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Isolation and characterization of nitrogen deficit *Rhizobium* isolates and their effect on growth of haricot bean

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Nitrogen deficiency in recognized as the limiting factor for reduction in the yield of haricot bean in Ethiopia. The nitrogen fixing bacteria have the ability to enhance the plant growth and yield by fixing the atmospheric nitrogen and also play a significant role to conquer this problem. The aim of study was to isolate and characterize the haricot bean nodulating *Rhizobium* isolates and also to evaluate their effect on the growth of haricot bean. Out of total, twelve isolates were selected as representative samples and were screened for morphological, physiological and biochemical characteristics. On the basis of screening, eight potential isolates (MJUML1, MJUML3, MJUML4, MJUML6, MJUML8, MJUML10, MJUML11 and MJUML12) were selected for the further greenhouse experiment. The result indicated that inoculation of plants with *Rhizobium* isolates have significant effect on shoot dry weight, shoot nitrogen contents, nodule numbers, and nodule dry weight per root system ($p < 0.05$). Among all the tested *Rhizobium* isolates, $MJUML_{11}$, $MJUML_{12}$ and $MJUML_{1}$ were best isolates and significantly increased the growth of haricot bean in nitrogen deficit soils and also survive in the soil as saprophytes.

Key words: Biological nitrogen fixation, Nitrogen content, N-deficit soil, *Phaseoulus vulgaris*.

INTRODUCTION

Pulses are the natural supplement to cereal in terms of protein. They not only provide the fat and carbohydrates but also having the high amounts of minerals and vitamins essential for health (Porres et al., 2003). It has been cultivated on the approximately 13% of the agricultural land and contributes approximately 10% of the agricultural economy of Ethiopia. Amongst the various crops, haricot bean (*Phaseolus vulgaris* L.) is ranked third, usually grown in the central and eastern lowlands Rift valley of the country. Moreover, this crop is considered as the main cash crop and the least expensive source of protein for the poor farmers (Ayalew, 2011). Amongst the various limiting factors affecting plant growth and productivity, Nitrogen (N) deficiency is one of the most common factors for reduction in yield of legume crops particularly in Ethiopia (Adler, 2008). The commercially available N fertilizers are very expensive and also had adverse effect on the environment and human health. Therefore, there is an urgent need to realize a vital and cheaper source of fertilizers having

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eco-friendly approach.

The exploitation of biological nitrogen fixation (BNF) by legume-*Rhizobium* symbiosis is most prominent symbiosis found in nature (Peoples et al., 1995) and a very promising approach to enhance the fertility of soil. It provides about 65% of total biosphere nitrogen to the plant (Lodwig et al., 2003). The effectiveness of BNF may depend on survival of the *Rhizobium* spp. under different soil conditions like salinity, drought, acidity, soil temperature (Zahran, 1999). Andrade et al. (2002) reported decrease in the abundance and diversity of *Rhizobium* spp. with increase in soil acidity. However, O'Hara et al. (2002) found that the abundance and diversity of *Rhizobium* spp. provides a large resource of natural germplasm with high adaptability under different soil conditions. The presence of nodules and the rate of N_2-fixation are the determinative criteria to measure the effectiveness of *Rhizobium* spp. present in the soil (Amijii and Giller (1998). Earlier reports on the diversity of *Rhizobia* associated with legumes indicated that the *Rhizobium etli* is most predominant species present in the nodules of *Phaseolus vulgaris* (Bernal and Graham, 2001; Martinez-Romero, 2003). In addition, the presence of other *Rhizobium* spp. (*Rhizobium leguminosarum bv. phaseoli, Rhizobium gallicum* and *Rhizobium giardinii*) were also reported (Martinez-Romero, 2003). However, Yadegari and Rahmani (2010) reported the inoculation of *Rhizobium* strains increased in yield characteristics, dry matter and protein content in bean. In the present study, attempts were made to isolate and characterize the haricot bean nodulating rhizobia in nitrogen deficit soil and also to evaluate their effect on the growth of haricot bean.

MATERIALS AND METHODS

Sampling sites and sample collection

The root samples were collected from the eight different (Bedabuna, Dedo, Serbo, Gimbe, Yebu, Haro, Kilicher and Sertsi) haricot bean growing districts of Jimma zone, South-Western, Ethiopia. In order to collect the root-nodule bacterium the root along with rhizospheric soil around the haricot bean was collected from each site up to the depth of 0 to 30 cm in the polyethylene bags. The samples were immediately brought to College of Agriculture and Veterinary Medicine, Jimma University, Ethiopia for further analysis. The pH of the soil was measured with the help of digital pH meter while, the nitrogen content was measured according to Kjeldahl method.

Isolation and maintenance of *Rhizobium* isolates

The collected root-samples of each district were washed gently with tap water to remove the adhering soil particles and placed separately on the blotting sheet to remove the excess moisture. After washing, clean and fresh nodules were detached from root with the help of sterilized forceps and immediately transferred to properly label sterilized Petri plates. The nodules were surface sterilized with 95% ethanol followed by 3% H_2O_2 and washed six times with sterile distilled water.

The clean and fresh root-nodules were crushed into sterilized Petri plates with the help of glass rot in a drop of sterile distilled water under laminar flow. A loop full of each nodule suspension was streaked onto the pre-solidified properly labeled Petri plates containing yeast extract mannitol agar (YEMA) medium (KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; yeast extract 0.5 g; NaCl 0.2 g; $MgSO_4.7H_2O$ 0.2 g; Mannitol 10.0 g; and agar 16.0 g in 1,000 ml of distilled water, pH 7.0). The Petri plates were incubated at 28 ± 2°C for 5 days. Single colony of each isolate was picked with the help of sterilized loop and transferred into test tubes containing 5 ml of sterilized yeast extract mannitol broth (YEMB). The test tubes were vortexes and placed on rotary shaker for 48 h at room temperature.

A loop-full of culture suspensions from each isolate was streaked on separate Petri plates containing YEMA medium and incubated at 28 ± 2°C for 5 days and pure culture of each isolates was maintained on YEMA slant containing 0.3% $CaCO_3$ (w/v) and incubated at 28 ± 2°C for 48 h. After sufficient growth the slants were preserved in the refrigerator at 4°C and designated as (Mulugeta Jimma University Microbiology Laboratory) MJUML isolates.

Confirmatory test of the *Rhizobium* isolates

After purification gram staining was performed to confirm the identity of the *Rhizobium* isolates followed by growth on Peptone-glucose agar medium and Cango red medium.

Gram staining

The isolates were subjected to gram staining technique and observed under light microscope at ×100 magnification using oil immersion. A single colony from the agar plates was transferred to a drop of water on glass slide and observed for cell motility.

Growth on the peptone-glucose agar medium

The peptone-glucose agar medium was prepared by dissolving peptone 10.0 g, glucose 5.0 g, agar 15.0 g, and 10 ml of Bromo-cresol purple in 1,000 ml of distilled water and the pH was adjusted to 6.8. Three days old *Rhizobium* isolates grown in YEMB was streaked onto peptone-glucose agar medium. The Petri plates were incubated at 28 ± 2°C for 5 days to observe the growth and change in the colour.

Congo red absorption test

About 10 ml of stock solution of Congo red (0.25 g of Congo Red in 100 ml distilled water) was added to 100 ml of YEMA medium and autoclaved. The three day old culture grown in YEMB was streaked on Petri plant containing cango red supplemented YEMA medium. The Petri plates were covered with black plastic sheet to prevent absorption of light and were incubated at 28 ± 2°C for 5 days. The colonies of *Rhizobium* isolates appears showed the absorption of Congo red.

Character identification

A loop full three day old culture of each Rhizobial isolate was streaked on the YEMA Petri plates for character identification and was incubated at 28 ± 2°C for 5 days. The diameter, shapes, colour and mucous production of the colonies were recorded. The growth of each *Rhizobium* isolates were also determined on tryptone lacking calcium (TY-Ca^{++}) medium YEMA medium containing 2%

urea and Luria Bertani agar medium.

Determination of growth rate

A single colony of each *Rhizobium* isolate was transferred into test tubes containing 10 ml YEMB and placed on a rotary shaker (125 rev/min) at room temperature for 48 h. One milliliter of suspension (1×10^8 cfu/ml) was transferred into 250 ml Erlenmeyer flasks containing 100 ml of YEMB. The turbidity was measured in terms of optical density at 540 nm using spectrophotometer (Jenway, 6405 UV/vis spectrophotometer) against the blank (sterilized un-inoculated YEM broth) and the mean generation time was calculated for each isolates.

Physiological and biochemical characterization

All the isolates were standardized by the method of McFarland and the concentration of the inoculum was adjusted to 1×10^8 cfu/ml. About 30 µl of the inoculums suspensions was streaked onto Petri plates containing YEMA plates and were incubated at 28 ± 2°C for 5 days. Each isolate was replicated thrice for each test. The growth of was recorded as (+) for slight growth, (++) for abundant growth and (-) for no growth.

Effect of pH tolerance

The ability of the *Rhizobium* isolates to grow in acidic or basic media was tested by streaking the each isolate on separate Petri plates on YEMA with pH adjusted to 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0.

Effect of salt tolerance

The ability of the *Rhizobium* isolates to grow in different concentrations of salt was tested by streaking each isolates separately onto YEMA supplemented with 1, 2, 3, 3.5 and 4% (w/v) NaCl.

Effect of temperature tolerance

Tolerance of *Rhizobium* isolates to high temperatures was tested by inoculating the each isolate onto Petri plates containing YEMA. The Petri plates were incubated at 37, 40, 45, 50 and 55°C.

Antibiotic resistance test

Resistance to antibiotics of *Rhizobium* isolates was determined by inoculating each isolate on Petri plates containing YEMA supplemented with freshly prepared filter sterilized antibiotics of different concentration (10.0 and 20.0 µg/ml). The stock solution of antibiotics (Ampicilline, chloramphenicol, erythromycin, neomycin sulfate, tetracycline and penicillin) were prepared according to method of Somasegaran and Hoben (1994). Chloramphenicol and Erythromycin was dissolved in NaOH and ethanol respectively while, the rest were soluble in distilled water. The stock solution of each antibiotic was prepared by dissolving 2.0 g of antibiotics in 100 ml of distilled water.

Carbohydrate utilization

All the *Rhizobium* isolates were tested for their ability to utilize the various carbon sources in carbohydrate free basal medium. About eleven carbon sources such as monosaccharides (D-glucose, D-fructose, inositol, mannose and xylose), disaccharides (maltose, D-sucrose), sugar alcohols (mannitol and glycerol) and polysaccharides (starch and dextrin) were used. The carbohydrate free basal medium was prepared by dissolving the $MgSO_4.7H_2O$ 0.2 g; NaCl 0.2 g; K_2HPO_4 0.5 g; KH_2PO_4 0.5 g; Yeast extract 0.05 g and agar 15.0 g in 1,000 ml of distilled water. The pH of the medium was adjusted to 7.0. The concentration of the carbon source was 1% (w/v). Each heat labile carbohydrate was filter sterilized using 0.2 µm Millipore and added to the autoclaved sterilized medium. The isolates were streaked on the Petri plates in three replicates. The presence or absence of growth was recorded after 5 days.

Phosphate solubilizing ability

Phosphate solubilizing ability was assessed on solid Sperber's basal medium (1958) (glucose 10.0 g; yeast extract 0.5 g; $CaCl_2$ 0.1 g; $MgSO_4.7H_2O$ 0.25 g; and agar 15.0 g in 1000 ml of distilled water) supplemented with 2.5 g/L $Ca_3(PO_4)_2$. The pH of the medium was adjusted to 7.2. The each *Rhizobium* isolate was inoculated to separate Petri plates incubated at 28 ± 2°C for 5 days. The diameter of clear (halo) zone of around each isolates was measured (Edi-Premono et al., 1996).

Greenhouse experiment

Out of twelve, eight *Rhizobium* isolates were recognized as the potential isolates and were selected for greenhouse experiment. The greenhouse was conducted at College of Agriculture and Veterinary Medicine, Jimma University, Jimma Ethiopia. Surface-sterilized plastic pots (3 kg capacity) were filled with sterilized sand and autoclaved at 121°C for 15 min.

The uniform, white colored, healthy seeds of haricot bean were surface sterilized with 95% ethanol for 10 s followed by 0.3% H_2O_2 for 60 s and rinsed 6 times in sterile distilled water. The four surface-sterilized seeds were germinated on sterilized water agar plates (7.5 g of agar in 1,000 ml of distilled water) incubated at 27°C for 48 h. The germinated seeds were transferred into plastic pot containing sterilized sand with the help of a sterile forceps. One week after germination the seedlings were thinned to maintain three healthy seedlings per pot.

Inoculation techniques

About 1 ml of each *Rhizobium* isolates (1×10^8 cfu/ml) was poured around the root using sterile pipette and covered with the layer of sand. All pots were fertilized with quarter strength of N-free medium (Broughton and Dilworth, 1970). Initially, all the plants were received 100 ml of 0.05% KNO_3 (w/v) as nitrogen source. The positive control was irrigated with 100 ml of 0.05% KNO_3 (w/v) solution and also 100 ml of quarter-strength N-free nutrient solution was supplied to each pot once in a week. The negative control was treated with same amount of sterile distilled water and the plants were watered every alternate day.

Experimental design

The experiment carried out in complete randomized block design with eight *Rhizobium* isolates, positive (with N-free medium) and negative (without N-free medium) control. Each treatment was replicated three times (10 × 3 = 30 treatments).

Table 1. Description of sampling sites, soil pH, and nitrogen content in soil (%) of *Rhizobium* isolates collected from different Jimma zone, South-Western, Ethiopia.

Isolates	Sampling sites	Soil pH	N content in soil (%)
$MJUML_1$	Serbo	4.75	0.037
$MJUML_2$	Serbo	4.74	0.036
$MJUML_3$	Haro	6.22	0.130
$MJUML_4$	Dedo	7.22	0.149
$MJUML_5$	Dedo	7.24	0.151
$MJUML_6$	Bedabuna	5.40	0.107
$MJUML_7$	Bedabuna	5.41	0.104
$MJUML_8$	Gimbe	5.71	0.173
$MJUML_9$	Gimbe	5.70	0.172
$MJUML_{10}$	Yebu	5.97	0.112
$MJUML_{11}$	Sertse	4.60	0.168
$MJUML_{12}$	Kilicher	5.18	0.167

Observations

The plants were harvested 45 days after inoculation. Data were recorded on shoots dry weight, shoot nitrogen content, number of nodules, nodule dry weight per root system. The shoot nitrogen content was determined by Kjeldahl method.

Statistical analysis

Data were statistically analyzed using one way ANOVA using statistical software SPSS 17.0 at $p = 0.05$. Duncan multiple range test was performed to denote the significant difference between the treatments. Standard errors were also calculated and graphs were prepared using sigma plot version 10.0.

RESULTS

Forty eight isolates belonging to *Rhizobium* spp. were isolated from soil samples collected from eight different haricot bean growing districts of Jimma zone, South-Western, Ethiopia. Out of total, twelve isolates were selected as a representative of the eight sampling sites and were screened for the further confirmatory tests. The pH and nitrogen content in the soil of the sampling site is listed in Table 1.

Morphology and cultural characteristics of isolates

All the isolates were failed to absorb congo red pigment when grown on YEMA-CR medium. The isolates change the colour of Bromocresol purple, when grown on glucose peptone agar medium and showed a poor growth and changed the YEMA-BTB medium. All isolates were Gram-negative and rod shaped and also exhibited different generation time (Figure 1). All the isolates showed the presence of creamish colonies on PY-medium except $MJUML_4$, $MJUML_8$ and $MJUML_{11}$. Among all the isolates $MJUML_1$, $MJUML_5$, $MJUML_6$, $MJUML_7$, $MJUML_8$, $MJUML_9$, and $MJUML_{11}$, showed large mucoid growth on YEMA medium, isolates $MJUML_1$, $MJUML_2$, $MJUML_3$, and $MJUML_{12}$, showed small mucoid, while isolate $MJUML_4$ small dry growth on YEMA media (Table 2).

Almost all isolates except $MJUML_3$ exhibits high amount of exo-polysaccharide on the medium showing the colony diameters in the range of 2.0 to 5.0 mm. The largest colony diameter (5.0 mm) was recorded in isolates $MJUML_6$ and $MJUML_{11}$ on YEMA medium (Table 2). All isolates were well grown on TY-Ca^{++}, Luria Bertani agar medium and YEMA medium supplemented with 2.0% urea (Table 3). However, isolates $MJUML_8$ were not able to grow on Luria Bertani agar medium and YEMA medium supplemented with 2.0% urea (Table 3).

Physiological and biochemical tests

Effect of pH tolerance

All the isolates showed profuse growth at pH 5.0 to 10.0 (Table 4). The growth was also observed at pH 4.5 except $MJUML_9$ and $MJUML_{10}$. Isolates $MJUML_1$ and $MJUML_2$ and $MJUML_6$ showed abundant growth at pH 4.0, however, isolates $MJUML_7$ and $MJUML_{11}$ showed poor growth at this pH. Out of twelve isolates, 9.0 grow at pH 11.0 and almost all isolates were not able to grow at pH 12.0.

Effect of salt tolerance

All isolates showed disparity in tolerance to different concentrations of salt (Table 5). Almost all of the isolates were grown in medium having 1.0 and 2.0% concentration of NaCl. On increasing the concentration of the salts the growth of the isolates were decreased. Isolates $MJUML_5$ and $MJUML_{12}$ were ability to grow at

Table 2. Growth, morphological and cultural characteristics of the *Rhizobium* isolates.

Isolates	Growth on YEMA	Morphological and cultural characteristics of isolates			
		Colony diameter (mm)	Colony morphology	Colony color	Mucous production
$MJUML_1$	Large mucoid	2-4	Creamy	Whitish	High
$MJUML_2$	Small mucoid	2-3	Creamy	Yellowish	Medium
$MJUML_3$	Small mucoid	1-2	Creamy	Yellowish	Medium
$MJUML_4$	Small dry	2-3	Rough	Whitish	Low
$MJUML_5$	Large mucoid	3-4	Creamy	Yellowish	High
$MJUML_6$	Large mucoid	4-5	Creamy	Whitish	High
$MJUML_7$	Large mucoid	3-4	Creamy	Whitish	High
$MJUML_8$	Large mucoid	2-3	Smooth	Whitish	High
$MJUML_9$	Large mucoid	3-4	Creamy	Yellowish	High
$MJUML_{10}$	Small mucoid	2-3	Creamy	Whitish	Medium
$MJUML_{11}$	Large mucoid	4-5	Rough	Yellowish	High
$MJUML_{12}$	Small mucoid	2-3	Creamy	Whitish	Medium

Table 3. Growth of *Rhizobium* isolates on different culture medium.

Isolates	TY-Ca^{++}	YEMA+ 2% Urea	LB
$MJUML_1$	++	++	++
$MJUML_2$	++	++	++
$MJUML_3$	++	+	+
$MJUML_4$	++	+	++
$MJUML_5$	++	+	++
$MJUML_6$	++	++	++
$MJUML_7$	++	+	++
$MJUML_8$	++	–	–
$MJUML_9$	++	+	+
$MJUML_{10}$	++	+	+
$MJUML_{11}$	++	++	++
$MJUML_{12}$	++	++	++

+ = Slight growth; ++ = abundant growth; − = no growth; TY-Ca^{++} = Tryptone lacking calcium medium; YEMA = Yeast extract mannitol agar medium; LB = Luria Bertani agar medium.

3.0% NaCl concentration, while isolates $MJUML_4$ and $MJUML_{11}$ have the ability to grow at 4.0% NaCl concentrations (Table 5).

Effect of temperature tolerance

All the isolates were able to grow at 37 to 40°C and none of them grow at 55°C (Table 6). All the isolates except $MJUML_1$, $MJUML_2$, $MJUML_4$, $MJUML_5$ and $MJUML_7$ were found to be tolerant to temperature of 45°C but only isolates $MJUML_6$ and $MJUML_{11}$ were found to be tolerant to 50°C (Table 6).

Antibiotic resistance test

Antibiotic resistance test indicated that all the isolates showed less variation in tolerance to different types and concentrations of antibiotics (Table 7). All the isolates grow well at both concentrations (10.0 and 20.0 µg/ml) of ampicillin. Similar, result were also obtained in Tetracycline for both concentration except $MJUML_5$ and $MJUML_8$ (Table 7). Most of the isolates were resistant to tetracycline, erythromycin, penicillin and ampicillin but sensitive to chloramphenicol and neomycin sulfate. None of them grow on chloramphenicol and neomycin sulfate at 20.0 µg/ml concentration. Isolates $MJUML_3$, $MJUML_5$ and $MJUML_{12}$ were sensitive to neomycin sulfate at 10.0 µg/ml concentration (Table 7).

Carbohydrate utilization

The isolates showed better growth on polysaccharides

Table 4. Effect of *Rhizobium* isolates on the pH tolerance.

Isolates	pH tolerance								
	pH =4.0	pH=4.5	pH =5.0	pH=5.5	pH=8.0	pH =9.0	pH=10.0	pH=11.0	pH=12.0
$MJUML_1$	++	++	++	++	++	++	++	++	−
$MJUML_2$	++	++	++	++	++	++	++	++	−
$MJUML_3$	−	+	+	+	++	++	++	−	−
$MJUML_4$	−	+	++	++	++	++	++	++	−
$MJUML_5$	−	+	+	++	++	++	++	++	−
$MJUML_6$	++	++	++	++	++	++	++	++	−
$MJUML_7$	+	+	+	++	++	++	++	+	−
$MJUML_8$	−	+	+	+	++	++	++	−	−
$MJUML_9$	−	−	+	+	++	++	++	+	−
$MJUML_{10}$	−	−	+	++	++	++	++	−	−
$MJUML_{11}$	+	++	++	++	++	++	++	++	−
$MJUML_{12}$	−	+	++	++	++	++	++	++	−

+ = Slight growth; ++ = abundant growth; − = no growth.

Table 5. Effect of *Rhizobium* isolates on the salt tolerance.

Isolates	Salt tolerance				
	1.0% NaCl	2.0% NaCl	3.0% NaCl	3.5% NaCl	4.0% NaCl
$MJUML_1$	++	++	−	−	−
$MJUML_2$	++	++	−	−	−
$MJUML_3$	++	++	−	−	−
$MJUML_4$	++	++	++	++	+
$MJUML_5$	++	++	+	−	−
$MJUML_6$	++	++	−	−	−
$MJUML_7$	++	++	−	−	−
$MJUML_8$	++	++	−	−	−
$MJUML_9$	++	++	−	−	−
$MJUML_{10}$	++	++	−	−	−
$MJUML_{11}$	++	++	++	++	++
$MJUML_{12}$	++	++	+	−	−

+ = Slight growth; ++ = abundant growth; − = no growth.

and sugar alcohols followed by disaccharides and monosaccharides (Table 8). In general, the isolates showed better growth on polysaccharides and sugar alcohols than monosaccharides except D-sucrose and maltose (Table 8).

Phosphate solubilizing ability

In our study, no isolates had shown the phosphate solubilizing ability on Soild Sperber media.

Greenhouse experiment

Out of twelve, eight potential *Rhizobium* isolates were selected for greenhouse experiment (Table 9). All the isolates have the ability to form the nodules on the roots of haricot bean (Figure 2). *Rhizobium* inoculated plants showed clear difference compared negative control (Figure 3). The negative control plants were shorter and thinner compared to plants inoculated with *Rhizobium* isolates, which have the profound green leaves (Figure 3). The positive control and *Rhizobium* inoculated plants having the similar physical appearance but the inoculated plants showed pink colored nodules in the root system. Shoot dry weight, shoot nitrogen content, number of nodules, nodules dry weight per root system and symbiotic effectiveness of isolates showed clear variations ($p < 0.05$) among the isolates (Table 9). Plants inoculated with isolate $MJUML_{11}$ showed the highest number of nodules followed by isolate $MJUML_1$ and

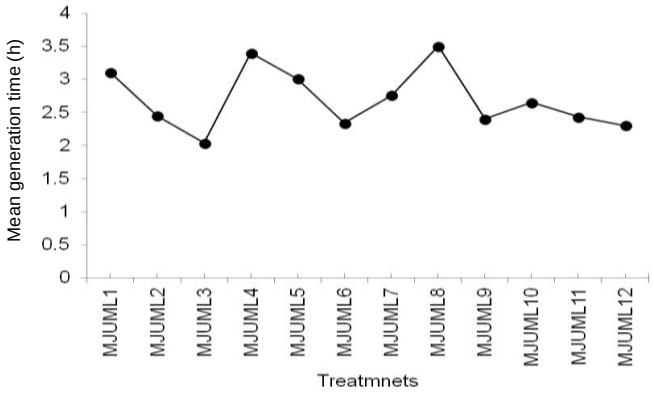

Figure 1. Effect of *Rhizobium* isolates on the generation mean time.

Table 6. Effect of *Rhizobium* isolates on the temperature tolerance.

Isolates	Temperature tolerance				
	35°C	40°C	45°C	50°C	55°C
$MJUML_1$	++	++	−	−	−
$MJUML_2$	++	++	−	−	−
$MJUML_3$	++	++	+	−	−
$MJUML_4$	++	++	−	−	−
$MJUML_5$	++	++	−	−	−
$MJUML_6$	++	++	+	+	−
$MJUML_7$	++	++	−	−	−
$MJUML_8$	++	++	+	−	−
$MJUM_9$	++	++	+	−	−
$MJUML_{10}$	++	++	+	−	−
$MJUML_{11}$	++	++	+	+	−
$MJUML_{12}$	++	++	+	−	−

+ = Slight growth; ++ = abundant growth; − = no growth.

Table 7. Effect of *Rhizobium* isolates on the antibiotic sensitivity.

Isolates	Antibiotics sensitivity											
	Ampicilline		Chloram-phenicol		Erythromycin		Tetracycline		Neomycin sulfate		Penicillin	
	10.0 µg/ml	20.0 µg/ml	10.0 µg/ml	20.0 µg/ml	10.0 µg/ml	20.0 µg/ml	10.0 µg/ml	20.0 µg/ml	10.0 µg/ml	20.0 µg/ml	10.0 µg/ml	20.0 µg/ml
$MJUML_1$	++	++	+	−	++	++	++	+	+	−	++	+
$MJUML_2$	++	++	+	−	++	++	++	+	+	−	++	+
$MJUML_3$	++	++	+	−	−	−	++	+	−	−	++	+
$MJUML_4$	++	++	+	−	++	++	++	+	+	−	++	+
$MJUML_5$	++	++	++	−	++	++	++	−	−	−	++	+
$MJUML_6$	++	++	+	−	++	++	++	+	+	−	++	−
$MJUML_7$	++	++	++	−	++	++	++	++	++	−	++	+
$MJUML_8$	++	++	+	−	+	−	++	−	+	−	+	−
$MJUML_9$	++	+	+	−	++	−	++	+	+	−	++	+
$MJUML_{10}$	++	++	++	−	+	−	++	+	+	−	++	+
$MJUML_{11}$	++	++	+	−	++	++	+	+	+	−	+	−
$MJUML_{12}$	++	++	+	−	++	++	++	+	−	−	+	−

+ = Slight growth; ++ = abundant growth; − = no growth.

Table 8. Effect of *Rhizobium* isolates on the utilization of carbohydrate.

Isolates	Carbohydrate utilization										
	D-mannitol	D-sucrose	Dextrin	Maltose	Inositol	Glucose	D-fructose	Starch	Mannose	Xylose	Glycerol
$MJUML_1$	++	++	++	++	+	+	+	+	+	+	−
$MJUML_2$	++	++	++	+	+	−	+	+	−	−	+
$MJUML_3$	++	+	+	+	−	−	−	+	+	+	+
$MJUML_4$	−	++	−	−	++	+	+	−	−	+	+
$MJUML_5$	+	++	+	+	+	−	−	+	+	−	+
$MJUML_6$	++	++	++	+	−	−	−	+	−	−	−
$MJUML_7$	+	++	+	−	−	−	−	+	−	−	+
$MJUML_8$	−	−	−	−	−	+	++	−	+	−	+
$MJUML_9$	+	−	+	+	−	−	−	+	−	+	−
$MJUML_{10}$	+	+	+	++	−	−	−	+	−	−	+
$MJUML_{11}$	+	++	−	+	+	++	−	+	−	−	+
$MJUML_{12}$	++	++	+	+	+	++	−	+	−	−	+

+ = Slight growth; ++ = abundant growth; − = no growth.

$MJUML_{12}$, respectively (Table 9). However, the least number of nodules was recorded in plants inoculated with isolate $MJUML_3$. The highest nodule dry weight was recorded in plants inoculated with isolate $MJUML_1$. Nevertheless, the lowest nodule dry weight was observed in plants inoculated with isolate $MJUML_{12}$ (Table 9).

Moreover, the highest shoot dry weights were recorded from plants inoculated with isolate $MJUML_{11}$ and $MJUML_1$ while the least dry weight was recorded in plants inoculated with isolate $MJUML_3$ (Table 9). The highest symbiotic effectiveness was recorded in isolate $MJUML_1$, while the lowest was recorded in $MJUML_3$. Regression analysis between shoot nitrogen content, shoot dry weight and nodule dry weight per root system of haricot bean showed a positive correlation (R^2 = 0.98, $p < 0.05$) was between shoot nitrogen content and shoot dry weight, while a poor correlation (R^2 = 0.45, $p = 0.05$) was observed between nodule dry weight and shoot dry weight of haricot bean (Figure 4A and B).

DISCUSSION

Our results indicate that all the isolates were gram negative rod and were failed to absorb Congo red pigments showing the characteristics features of the family *Rhizobiaceae*. The isolates also were not capable to change the color of Bromo-cresol purple in glucose peptone agar medium and exhibiting a poor growth on the medium and exhibiting a poor growth on the medium. Isolates except $MJUML_4$, $MJUML_8$ and $MJUML_{11}$ showed creamy growth on peptone yeast medium exhibiting the typical characteristic of *Rhizobium tropici* (Martinez-Romero et al., 1991). The isolates $MJUML_4$ and $MJUML_{11}$ showed the rough colony morphology, may belongs to *Rhizobium gallicum*, while isolate $MJUML_{11}$ showed the smooth colony morphology may be belongs to *R. leguminosarum* bv *phaseoli* (Silva et al., 2003). Workalemahu (2006) reported that almost all common bean nodulating rhizobia exhibited smooth gummy colony except AUPR9 and AUPR10 isolate, which showed rough colony morphology. However, isolate AUPR8 isolate displayed creamy colony morphology on peptone yeast agar medium. Andrade et al. (2002) reported that isolates having the ability to grow on LB, TY-Ca^{++} and YEMA containing 2% urea were characterized as *R. tropici*. Our results are in agreement with the earlier finding because almost all the isolates were grown on LB, TY- Ca^{++} and YEMA containing 2% urea except isolate $MJUML_8$ (Andrade et al., 2002).

The response of various isolates against the different concentration of NaCl indicated that almost all the isolates having the capacity to tolerate against the 1 and 2% NaCl concentration. Isolate $MJUML_4$ and $MJUML_{11}$ showed maximum growth compared to other isolates in response to

Figure 2. Root nodules of haricot bean inoculated with $MJUML_1$ and $MJUML_{11}$ isolates.

Figure 3. Comparison of negative and positive control treatments with $MJUML_1$, and $MJUML_{11}$ isolates.

Table 9. Effect of *Rhizobium* isolates on the shoot dry weight, nitrogen content, number of nodules and nodule dry weight per root system of haricot bean.

Treatments	Shoot dry weight per plant	Shoot N content (%) per plant	Number of nodule per root system	Nodule dry weight per root system
Control+ve	2.91±0.13^{e}	1.52±0.06^{d}	-	-
Control-ve	2.27±0.19^{f}	0.80±0.19^{e}	-	-
$MJUML_1$	4.59±0.09^{b}	3.20±0.07^{b}	129.6±6.95^{b}	0.78±0.05^{a}
$MJUML_3$	3.27±0.07^{de}	1.67±0.17^{d}	46.2±2.54^{g}	0.24±0.02^{cd}
$MJUML_4$	3.65±0.15^{cd}	2.39±0.16^{c}	98.4±3.97^{d}	0.64±0.05^{b}
$MJUML_6$	3.56±0.22^{cd}	2.28±0.09^{c}	84.8±5.12^{e}	0.28±0.02^{c}
$MJUML_8$	3.52±0.12^{cd}	2.20±0.08^{c}	62.6±3.28^{f}	0.72±0.03^{ab}
$MJUML_{10}$	3.81±0.29^{c}	2.23±0.05^{c}	118.0±4.60^{c}	0.32±0.02^{c}
$MJUML_{11}$	5.27±0.21^{a}	3.92±0.18^{a}	141.6±5.49^{a}	0.68±0.03^{b}
$MJUML_{12}$	4.33±0.11^{b}	3.08±0.11^{b}	128.8±6.94^{bc}	0.18±0.02^{d}

*Values within each column followed by the same letter are not significantly different (DMRT, $p<0.05$). Data represent ± standard error (n = 5)

NaCl concentrations at higher NaCl concentration. Our results are in line with the finding Zerhari et al. (2000) that reported that the fast growing *Rhizobium* generally grew well at 3 to 4% NaCl concentration. Similarly, Zahran

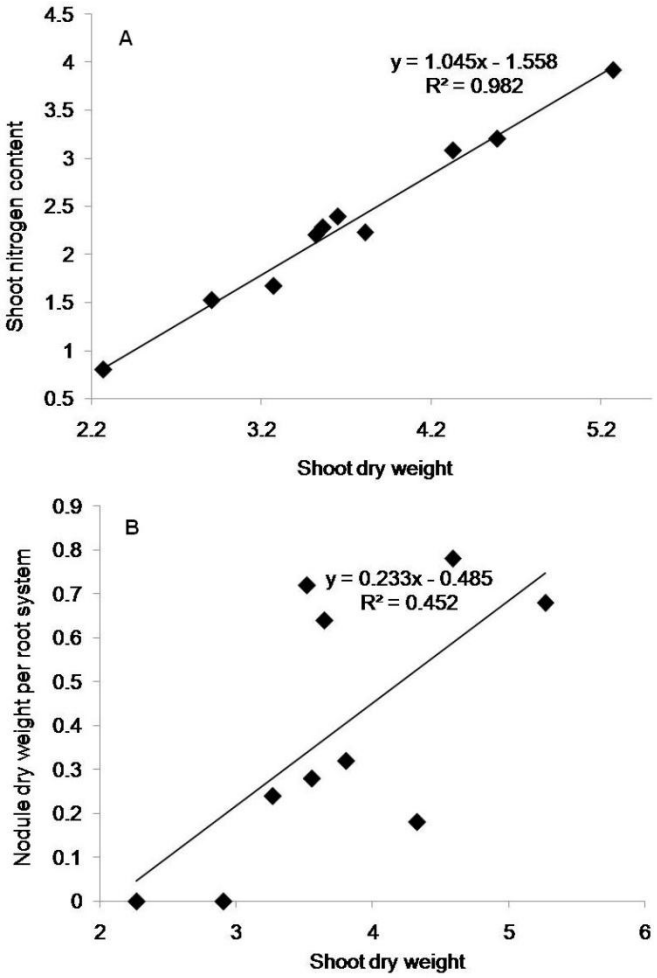

Figure 4. Regression analysis between shoot nitrogen content, shoot dry weight and nodule dry weight per root system of haricot bean (A) Regression analysis between shoot nitrogen content and shoot dry weight of haricot bean (B) Regression analysis between nodule dry weight and shoot dry weight of haricot bean (n = 5).

(1999) also reported that mostly fast growing rhizobial isolates could tolerate more than 2% NaCl concentration than slow growing isolates which were highly sensitive to salt at less than 1% NaCl. However, Hussain et al. (2002) reported that the inoculation of salt tolerant *Rhizobium* to saline soil improved the shoot dry weight, nitrogen fixation and number of nodules per root system.

The results of carbohydrate utilization showed that isolates having better growth on polysaccharides and sugar alcohols compared to di- and monosaccharide because the catabolism of mono and disaccharides are widespread in fast growing Rhizobia. In the present study most of the isolates utilized dextrin as a carbon source, which indicate that they belongs to *R. tropici* because other species of the *Rhizobium* were not able to utilize the dextrin as carbon source (Hungria and Vargas, 2000). It was revealed from our results that most of the isolates grow well at both at low and high pH. The isolates $MJUML_1$, $MJUML_2$, $MJUML_6$, $MJUML_7$ and $MJUML_{11}$ grow well in between pH 4.0 and 11.0. This indicates that these isolates have the potential to be used as inoculants in acidic soils. It was evident from the previous reports that the soil acidity is the major limiting factors for the production of haricot bean in Ethiopia (Ayalew, 2011). Therefore, these acid tolerant *Rhizobium* isolates may play a great role in improving the yield and production of haricot bean in this region and also having the utmost agronomic and ecological importance in relation to development of sustainable agricultural system.

Broad range of antibiotics was tested for the competitive ability of the *Rhizobium* isolates in the soil causing effective nodulation in plants. Our results indicate that the inoculation of *Rhizobium* isolates to plant increased the plant growth, nodule numbers showing their competitive ability in soil as indicated by Brockwell et al. (1995) in their experiment. The highest competition ability may directly influence the N_2-fixing ability of the plant correlated with the increase in the yield (Thies et al., 2001). Almost, all the isolates exhibit their growth between 35 and 40°C except few showed the slight growth between 45 and 50°C. This result may strengthen the findings of earlier reports (Zahran, 1999; Hungria and Vargas, 2000). However, Argaw (2007) recovered the high temperature (50°C) tolerant strains from the soil of eastern Ethiopia. The high soil temperature was a major problem for biological nitrogen fixation in leguminous crops because the high temperature may check the bacterial infection in host plant. In general, the optimum temperature for the growth of rhizobia ranges from 28 to 30°C (Graham, 1992). However, if the rhizobia grow at high temperatures it does not mean that it is an efficient N_2-fixer but rather than this the survival of the strains has been increased during thermal stress (Michiels et al., 1994).

It is evident from our results that there is a significant increase in the shoot dry weight of plants inoculated with *Rhizobium* isolates compared to un-inoculated control. The isolates which gave the least number of nodules produced 12.3% more dry matter than the un-inoculated control. Similarly, there is significant difference in shoot N content compared to positive and negative except $MJUML_3$. However, isolate $MJUML_{11}$ fix more N than other treatments. As compared to different parameters tested, two isolates $MJUML_{11,}$ and and $MJUML_1$ found best in terms of shoot dry weight, nitrogen content nodule dry weight and number of nodule per root system. Our result is in agreement with the previous results previous reports (Adamu et al., 2001) showed the significant variations in shoot dry weight and nodule dry weight, number of nodule and shoot N content of faba bean between inoculated and control treatments. The results indicated that there is good correlation between shoot nitrogen content and shoot dry weight and a poor correlation between shoot dry weight and nodules dry

weight. Thus, it has been concluded from our study that most of the isolates have the ability to grow over a wider range of pH, salinity, temperature, antibiotic resistance ability, and carbon sources utilization. These characteristic may be responsible for their saprophytic persistence in the soil. Isolates $MJUML_{11,}$ $MJUML_{12}$ and $MJUML_{1}$ significantly increased the plant growth and nitrogen content of haricot bean in nitrogen deficient soils but further, more studies are needed to confirm these results under field conditions.

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