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Isolation and Characterization of Nitrogen Fixing Bacteria from Rhizospher of Chickpea and Peanodules, Hossana, Ethiopia

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Authors' contributions

This work was carried out in collaboration among all authors. Author DSW designing study, performing analysis, writing the first draft of the manuscript; reading and approving the final manuscript. Authors MAB, EKE, RKN and GBB performing Lab activities, follow up the research, managing the analysis and searching all possible literatures, reading and approving the final manuscript. Author SSA designing variables, analyzing data and interpreting the result using SPSS software during the research study. All authors read and approved the final manuscript.

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ABSTRACT

One of the ways to increase the competitive survivability of rhizobial bio fertilizers and achieve better plant growth under such conditions is by modifying the rhizospheric environment or community by addition of non rhizobial nodule-associated bacteria (NAB) that cause better nodulation and plant growth when co-inoculated with rhizobia. To achieve this objective experimental design was carried out. Rhizobium is the root of legumes host nitrogen fixing bacteria which can invade root and get sugars from the plant. A study was performed to investigate the most commonly associated nodule-associated bacteria and the rhizospheric microorganisms associated with the legume plant. Present study on Isolation and characterization of Nitrogen Fixing Bacteria from agricultural rhizosphere was carried out with the aim of Isolation and characterization of Nitrogen Fixing Bacteria. The occurrence of Nitrogen Fixing Bacteria from root nodule of pea, white chickpea and red chickpea were investigated. Nitrogen fixing bacteria was isolated from root nodule of pea, white chickpea and red chickpea. The isolates were grown on yeast extract – mannitol agar (YEMA), and the morphologic characterization was done by Gram stain. A total of 21 isolates were isolated on Yeast Extract Mannitol (YEMA) agar medium. The isolates were circular, and irregular in form with smooth surface; entire, colony margins; with light pink coloured. The identification of isolated pure cultures through colony morphology analysis, cellular morphology and biochemical properties are including gram staining (negative), catalase test (negative), Methyl red test (negative), voges proskaeur test (positive), citrate utilization test (positive), starch hydrolysis test (positive) and mannitol salt agar (positive). Among the 21 bacterial isolates isolated, 18 were shown the characteristic of rhizobia. The effect of these 18 isolates those shows the characteristics of rhizobia were performed and all of them are significant, while compared with controls. These isolates will be useful to produce efficient bio fertilizers for Agriculture.

Keywords: Chickpea; isolation; nitrogen fixation; nodule associated bacteria; pea; rhizobia.

1. INTRODUCTION

1.1 Background of the Study

"Soil microorganisms constitute the world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. The activity of nitrogen-fixing microorganisms depends greatly upon excessive amount of carbon compounds and adequately low level of combined nitrogen" [1]. "Although synthetic fertilizers give short sustain high yield product, it causes long-term negative impact on the farmlands" [2].

"Nitrogen (N_2) is an element essential for the support of all forms of life. It is found in amino acids and proteins and many other organic compounds are derived from the nitrogen fixation process" [3]. "N_2 is the most abundant gas in Earth's atmosphere; it is extremely unreactive" (Egamberdieva, D. and Z. Kucharova., 2008.). "Nitrogen is the most limiting nutrient for growth of leguminous plants like Common beans, Soya beans, Cow peas and Garden peas because that present in the soil cannot support growth" [4]. "Biological nitrogen fixation is an important part of the microbial processes" [5]. "Nitrogen is a major limiting factor in agricultural production even if it represents almost 80% of the atmosphere" [3].

"The symbiotic interaction between two symbiotic components, legume and rhizobium is highly specific and is determined via signal changes between the crop and bacterium at different stages. At first, the host legume releases signal compounds (mainly flavonoids) within the rhizosphere and rhizobium reacts to these signals by producing combinations which are called nodulation factors. Nitrogen is one of the most important elements which limit crops production. Members of the Leguminosae form the largest plant family on Earth, with around 18,000 species. The success of legumes can largely be attributed to their ability to form a nitrogen-fixing symbiosis with specific bacteria known as rhizobia, manifested by the development of nodules on the plant roots in which the bacteria fix atmospheric nitrogen, a major contributor to the global nitrogen cycle. Then, nodulation factors are responsible for the growth of nodules in crops" [6]. "Rhizobium is the root of legumes host nitrogen fixing bacteria which can invade root and get sugars from the plant. In return, they convert large amounts of

dinitrogen (N2) from the atmosphere into forms that the plants can use" [7].

"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. Biological Nitrogen Fixation (BNF) is important in farming systems and can be ameliorated by simple and inexpensive inoculation procedures. Extended use of biological fertilizers would reduce the cost of chemical fertilizers ensuring that economic benefits accrue to the farmers while at the same time maintaining soil fertility and sustainability of agro-ecosystems" [8].

1.2 Statement of the Problem

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.

At present days, what encourages the developed countries to produce and use bio-fertilizers is their serious attention to environmental side effects resulting from the unbalanced and extreme use of chemical fertilizers. One of the most important ways to take advantage of useful interaction of microorganisms and to maintain diversity in agricultural ecosystems is to use useful soil microorganisms which are living in rhizosphere environment and are capable of improving plant nutrition and soil fertility through biological fixation of nitrogen. Chemical (Synthetic fertilizers) gives short sustain high yield product, but it causes a long term negative impact on the farm lands (soils) like increasing its acidity, erosion etc. So it can be eradicating by obtaining microbial products (bio fertilizer) as an alternative.

2. MATERIALS AND METHODS

2.1 Study Area and Period

The study was conducted during 2022 in the months between March- July. This study was carried out in Post graduate Microbiology laboratory of Wachemo University, Department of Biology, Hossana. The university is found in Hossana, Southern Ethiopia. Hossana town is located 230 kms far from Addis Ababa. The average annual temperature is 18.6 °C. The rainfall average is 1244mm.

2.2 Sample Collection and Preparation

Soil samples were collected from a soil where leguminous plants were grown with 10-15 cm depth (Hossana area). Seed samples (pea, red chickpea, and white chickpea) were bought from Hossana town market. Isolation of nitrogen fixing bacteria was performed from Rhizospher of White Chickpea, Red Chick pea and pea Nodule. The chickpea and pea was planted in green house. Chick pea and pea nodules were collected from the green house and kept in refrigerator at 4^{o}c until use.

Fig. 1. Root nodulation on pea

2.3 Media Preparation

Yeast extract mannitol agar (YEMA) media (mannitol 10.00 g, MgSO4 · 7H2O 0.20 g, NaCl0.10 g, K2HPO4 0.50 g, CaCl2 · 2 H2O 0.20 g, FeCl3 · 6 H2O 0.01 g, yeast extract 1.00 g, agar20.00 g and distilled water1000 ml) with the right calibration of pH (pH=6.8-7) was prepared. After autoclaved at 121^{o}c for 15 minute, it was dispensed into the Petri dish in the hood.

2.4 Preparation and Sterilization of Nodule

Initially, detached nodules were washed under running tap water to remove the adhering soil particles from nodule surface. Nodules were dipped in 5% of Sodium hypochlorite ($NAOCl_3$) solution for 1 minutes and later was washed successively ten times with sterilized distilled water to remove the traces of toxic $NaOCl_3$, surface sterilized nodules were transferred in test tube containing 5ml of sterilized distilled water. These nodules were crushed with the help of sterilized mortar and pestle to obtain a milky suspension of bacteriods. These were spreaded on Yeast Extract Mannitol Agar Media.

2.5 Isolation of Nitrogen Fixing Bacteria

The bacteria were isolated from Rhizospher of Chickpea and pea nodule at the dilution of 10^{-1} to 10^{-5} on YEMA media. These were spread on Yeast Extract Mannitol Agar Media. It was incubated for 24hrs at 32^0C. After the required bacteria was developed well bacterial culture was repeated by single colony streaking on YEMA medium. It was incubated again for 24hrs at 32^0C. Identification of the isolates were done by morphological & various biochemical methods.

2.6 Characterization of Nitrogen Fixing Bacteria

2.6.1 Morphological characterization

Isolates were characterized by cell (Gram stain) and colonies (shape, color, margin, texture,) were observed. The test isolates were grown on nutrient agar medium. After 48 hrs the colony shape, cell shape, colony color, margin of colony, surface of colony and texture of colony were recorded by Buchanan and Gibbons (1994).

2.6.2 Gram staining

Gram staining was done as the method provided by Beveridge [9]. Very small inoculum of each bacterial sample was smeared on clean glass slides using an inoculation loop. Samples were diluted with a drop of sterile distilled water, followed by heat fixing and air drying. The slides were stained with crystal violet solution for 1 minute. Then the slides were washed with tap water. Again the slides were stained with the iodine solution for 1 minute to fix the dye and rinsed with tap water. Then, the slides were decolorized with 95% ethanol until no more stain comes away and washed with tap water. These specimens were counter stained with the safranin solution for 1 minute and rinsed with tap water and air dried. Slides were examined under microscope after adding an oil immersion.

2.6.3 Biochemical characterization

Colonies morphologically confirmed colonies were grown in YEMA media were again confirmed by Biochemical characterization based on methyl red test, VP test, Catalase test (cover slip method), starch hydrolysis test, citrate utilization test and mannitol salt agar (MSA) test as the method given by Phyllis et al. [10].

2.6.4 Methyl red test

The MR-VP broth was prepared and 5 ml of the broth was poured into sterile test tube. The isolates were then inoculated separately into the tubes with MR-VP broth and were incubated at 32^oC for two days. After the incubation period, 5 ml of methyl red indicator was added to each tube.

2.6.5 Voges proskauer test (acetoin production)

The MR-VP was prepared. Five ml of the broth was poured into sterile test tubes. The isolates were then inoculated separately into the tubes and incubated at 32^oC for two days. After the incubation period, 5 ml of Barrits reagent A and B mixed together was added.

2.6.6 Starch hydrolysis test

In the starch hydrolysis test, the test bacteria were grown on agar plates containing starch.100ml starch media which contains (21gm nutrient agar and 0.2gm starch soluble) was prepared and it was dispensed in to Petri dish. The bacteria were cultured on the dispensed media on the hood and it was incubated at 32^oC for 48 hours. After colonies of the bacteria were visible, the plates were flooded with iodine solution.

2.6.7 Citrate utilization test

Simon's citrate agar medium was prepared and poured into the sterile test tubes. The rhizobial isolates were then inoculated separately into the test tube and incubated at 32^oC for 2days.

2.6.8 Catalase test

A clean slide was taken and a drop of test culture suspension was placed. Few drop of 3% hydrogen peroxide were then added to the culture.

2.6.9 Mannitol salt agar (MSA) test

MSA media contains Nutrient agar, Mannitol, Sodium Chloride and Phenol red indicator were prepared. The prepared media were dispensed on the plates immediately. The isolates were inoculated on MSA plate by loop using streak plate method and incubated for 24-48 hours.

2.7 Study Design

The study design was experimental study design. The experiment was carried out in Post graduate Microbiology Laboratory of Wachemo University. To observe the effect of bacteria isolates on Pea, white chickpea and red chickpea, each pea were planted on the pot in the green house and all isolated bacteria (18 isolates) were sprayed for each 3 plants separately. One control was planted for each 18 pots 0f plants. The shoot height, leaf number, and number of branch were measured and recorded after 10 days from planted day.

2.8 Data Analysis

All recorded data was subjected to analysis of variance using the SPSS statistical software version 26. Data was submitted to analysis of variance (ANOVA) and the means was compared by Turkeys' multiple range test ($P<0.05$) for significance analysis.

3. RESULTS AND DISCUSSION

3.1 Isolation of NFB

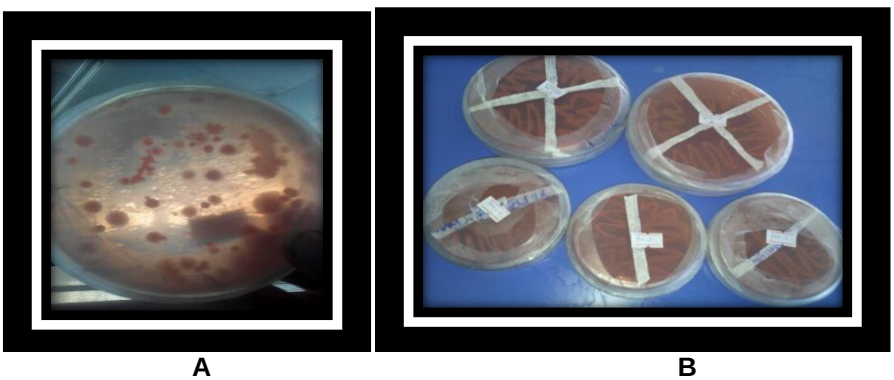
"The cooperative interaction between rhizobia and other plant root colonizing bacteria is of relevance in improvement of nodulation and N2 fixation in legume plants" [11]. The isolation procedure adopted involved through surface sterilization of nodules and was thus specifically aimed to eliminate nodule epiphytes. At present study root nodule associated bacteria were isolated from the nodules of pea, white chickpea, red chickpea plants from green house on the YEMA. The required bacteria were grown well on the media (Fig. 2a) and the grown colony were streaked and grown well. All isolates showed light pink on their growth on YEMA congored media (Fig. 2b). Colonies showing different morphological characteristics on the plates were selected for further characterization.

3.2 Colony, Morphology and Physiological Characteristics of the Isolates

"A study reported isolation of 114 bacterial isolates from 15 root nodules, of which nearly 60% were rhizobia while the remainder was identified as belonging to several other genera of which eight species were exclusively found only in root nodules" Sturz et al. [12]. In the present study we also had similar frequency wherein we isolate were characterized by cell (Gram stain), colony (shape, color**,** margin, surface of Colony and texture,) was observed (Table 1 and Fig. 2A and B). A total 21 were isolated with different morphological characteristics were studied. Among them18 isolates shown properties of Rhizobium Spp. Majority (85.7%) of the nodule associated bacteria (NAB) in this study belonged to gram negative isolates followed by 14.3% being gram positive.

3.3 Biochemical Characterization

Various biochemical tests performed on isolates for characterization according to Burgys Manual. Production of bubbles indicates a positive result for catalase test. All selected isolates were positive and negative for citrate utilization test and catalase, respectively (Fig. 5G and 3D), this is consistent with the previous study [13-17]. Each selected isolated were positive for starch hydrolysis (Fig. 5H) and Vp-test (Fig. 4F), this is consistent with the previous study (R.K. Suraj et al., 2013*).* Mannitol salt agar test for the bacteria's ability to tolerate mannitol salt agar media and ferment mannitol was performed and all isolates were grown well (Fig. 6I).



A **B**

Fig. 2. Colony characteristics which were grown on YEMA media for all isolates (A). Growth of all isolates streaked on YEMA media (B)

Table 1. Morphological characterization of bacterial isolates which was grown on YEMA media for all isolates from pea, white chickpea and red chickpea nodule

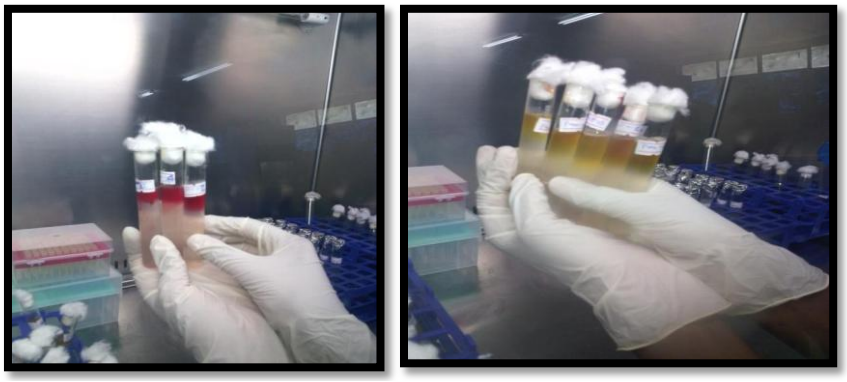
Isolates	Colony shape	Cell shape	Colony color	Margin of colony	Surface of colony	Texture of colony
Pea nodule	Circular	rod	Light pink	Entire	smooth	moist
white chickpea nodule	Circular	rod	Light pink	Entire	smooth	moist
red chickpea nodule	Circular	rod	Light pink	Entire	smooth	moist

Table 1. Biochemical characterization of bacterial isolate

Isolates	Gram staining	Methyl red test	Vp-test	Catalase test	Citrate utilization test	Starch hydrolysis test	SMA test
WCP4a	-	-	+	-	+	+	+
RCP2a	-	-	+	-	+	+	+
WCP2b	-	-	+	-	+	+	+
Pea3a	-	-	+	-	+	+	+
Pea1a	-	-	+	-	+	+	+
RCP2b	-	-	+	-	+	+	+
RCP4a	-	-	+	-	+	+	+
RCP4b	-	-	+	-	+	+	+
WCP4b	-	-	+	-	+	+	+
RCP5a	-	-	+	-	+	+	+
RCP5c	-	-	+	-	+	+	+
WCP4c	-	-	+	-	+	+	+
Pea4b	-	-	+	-	+	+	+
WCP2a	-	-	+	-	+	+	+
Pea3b	-	-	+	-	+	+	+
RCP5b	-	-	+	-	+	+	+
Pea2a	-	-	+	-	+	+	+
Pea4a	-	-	+	-	+	+	+
WCP2c	-	+					
WCP3a	-	+					
Pea5a	-	+					

C D

Fig. 3. Gram staining; the viscosity was formed on 3%KOH for all isolates(C) and Catalase test; the babble is not formed on 3% H2O2 for all isolates (D)

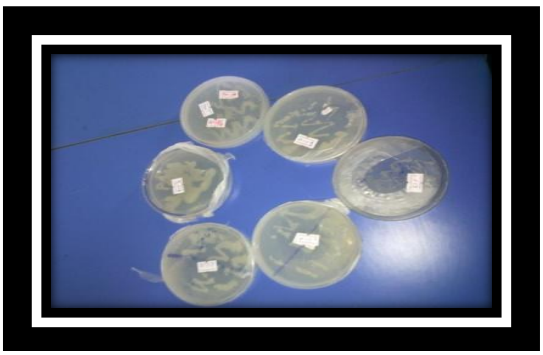


E F

Fig. 4. Result observed after incubation of inoculated isolate for methyl red test (E) and Result observed after incubation of inoculated isolate for voges – prokauser test on phosphate glucose broth (F)

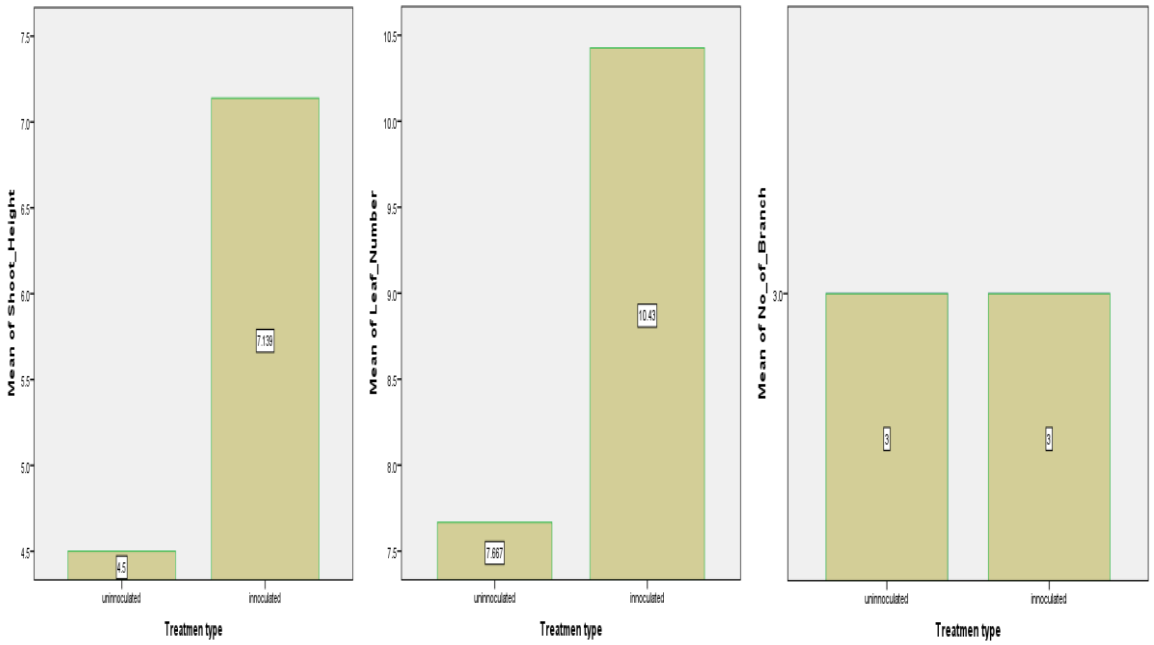
G H

Fig. 5. Result observed after incubation of inoculated isolate for citrate utilization simmon's citrate agar medium (G). Result observed after incubation of inoculated isolate for starch hydrolysis test on Lugol's iodine solution. (H)



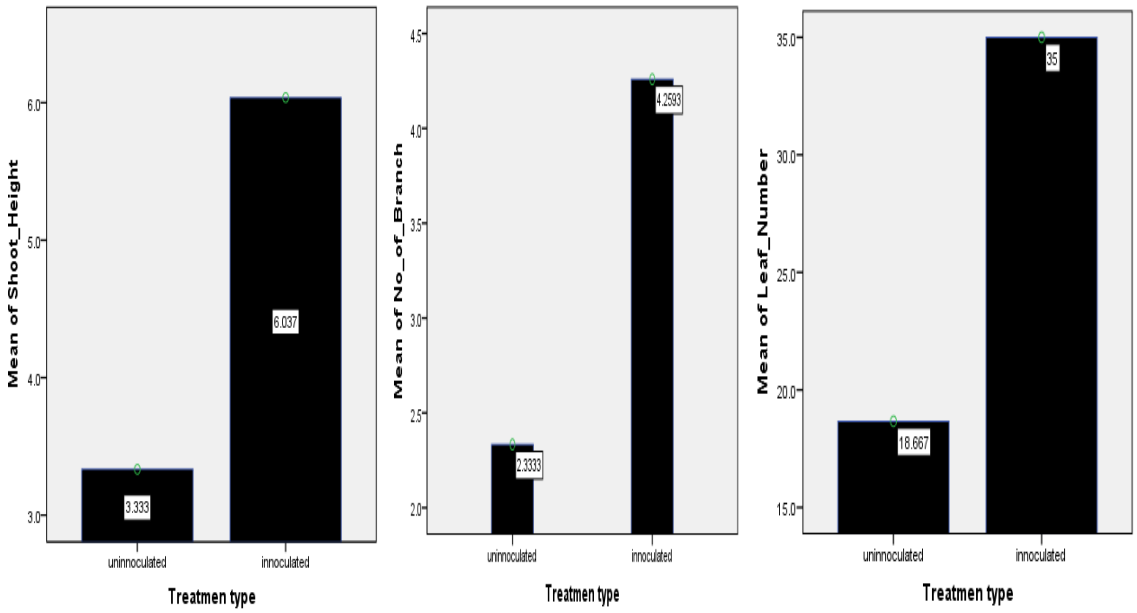
I

Fig. 6. Result observed after incubation of inoculated isolates for mannitol salt agar (I)



A B C

Fig. 7. Mean of shoot length (A) Mean of leaf number (B) and mean of branch (C) of pea



A B C

Fig. 8. Mean of shoot height of white chickpea (A) Mean of leaf number of white chickpea (B) Mean of number of branch of white chickpea (C)

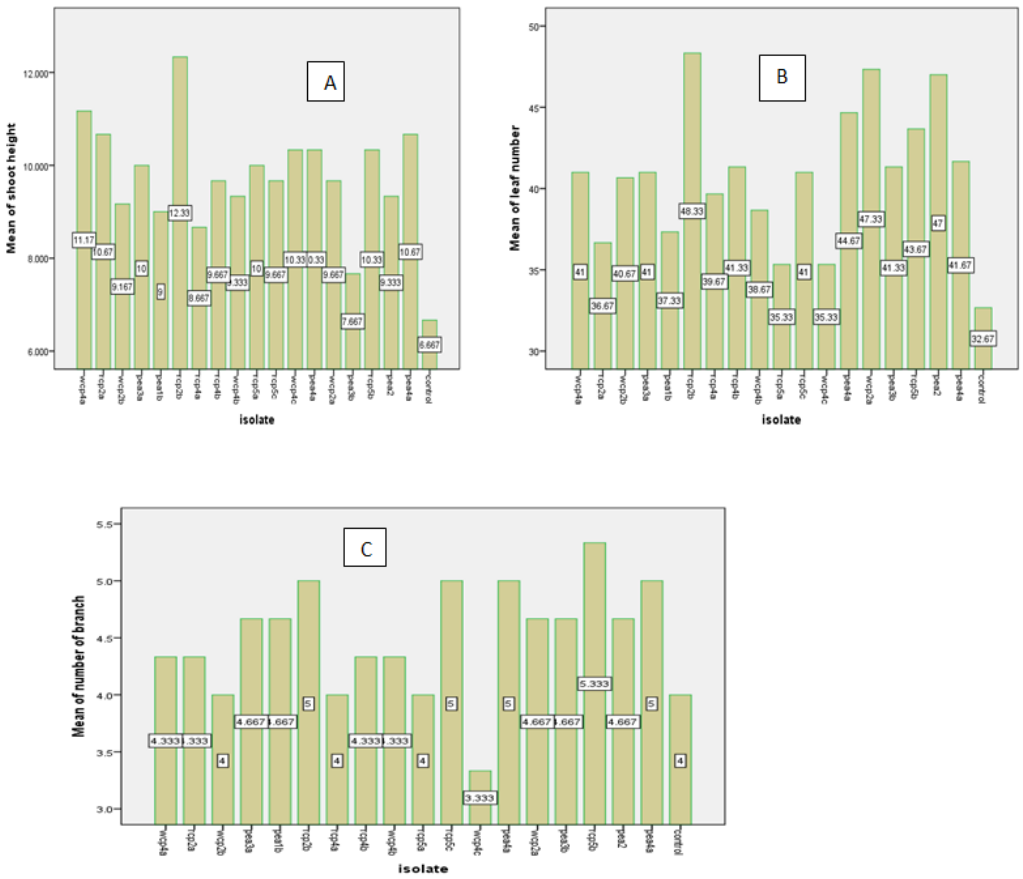


Fig. 9. Mean of shoot height of red chick pea (A) Mean of leaf number of red chick pea (B) Mean number of branch of red chick pea (C)

3.4 The Effect of Bacterial Isolates on Pea, White Chickpea, and Red Chickpea

The effect of selected isolates on the growth of plant was demonstrated by using simple pot experiments at the green house. The shoot height, leaf number, and number of branch was measured and recorded after 10 days [18-20]. The inoculated white chickpea and pea plants were compared with UN inoculated with their mean comparison (Table 2). All isolate bacteria were significant since the significance value of our output were <0.05 (Table 2). But, the means of each isolates were compared using the recorded data of red chickpea and among 18 isolates the rcp2b isolates were the most significant isolates [21-23].

4. CONCLUSION AND RECOMMENDATION

Goal of this study is to discover new and better strains for use in legume inoculants. This pursuit entails the collection of isolates, their characterization, and assessment of symbiotic capacity and comparison of isolates currently included within inoculants. In the study a total of 21 isolates were isolated on Yeast Extract Mannitol (YEMA) agar medium and the author found that among the 21 bacterial isolates isolated, 18 were shown the characteristic of rhizobia. The effect of these 18 isolates those shows the characteristics of rhizobia were performed and all of them are significant, while compared with controls. These isolates will be useful to produce efficient bio fertilizers for Agriculture. This study concludes that the selection of highly performed isolates employing distinct consecutive methods of identification and authentication are worthy of further exploration of field try for rhizobiological science. Symbiotic performance is critical, but rhizobia's ability to endure stress conditions or use less expensive growth media are also essential concerns. The process of Rhizobium discovery and characterisation is quite laborious, and efforts

must remain unbiasedly focused on relatively few legumes of interest, such that elite strains coming from a work are clearly superior.

These native rhizobia isolates are having high potency in nitrogen fixation so based on the above conclusion the following recommendations are forwarded:

- Further study them has become a major practice of significance in the field of agriculture and science
- Extending application of biological nitrogen fixation by any means is of huge importance
- Further work is needed on isolation and scaling - up of the modified isolation and characterization for optimization of bio - fertilizer production

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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