act for 30 sec. The slides were washed off with distilled water, drained and blotted dry with absorbent paper. When completely dried a drop of immersion oil was added and observed under the microscope which showed the pale green color of the spores if the isolate is spore producer.

Catalase Test

This test was undertaken to examine the presence of catalase enzyme in the tested organism. Catalase activity was examined by suspending one loopful of the organism in a drop of 3% hydrogen peroxide on a glass slide. This test was performed as per standard procedure (Pervin et al., 2017). Production of bubbles indicates a positive result for catalase test.

Citrate Utilization Test

This test was done to verify the test organism either utilize citrate as a source of carbon or not. It was done by incubating the test organism on Simmon's citrate agar slants. A distinct change in color from green to blue refers to as a positive result for citrate utilization test.

Starch Hydrolysis Test

This was done to prove the production of certain amylase enzymes from the test organism. It was done by incubating the test organism on a nutrient agar plate containing 1% starch for 48 h. After incubation, a drop of iodine solution was added and formation of clear zone indicates a positive result for starch hydrolysis test.

Congo Red Test

This was done to confirm the purity of rhizobial isolates.

Table 3. Morphological characterization of selected Isolates.

Isolate code	Medium used	Colony colour	Colony configuration	Colony margin	Elevation	Gram staining	Cell shape	Probable genus
LRR29	YEMA	Creamy White	Circular	Regular	Flat	Negative	Rod	*Rhizobium*
LNR16	YEMA	Creamy White	Circular	Regular	Flat	Negative	Rod	*Rhizobium*
LRA10	Azotobacter	Milky White	Circular	Regular	Raised	Negative	Rod	*Azotobacter*

The purity of the rhizobial isolates was detected by adding Congo red in YEMA media. Most rhizobia absorb the dye only weakly whereas others related bacteria will absorb strongly.

Spot Test for Assessment of Indole Production

Spot test for indole production was performed by growing selected isolates on Tryptone Soya Agar medium amended by adding glucose (10 g); K_2HPO_4 (0.5 g); $MgSO_4$.$7H_2O$ (0.2 gm); NaCl (0.1 g); yeast extract (1.0 g) for 1L medium (Mujahidy et al., 2013). A Whatman filter paper was placed on the bacterial growth. Then the filter papers were saturated with few drops of Salkawski reagent (1mL 0.5 M $FeCL_3$, 50 mL H_2SO_4). After two minutes, the appearance of pink color was observed which was an indicator of IAA production.

The Effect of Microbial Biomass on Plant Growth

To investigate the impact of selected isolates on the growth of corn plant, the inoculums were prepared by incubating each isolate in a liquid medium in a rotary shaker for three days. The microbial density (cell population) was measured by a spectrophotometer. Then, 10 ml of microbial biomass from each isolate was inoculated in the pot which contains sterilized soil for the growth of corn plant seed. Plant parameters like the length of root and shoot were used to assess the impact of selected isolates on the growth of the plant. A pot treated with 10ml pure water without microbial biomass was used as a control.

Data Analysis

The data were analyzed using basic statistical parameters mean, standard deviation and percentage. In addition to this, Microsoft Office Excel worksheet 2010 was used to construct tables and data presentation.

RESULTS AND DISCUSSION

Fifty-five nitrogen-fixing bacteria were isolated from the study area. Twenty-nine and sixteen Rhizobium were isolated from lab rhizosphere (LRR) and lab root nodules (LNR) respectively, and 10 Azotobacter from lab rhizosphere (LRR). Hence, the strains were identified as Rhizobium and Azotobacter. Out of fifty-five, three Nitrogen fixing bacterial isolates were selected for further examination. Isolation of nitrogen-fixing bacteria was carried out on selective media known as Yeast Extract Mannitol Agar selective media and Azotobacter medium. One of the main concerns about this investigation is isolation and characterization of nitrogen-fixing bacteria from soil sample. The growth of bacteria on Yeast Extract Mannitol Agar and Azotobacter medium indicate their ability to fix atmospheric nitrogen without association or symbiotically to the plants. The use of Yeast Extract Mannitol Agar and Azotobacter medium for the isolation of nitrogen-fixing bacteria has earlier been reported by some researchers (Rosemary et al., 2013; Pervin et al., 2017; Mujahidy et al., 2013; Rajpoot and Panwar, 2013). This implies that soil collected around lab rhizosphere has the potential for screening of nitrogen-fixing bacterial strain to be used and produce biofertilizers particularly for countries depending on agriculture.

Characterization of Selected Isolates

Characterizations of selected isolates were done using morphological appearance and various biochemical tests. Colonial structures of the isolates were visualized observed through a microscope. Accordingly, isolates LRR29 and LNR16 showed creamy white color, a circular configuration, flat and small-sized colonies on YEMA. Results are almost similar with that investigated by some researchers (Arora et al., 2001; Deshwal et al., 2003; Somasegaran and Hoben, 1994; Temam and Alemayehu, 2017). Among selected isolates, the colony of LRA10 was found to have a milky white color, raised elevation and rod-shaped cell. This result is consistent with the previous report of Rosemary et al. (2013). All the isolates were found to be Gram-negative, motile and rod-shaped. Similar results were reported in the previous studies (Rosemary et al., 2013; Pervin et al., 2017). Morphological characteristics of selected isolates are presented in Table 3. The results of biochemical characteristics for selected isolates were presented in Table 4 and Figure 1. Isolates LRR29 and LNR16 were showed negative result for methyl red and starch hydrolysis test. Thus, they cannot produce amylase enzyme to hydrolyze the starch present in the medium. All selected isolates were found to be positive and negative for catalase and citrate utilization test, respectively, this is consistent with the previous study (Pervin et al., 2017).

The biochemical characteristics of the LRA10 isolate are

Table 4. Biochemical characterization of selected isolates.

Selected Isolates	Biochemical tests								
	Congo Red	Methyl Red	Motility	Endospore	VP	Catalase	Starch Hydrolysis	Citrate Utilization	Indole
LRR29	Pink	-Ve	+Ve	-Ve	+Ve	+Ve	-Ve	-Ve	+Ve
LNR16	Pink	-Ve	+Ve	-Ve	+Ve	+Ve	-Ve	-Ve	+Ve
LRA10	Red	-Ve	+Ve	-Ve	+Ve	+Ve	-Ve	-Ve	+Ve

(Key: - +Ve = Positive; -Ve = Negative).

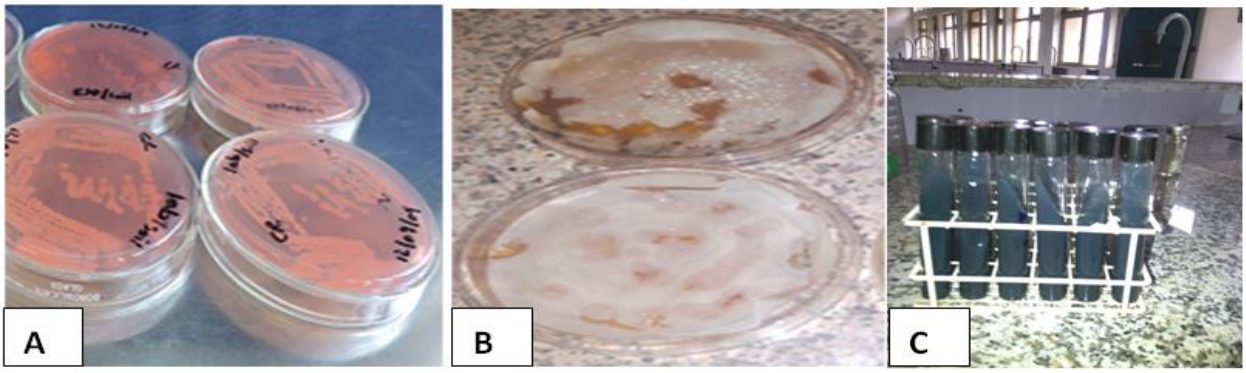

Figure 1. Result of biochemical tests of LRR29: (A) growth on YEMA with Congo red at 32°C (B) Positive indole test and (C) Negative citrate utilization test.

almost similar with that of LRR29 and LNR16 isolates (Table 4). In Congo red supplemented media, the entire isolates showed pink color except for LRA10 isolate. The appearance of this pink color is due to poor absorption of dye Cong red present in the medium, this indicates the purity of rhizobial isolates. The same results appeared in other studies (Rosemary et al., 2013; Pervin et al., 2017; Somasegaran and Hoben, 1994; Temam and Alemayehu, 2017). Based on the above morphological and biochemical characteristics, the isolates LRR29 and LNR16 were identified as Rhizobium Sp. whereas LRA10 isolate was found to be Azotobacter Sp. Amended tryptone soya agar media was used to investigate the production of indole by nitrogen-fixing bacteria isolated in the present study. When Salkawski reagent was added to the culture covered with Whatman filter paper, the appearance of pink color was the indicator of positive result of indole production. Accordingly, all the selected bacterial isolates were found to be positive for indole production test (Figure 1B and Table 4). Microbial isolate as inoculum known to be good plant growth promotors (Villarreal et al., 2003). These strains may be considered as effective plant growth promoting bacterial strains. A similar observation has been reported by some researchers (Mujahidy et al., 2013; Rajpoot and Panwar, 2013; Villarreal et al., 2003). The impact of selected isolates on the growth of plant was demonstrated by using simple pot experiments at the Garden of Arba Minch University, Abaya campus. The seeds of corn plant were treated with 10 ml of microbial biomass from each isolate in the pot containing sterilized soil.

The initial microbial density was determined using spectrophotometer which is described in Table 5. The entire container had similar treatment except for the control, it was treated with 10 ml of pure water instead of microbial biomass. After 15 Days, a pot treated with microbial biomass from LRA10 showed better results rather than two strains and control also. It has relatively good characteristics of plant parameters like root and soot length when compared to the other strains and control. In addition, broad leaf and thick stem were visualized. Results are shown in Table 5 and Figure 2. This is because LRA10 isolates can fix nitrogen without the requirement of any association since corn is non-leguminous plant and LRA10 belong to Azotobacter which is free nitrogen fixer. Similar results were reported by some researchers (Deshwal et al., 2003; Sadik et al., 2016). The microbial biomass produced from Rhizobia and Azotobacter bacteria isolated in the present study have plant growth promoting activity. Accordingly, treatment of corn seed with 10 ml of microbial biomass from LRA10 isolate have enhanced the root length up to 47% and the shoot length up to 42% over water treated control (Table 5).

This study is consistent with the previous reports (Rajpoot and Panwar, 2013; Ahmad et al., 2008). In the present study revealed that the treatment of corn plant seeds with Azotobacter promote growth (root and shoot length) more than Rhizobacteria. Nitrogen fixation by means of microorganism which exist naturally in the soil plays a great role by minimizing the input and effect of the hazardous chemical fertilizer in the field. In addition to

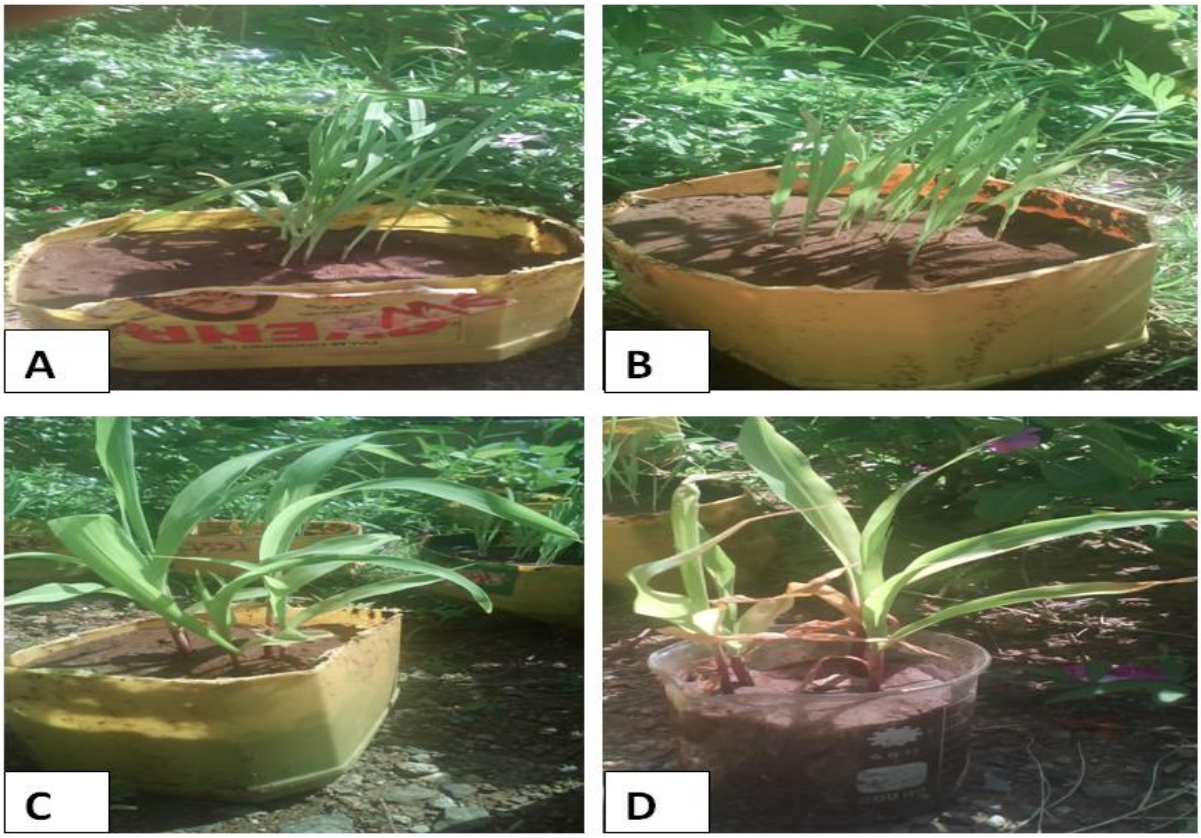

Figure 2. Sample picture: the effect of isolates on the growth of corn plant: (A) Control (Soil with 10 ml pure water (without microbial biomass); (B) Soil + 10ml microbial biomass from LNR16; (C) Soil with 10ml microbial biomass from LRA10; and (D) Soil with 10 ml microbial biomass from LRR29.

Table 5. Effect of selected isolates on the growth corn plant after 15 days.

Treatment		Root Length (cm)	Shoot Length (cm)	Root Dry Weight (gm)	Shoot Dry Weight (gm)
Strain	Optical Density				
LRR29	0.761	5.1±0.028	14.7±0.088	0.009±0.0068	0.026±0.003
LNR16	0.880	4.9±0.017	13.9±0.052	0.008±0.005	0.025±0.002
LRA10	0.668	5.6±0.031	15.8±0.140	0.014±0.007	0.028±0.006
Control	0.000	3.8±0.009	11.1±0.008	0.004±0.002	0.016±0.001

this, it has a positive impact on sustainable agriculture development particularly for countries like Ethiopia which largely rely on agriculture as a major source of the national economy. Considering this, the present investigation is expected to reveal isolation and characterization of nitrogen-fixing bacterial strains native to Ethiopia and the result showed that nitrogen-fixing bacteria are present on rhizosphere and root nodules of leguminous plant. A similar idea was reported in the earlier study (Pervin et al., 2017).

CONCLUSIONS

The result of the present study indicates that nitrogen-fixing bacteria are concentrated and are accumulated on rhizosphere and root nodules of leguminous plant. The genera Azotobacter and Rhizobium are present in the soil and these organisms were isolated using Azotobacter medium and YEMA medium, respectively. These bacteria were isolated and characterized in the present investigation based on their morphological and

biochemical properties. Bacteria isolated in the present study are found to have plant growth promoting characters, which enhance root and shoot length of plant. However, it requires further study particularly in the yield and seed quality to use this isolate as biofertilizer. This is more sensitive for developing countries like Ethiopia which rely on agriculture as the major sources of national economy.

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REFERENCES

Affourtit J, Zehr J and Paerl H (2001). Distribution of nitrogen-fixing microorganisms along the Neuse River Estuary, North Carolina. Microb. Ecol. 41(2):114-123.

Ahmad F, Ahmad I, Khan MS (2008). Screening of free living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res. 163:173-181.

Aneja MK, Sharma S, Fleischmann F, Stich S, Heller W, Bahnweg G, Schloter M (2006). Microbial colonization of beech and spruce litter—influence of decomposition site and plant litter species on the diversity of microbial community. Microbial Ecology. 52(1):127-135.

Arora NK, Kang SC and Maheshwari DK (2001). Isolation of siderophore producing strains of Rhizobium meliloti and their biocontrol potential against Macrophomina phaseolina that causes charcoal rot of groundnut. Curric. Sci. 81:673-677.

Berman-Frank I, Lundgren P and Falkowski P (2003). Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Res. Microbiol. 154(3):157-164.

Church MJ, et al. (2008). Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean. Limnol Oceanograph. 53(1):63-77.

Deshwal VK, Dubey RC and Maheshwari DK (2003). Solation of plant growth promoting strains of Bradyrhizobium Arachis sp. with biocontrol potential against Macrophomina phaseolina causing charcoal rot of peanut, Curric Sci. 84(3):443-448.

Egamberdieva D and Kucharova Z (2008). Cropping effects on microbial population and nitrogenase activity in saline arid soil. Turkish J. Biol. 32(2):85-90.

Gonzalez-Lopez J, et al. (2005). Liberation of amino acids by heterotrophic nitrogen fixing bacteria. Amino Acids. 28(4):363-367.

Gyorgy E, Mara G, Mathe I, Laslo E, Marialigeti K, Albert B, Oancea F and Lanyi S (2010). Characterization and diversity of the nitrogen fixing microbiota from a specific grassland habitat in the Ciuc Mountains. Romanian Biotechnol. Lett. 15 (4):5474-5481.

Johnston AW, Todd JD, Curson AR, Lei S, Nikolaidou-Katsaridou N, Gelfand MS, Rodionov DA (2007). Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium Rhizobium and other α-proteobacteria. Biometals. 20(3-4): 501-511.

Lombard N, Prestat E, van Elsas JD, Simonet P (2011). Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. FEMS microbiology ecology. 78 (1):31-49.

Mujahidy J, Hassan M, Rahman M and Rashid ANM (2013). Isolation and characterization of Rhizobium spp. and determination of their potency for growth factor production. Int. Res. J. Biotechnol. 4(7):117-123.

Nghia N and Gyurján I (1987). Problems and perspectives in establishment of nitrogen-fixing symbioses and endosymbioses. Endocytobiosis and Cell Res. 4(2):131-141.

Pervin S, Jannat B, Sanjee S and Farzana T (2017). Characterization of Rhizobia From Root Nodule And Rhizosphere Of Lablab Purpureus And Vigna Sinensis In Bangladesh. Turkish J. Agric. -Food Sci. Technol. 5(1):14-17.

Rajendhran J and Gunasekaran P (2008). Strategies for accessing soil metagenome for desired applications. Biotechnology advances. 26(6):576-590.

Rajpoot P and Panwar KS (2013). Isolation & Characterization of Rhizobia and their Effect on Vigna radiata Plant. Octa J. Biosci. 1(1):69-76.

Reghuvaran A, Kala KJ and Ravindranath AD (2012). Isolation and characterization of nitrogen fixing bacteria from raw coir pith. Afr. J. Biotechnol. 11(27):7063-7071.

Requena N, Baca T and Azcon R (1997). Evolution of humic substances from unripe compost during incubation with lignolytic or cellulolytic microorganisms and effects on the lettuce growth promotion mediated by Azotobacter chroococcum. Biol. Fertil. Soils. 24(1):59-65.

Rosemary CO, Gloria TO and Cecilia CI (2013). Isolation and Characterization of Nitrogen-Fixing Bacteria In The Soil. Int. J.Life Sci. Biotechnol. Pharma. Res. 2(3):438-445.

Sadik AS, Noof AE and Sonya HM (2016). Isolation and Characterization of Free-Nitrogen Fixer Bacterial Strains (Azotobacter Sp.) and Their Phages from Maize Rhizosphere Soil at TAIF. Pak. J. Biotechnol. 13(1):31-37.

Simon C and Daniel R (2011). Metagenomic analyses: past and future trends. Applied and environmental microbiology. 77(4): 1153-1161.

Somasegaran P and Hoben HJ (1994). Handbook for Rhzobia: Methods in Legume-Rhizobium technology, Springer-Verlag Publisher, New York. p. 450.

Temam AH and Alemayehu LA (2017). Isolation and Characterization of Rhizobia from Rhizospher and Root Nodule of Cowpea, Elephant and Lab Plants. Int. J. Novel Res. Interdiscipl. Stud. 4(4):1-7.

Torsvik V and Ovreas L (2002). Microbial diversity and function in soil: from genes to ecosystems. Current opinion in microbiology. 5(3): 240-245.

Villarreal SJ, Ilyina A, Mendez L, Torres V, Rodriguez R, Lopez B, Martinez J (2003). Isolation of microbial groups from a seaweed extract and comparison of their effects on a growth of pepper culture (Capsicum annuum L.). Aecth. Mock. 44:1.