**ORIGINAL ARTICLES**

# Phenotypic and Symbiotic Effectiveness of *Rhizobium Leguminosarum Bv. Viciae* Nodulating Lentil (*Lens Culinaris Medik)* From Highlands of Shewa, Ethiopia

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## ABSTRACT

Screening of symbiotically efficient rhizobial strains is the prerequisite in developing rhizobial inoculants for enhanced productivity of legumes. In this study, composite soil samples were collected from different lentil growing areas of Shewa in 2010. In 2011 and 2012, lentil nodulating rhizobial isolates were characterized for morphological, cultural and physiological properties under laboratory condition and evaluated for symbiotic effectiveness under greenhouse condition. In sand culture, the isolates showed significant differences for shoot dry weight, nodule number, nodule dry weight, percentage of symbiotic effectiveness and percentage of total nitrogen and were superior to the negative control. 5% of the isolates were highly effective and 59% were effective. The low effective and ineffective isolates accounted for 33% and 1%, respectively. The isolates showed diversity in tolerance to salinity, different pH, temperature, antibiotics, heavy metals as well as utilization of carbon and nitrogen sources. Hence, the isolates are competitive enough to colonize the rhizosphere under different edaphic and environmental conditions.

**Key words:** Lentil, Nitrogen fixation, Rhizobial inoculants

## INTRODUCTION

Lentil is among the first domesticated pulse crops worldwide and it is an important food-legume in the farming systems of many developing and developed countries (Zafar-ul-hye, 2008). The average lentil production of the world has increased from 611 kg ha$^{-1}$(in early 1970) to 966 kg ha$^{-1}$(in early 2000) and total production from 1.3 million tones to 3.8 million tones mainly due to the adoption of improved varieties in combination with application of modern technologies (Sarker and Erskine, 2006).

Lentil is one of the pulse crops grown in the highlands of Ethiopia and widely used as whole, split in stews, soups and various forms of sandwiches. As a result, it is a popular ingredient of every day diet in the majority of household. So that local consumption and price are higher than most pulse crops.

Nitrogen is the most important nutrient for plant growth and its availability has a major influence on both crop yield and product quality in agriculture (Stougaard, 2000). Though it is the most abundant element in the atmosphere, plants do not directly utilize atmospheric nitrogen because they lack the genes encoding the nitrogenase enzyme that reduce dinitrogen ($N_2$) and transform it into biologically utilizable compounds. Nitrogen is converted into utilizable form of ammonia and nitrate through industrial fertilizer production and biological nitrogen fixation (Postgate, 1998). Biological nitrogen fixation is under taken by prokaryotic microorganisms that live freely or associated loosely or endosymbiotically with certain group of leguminous and actinorzial plants (Sprent, 2001). The symbiotic association between rhizobia and legumes play a significant role in word agricultural productivity by annually converting approximately 120 million tones of atmospheric nitrogen in to ammonia (Freiberg *et al*., 1997).

Legumes belong to the most important and third largest plant family (Leguminosae). They are the primary source of nitrogen for many cropping systems as well as providing food for humans and domestic animals. Many legumes have the ability to form nitrogen fixing root nodules with rhizobia and contribute to the biological fixation of nitrogen (Sprent, 2001). Thus, legumes provide a relatively low-cost method of replacing nitrogen in the soil, enhancing soil fertility and boosting subsequent crop yields.

Lentil is one of the break-crops in the Ethiopian agriculture grown in rotation with cereals to replenish soil fertility. This is due to its capacity to fix nitrogen symbiotically with a root nodule bacterium known as *Rhizobium leguminosarum var viceae* (Vincent, 1974).Hence, the ability to fix atmospheric nitrogen makes lentil suitable in sustainable cropping systems as it improves the soil nitrogen status (Zafar-ul-hye, 2008).

Researches carried out so far proved that lentil has the potential to fix nitrogen by forming endosymbiotic association with *Rhizobium leguminosurum* (Zafar-ul-hye, 2008; Shewakena Belayneh, 2009). Holeta Agricultural Research Center and National Soil Testing Center isolated the bacteria from different lentil growing areas and evaluated their symbiotic effectiveness under the greenhouse and field conditions (personal communication with Anteneh Argaw and Daniel Muleta, 2012). Shewakena Belayneh (2009) also isolated 40 isolates of lentil nodulating rhizobia from Northern and Eastern Shewa and showed that inoculation of lentil by rhizobial isolates increased the shoot dry weight, percentage of total nitrogen, nodule number and nodule dry weight both on sand and soil cultures under the greenhouse condition. However, only a few works were done on lentil nodulating rhizobia as compared to other pulse crops (faba bean, field pea, haricot bean and soybean).

Therefore, this study aimed at collection, isolation and characterization of lentil nodulating bacterial isolates from the major growing areas of highlands of Shewa.

## MATERIALS AND METHODS

### Study sites

The study site covers lentil growing areas in the highlands of Western, Eastern, Northern, North Eastern and South Western Shewa. Geo-referenced soil samples were collected in November 2010 while the crop was growing in the field. Sample collection was made from areas where lentil has been growing for a long period having no previous history of inoculation with the crop.

### Sample collection and isolation of *Rhizobium*

About five kg composite soil samples were collected from the top 15-20cm of the top soil of the rhizosphere region of mature lentil crops from farmers' field and kept separately in surface sterilized plastic bags (70% ethanol was used).

Induction of nodulation was under taken using plant trap method (Vincent, 1970). Three kg of soil from each site was weighed and added into five kg capacity surface sterilized plastic pots (70% ethanol was used). Undamaged and uniform lentil seeds (variety: Alemeya) obtained from Debrezeit Agricultural Research Center. The seeds were surface sterilized with 70% ethanol for three minutes followed by 3% hydrogen per oxide for three minutes (Somasegaran and Hoben, 1994). The seeds were rinsed in five changes of sterile distilled water to remove the effect of sterilizing chemicals. Five seeds per pot were sown and allowed to germinate and latter thinned down to three and allowed to grow for 45 days with regular watering.

Forty five days after planting, plants were uprooted, the soil adhering to the roots removed and undamaged nodules were carefully collected and kept in 50ml capacity beakers for immediate isolation.

Nodules were surface sterilized according to Somasegaran and Hoben (1994) procedures. They were first immersed in 70% (v/v) ethanol for 1 minute, followed by 3% (v/v) hydrogen per oxide for 1 minute. Surface sterilized root nodules were rinsed in sterile distilled water in five changes to remove the effect of surface sterilizing chemicals. The sterilized

root nodules were crushed aseptically in 50ml capacity beaker in laminar air flow hood by sterile crushing glass rods using a drop of sterilized normal saline (0.85% NaCl) solution. Loop-full of the suspensions of the crushed root nodules were streaked with sterile inoculating loop on Yeast Extract Mannitol Agar (YEMA) plates containing 0.025% (w/v) congo red and incubated at $28^0$C for 4 days (Somasegaran and Hoben, 1994). After 4 days, a single colony from each isolate was selected and re-streaked on new YEMA plates for further purification of the isolates. One reference isolate (EAL- 600) from National Soil Testing Center was used for comparison.

**Presumptive tests of the isolates**

The purity of the isolates was determined based on different morphological characteristics of colonies and absorption of congo red in dark incubation (Somasegaran and Hoben, 1994). Gram staining techniques and the growth of colonies on Peptone Glucose Agar (PGA) medium containing 5g of glucose, 10g of peptone, 15g of agar and 10ml stock solution of bromocresol purple in a liter of distilled water were also examined for the confirmatory test of the isolates as root nodulating rhizobia (Lupwayi and Haque, 1994).

**Cultural characteristics**

A loop-full of each of the test isolates from 48 hours old Yeast Extract Mannitol Broth (YEMB) culture approximately containing $10^8$ cells of bacteria per 1ml of culture was inoculated into YEMA to examine the cultural characteristics of the isolates such as colony shape, size, appearance, color, gum production and consistency (Lupwayi and Haque, 1994).

The production of acidity or alkalinity of the isolates were tested by incorporating 25$\mu gml^{-1}$ of bromothymol blue (BTB) indicator in YEMA plate at pH 6.8. A loop-full of 48 hours old culture was streaked on the medium and incubated at $28^0$C for 4 days. Color change to blue or yellow was recorded according to Jordan (1984).

A single colony of each of the isolates was transferred into test tubes containing 10ml YEMB vortexed and incubated on shaker incubator at 120 revolutions per minute (rpm) for 48 hours at $28^0$C. Then, 1ml of each broth culture of the isolate was transferred into 100ml sterilized YEMB in 250ml erlenmeyer flask to allow growth for 48 hours on shaker incubator at 120 rpm at $28^0$C. Immediately after transfer of 1ml broth culture of the isolates, the samples were serially diluted to examine countable colony forming units (CFU) by spread plating method at least from two serial dilutions on YEMA plates and incubated at $28^0$C for 4 days and the process was continued every 4 hours interval for 48 hours. Finally, the generation time (G) was calculated from the logarithmic phase using the formula described by White (1995) as:

$G = \frac{t}{3.3 \log \frac{b}{B}}$; Where: t= time interval

B= number of bacteria at the beginning of a time interval

b= number of bacteria at the end of a time interval

**Evaluation of symbiotic effectiveness of the isolates**

Fifty kg river sand was first soaked for 24 hours with 5L 28.5% sulphuric acid (Lupwayi and Haque, 1994). Then, the sand was rinsed until the water run clear and the pH of the sand water solution became neutral. This acid washing is important to remove all organic nitrogen from the sand. The acid washed sand was then sterilized by autoclaving at $121^0$C for two hours and 3kg of the sterilized sand was added to 70% ethanol surface sterilized 5kg capacity plastic pots containing a hole at the bottom to prevent water logging during growth. To prevent rapid leakage of water and nutrients, the bottom of each pot was lined with sterile absorbent paper before the sand added into the pot.

The seeds of lentil (variety: Alemeya) were used for symbiotic effectiveness evaluation of the isolates on sand culture. Undamaged seeds of uniform size were selected and surface sterilized by first soaking in 70% ethanol for 3 minutes followed by 3% hydrogen per oxide for 3 minutes (Somasegaran and Hoben, 1994). The seeds were rinsed as before with five changes of sterile distilled water to remove traces of sterilizing chemicals. The surface sterilized seeds were placed in sterilized petri dishes containing filter paper inside. Sterile distilled water was added to each petri dish containing seeds to facilitate germination and seeds were incubated at $28^0$C. Six pre-germinated seeds were transferred into each pot by using sterile forceps. Each rhizobial isolate was grown in 50ml capacity erlenmeyer flasks containing 25ml YEMB medium on shaker incubator adjusted at $28^0$C and 120 rpm for 3 days. Finally, each seedling was inoculated with 1ml containing $10^8$ cells/ml broth culture of each isolate from the logarithmic phase by using micropipettes with sterilized tips.

The experiment was statistically laid out with three replications using a complete random block design (CRBD) in the greenhouse, with approximately 12 hours illumination and average day and night temperature of $27^0$C and $10^0$C, respectively. Each block contained a negative control in which nitrogen free chemical nutrient is applied without inoculation and a positive control in which application of nitrogen chemical nutrient in the form of $KNO_3$ 0.05% (w/v) per week without inoculation. In order to maintain the moisture, all pots were supplied with 400ml water every day on the basement of the pots and fertilized with 100ml quarter strength of Broughton and Dilworth N-free medium per week as described in Somasegaran and Hoben (1994).

The plants were harvested after eight weeks of growth and measurements such as nodule number, nodule dry weight and shoot dry weight of the host plants were recorded after oven drying of the samples at $60^0$C for 48 hours.

The symbiotic effectiveness of the isolates was calculated according to the equation proposed by Date *et al*. (1993). The nitrogen fixing effectiveness was classified as highly effective when greater than 80%, effective when 50-80%, lowly effective when 35-50% and ineffective when less than 35%.

$\%\ SE = \frac{SDWPITS}{SDWPSN} * 100$; Where: %SE = Percentage of symbiotic effectiveness; SDWPITS= shoot dry weight of plants inoculated with test strain; SDWPSN= shoot dry weight of plants supplied with nitrogen

**Physiological and biochemical characteristics**

Physiological and biochemical tests were carried out by streaking a loop-full of overnight incubated broth culture of the isolates on a test plate media that were divided into 16 equal parts. All tests were carried out in triplicates at an incubation temperature of $28^0$C for 4 days. Bacterial growth was compared with controls for each test and the results were scored qualitatively either as + for growth or – for no growth (Somasegaran and Hoben, 1994).

**Carbohydrate sources utilization**

The growth of the isolates was tested on fourteen different carbon substrates. Glucose and lactose were among heat stable carbohydrates while galactose, maltose, fructose, sorbitol, manose, xylose, cellobiose, raffinose, succinate, arabinose, citrate and starch were among heat liable carbohydrates. The test was carried out on basal medium containing (per liter) 1g of $K_2HPO_4$, 1g of $KH_2PO_4$, 0.01g of $FeCl_3.6H_2O$, 0.2g of $MgSO_4.7H_2O$, 0.1g of $CaCl_2$, 1g of $(NH_4)_2SO_4$ and 15g of agar. Then, 1g of the carbon sources used for test was added to the basal medium (Amarger *et al*., 1997). The heat stable carbohydrates were autoclaved with the basal medium, while the heat liable carbohydrates were filter sterilized by using sterile 0.2µm pore size disposable membrane filter and added to the medium after it was autoclaved and cooled to approximately $45^0$C.

**Nitrogen sources utilization**

The nitrogen source utilization of the isolates were determined by using fourteen different nitrogen sources, such as lysine, isoleucine, tyrosine, alanine, valine, asparagine, tryptophan, arginine, leucine, phenylalanine, tryptose, glutamine, glycine and urea. They were filter sterilized like heat liable carbon substrates and added at the concentration of 0.5g/l to a similar basal medium used for carbon substrate utilization except omitting ammonium sulfate and supplementing mannitol at a concentration of 1g/l (Amarger *et al*., 1997).

**Salt tolerance**

Salt tolerance of the isolates were determined by inoculating the isolates on YEMA plates supplemented with 0.1%, 0.5%, 1%, 2%, 3%, 4% and 5% (w/v) of NaCl (Maatallah *et al*., 2002).

**pH tolerance**

The pH tolerance of the isolates were tested by adjusting YEMA medium at pH levels of 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, and 10.5 (Bernal and Graham, 2001).

**Temperature tolerance**

The growth of the isolates at different temperature was determined by inoculating a loop-full of suspension cultures on YEMA plates and incubating at the temperatures of 20, 30, 35, 40 and $45^0$C (Maatallah *et al*., 2002).

**Intrinsic antibiotics resistance**

The intrinsic antibiotic resistance of the isolates was tested on YEMA plates containing filter sterilized antibiotics by using 0.2µm pore size disposable membrane filter papers. The antibiotics were incorporated in to YEMA plates at concentrations of (µgml$^{-1}$): tetracycline (5, 10), erythromycin (5, 10), streptomycin (5, 10), novobiocin (5, 10), ampicillin (5, 10), kanamycin (5, 10), neomycin (5, 10). The filter sterilized antibiotics were aseptically added to YEMA medium after autoclaved and cooled approximately to the temperature of $45^0$C (Somasegaran and Hoben, 1994).

**Intrinsic heavy metals resistance**

Resistance to heavy metals was also tested on YEMA plates containing filter sterilized heavy metals by using 0.2µm pore size disposable membrane filter papers in µgml$^{-1}$: $HgCl_2$ (5, 10), $ZnCl_2$ (50,100), $CoCl_2$ (20), $CuCl_2$ (50, 100) and $MnCl_2.4H_2O$ (250, 500). The filter sterilized heavy metals were also added to YEMA medium in similar manner as before (Somasegaran and Hoben, 1994).

**Total nitrogen content**

The total nitrogen content of plant tissue was determined by the modified Kjeldahl method (Sahlemedhin and Taye, 2000). Oven dried shoot samples were ground by using pestle and mortar and used for total nitrogen determination.

**Data analysis**

All data were subjected to one way analysis of variance (ANOVA) using statistical analysis system (SAS) software version 9.00 with generalized linear model. Treatment means were compared using the least significant difference (LSD) test at $P \leq 0.05$.

## RESULT AND DISCUSSION

**Isolation of *Rhizobium***

A total of sixty-three lentil nodulating rhizobia were isolated from four Zones of Shewa: East Shewa (16), North East Shewa (12), North Shewa (10), West Shewa (16) and South West Shewa (9).The isolates were compared to the reference isolate (EAL 600) obtained from the National Soil Testing Center. Some of the isolates showed higher symbiotic effectiveness as compared to the reference isolate.

**Presumptive tests of the isolates**

All the isolates were gram negative rod shape and did not absorb congo red on dark incubation (data not shown). However, six isolates (NSLR-7, NSLR-54, NSLR-62, NSLR-64, NSLR-69 and NSLR-73) showed

slight growth on PGA medium (data not shown). Since all the isolates induced nodulation on the host, they were authenticated as root nodule bacteria. As described by Brockwell (1998) the ability to form nodules along with the subsequent capacity of fixing nitrogen are the widely used means of evaluating the inherent links between the rhizobia and their respective host.

**Cultural characteristics**

The isolates showed different cultural characteristics indicating the existence of diversity among *Rhizobium* nodulating lentil. All the tested isolates displayed fast growth of large mucoid and watery colonies with colony diameter ranging from 2 – 7.5mm at the fourth day of incubation. The largest colony diameter (7.5 mm) was for isolate NSLR-53 from North Shewa of Girar Jarso Wereda and the smallest (2mm) for isolates NSLR-23 (South West Shewa), NSLR-62 and NSLR-72 from West Shewa. The isolates also displayed raised dome and flat shaped with buttery texture colonies. Three highly effective (NSLR-17, NSLR-27 and NSLR-69) and five effective (NSLR-6, NSLR-8, NSLR-12, NSLR-54 and NSLR-65) isolates showed differential generation time ranging from 0.74 hour to 2.69 hours and all the isolates produced acid on YEMA-BTB medium indicating that all isolates were fast growing and can be grouped under the cross inoculation group of *R. leguminosurum var vaceae* (Jordan, 1984).

**Evaluation of symbiotic effectiveness of the isolates**

The sand culture study on nodule induction and symbiotic effectiveness showed significant differences in nodule number, nodule dry weight, shoot dry weight and percentage of total nitrogen. The highest (167) and the lowest (46) nodule number per plant was obtained from plants inoculated with isolates NSLR-2 of East Shewa andNSLR-60 of West Shewa, respectively (Table1). These values are higher than that reported by Shewakena Belayneh (2009) in which he reported highest (45) and lowest (14) nodule number per plant. Isolate NSLR-2 displayed the maximum nodule number per plant. However, its symbiotic effectiveness was low compared to the highly effective and effective isolates.

Nodule dry weight showed significant differences between plants inoculated with different isolates. The maximum and minimum nodule dry weight per plant was 38.4mg for isolate NSLR-69 (from West Shewa) and 15.4mg for isolate NSLR-66 of the same area, respectively Shewakena Belayneh (2009) reported the highest (11 mg) and the lowest 2 mg of nodule dry weight per plant. These indicate that the isolates have larger nodule size as compared to the previous report.

The highest shoot dry weight (0.447g per plant) was recorded from plants inoculated with isolate NSLR-17 from East Shewa and the lowest (0.129g per plant) was from plants inoculated with isolate NSLR-31 from South West Shewa. All the inoculated plants had a higher shoot dry weight compared to the negative control but lower than the positive control. The results were in agreement with Shewakena Belayneh (2009) who reported that shoot dry weight of from the positive control was greater than all the inoculated plants.

The highest value of symbiotic efficiency (92%) was recorded from the isolate NSLR-17 collected from Gimbichu Wereda of East Shewa followed by isolates NSLR-27 (91%) and NSLR-69 (84%) that were collected from Dawo Wereda of South West Shewa and Ambo Wereda of West Shewa, respectively. Fifty nine percent of the isolates tested were found to be effective with symbiotic efficiency of 50 - 80% and 33% of them had low effectivity with symbiotic efficiency of 35 – 50%. Two isolates (NSLR-31 and NSLR-46) were ineffective with symbiotic efficiency of 27 and 32%, respectively. The results were comparable with that reported by Shewakena Belayneh (2009).

**Table 1:** The effect of rhizobia nodulating lentil on nodule number, nodule dry weight, shoot dry weight and plant total nitrogen of lentil on sand culture.

| Isolates | NN plant$^{-1}$ | NDW (mg plant$^{-1}$) | SDW (gm plant$^{-1}$) | %SE | TN (%) |
|---|---|---|---|---|---|
| NSLR-1 | 61±6$^{s-v}$ | 20.78±5.36$^{j-s}$ | 0.254±0.024$^{m-r}$ | 53 | 2.93±0.19$^{a-n}$ |
| NSLR-2 | 167±39$^{a}$ | 21.28±1.67$^{i-j}$ | 0.243±0.035$^{p-t}$ | 50 | 2.65±0.18$^{l-v}$ |
| NSLR-3 | 132±56$^{a-m}$ | 25.83±10.09$^{d-n}$ | 0.193±0.006$^{v-x}$ | 40 | 2.51±0.34$^{p-w}$ |
| NSLR-4 | 154±12$^{a-f}$ | 29.11±1.84$^{b-l}$ | 0.251±0.029$^{m-r}$ | 52 | 2.84±0.16$^{d-p}$ |
| NSLR-5 | 78±8$^{m-v}$ | 25.00±0.60$^{f-r}$ | 0.196±0.017$^{vwx}$ | 41 | 2.35±0.17$^{s-w}$ |
| NSLR-6 | 131±19$^{a-m}$ | 23.22±1.34$^{g-s}$ | 0.346±0.019$^{de}$ | 72 | 3.09±0.18$^{a-i}$ |
| NSLR-7 | 164±53$^{ab}$ | 31.78±3.5$^{a-g}$ | 0.271±0.037$^{i-p}$ | 56 | 2.80±0.15$^{f-q}$ |
| NSLR-8 | 155±9$^{a-e}$ | 34.39±5.66$^{a-e}$ | 0.345±0.053$^{def}$ | 71 | 2.95±0.07$^{a-n}$ |
| NSLR-9 | 102±29$^{e-u}$ | 18.34±1.76$^{o-s}$ | 0.189±0.006$^{vwx}$ | 39 | 2.84±0.13$^{e-p}$ |
| NSLR-10 | 110±22$^{b-t}$ | 26.28±2.01$^{d-q}$ | 0.242±0.027$^{p-u}$ | 50 | 2.99±0.15$^{a-l}$ |
| NSLR-11 | 107±63$^{d-t}$ | 22.72±4.72$^{g-s}$ | 0.186±0.007$^{vwx}$ | 38 | 2.87±0.04$^{d-p}$ |
| NSLR-12 | 149±22$^{a-g}$ | 27.94±4.57$^{b-n}$ | 0.385±0.028$^{cd}$ | 80 | 3.20±0.09$^{a-e}$ |
| NSLR-13 | 140±50$^{a-j}$ | 22.11±5.11$^{i-s}$ | 0.247±0.022$^{o-s}$ | 51 | 2.69±0.13$^{k-u}$ |
| NSLR-15 | 117±57$^{a-r}$ | 28.17±3.50$^{b-n}$ | 0.204±0.010$^{s-w}$ | 42 | 2.61±0.13$^{m-w}$ |
| NSLR-16 | 161±3$^{abc}$ | 29.39±6.85$^{a-j}$ | 0.253±0.030$^{m-r}$ | 52 | 2.93±0.08$^{a-n}$ |
| NSLR-17 | 102±28$^{e-u}$ | 34.78±1.07$^{abcd}$ | 0.447±0.086$^{ab}$ | 92 | 3.26±0.22$^{ab}$ |
| NSLR-18 | 88±15$^{j-v}$ | 36.78±4.34$^{ab}$ | 0.249±0.024$^{m-s}$ | 51 | 2.83±0.42$^{e-q}$ |

| | | | | | |
|---|---|---|---|---|---|
| NSLR-19 | 75±22$^{n-v}$ | 19.89±4$^{m-s}$ | 0.185±0.015$^{vwx}$ | 38 | 2.60±0.04$^{n-w}$ |
| NSLR-20 | 108±31$^{c-t}$ | 26.61±7.48$^{d-p}$ | 0.263±0.014$^{k-q}$ | 54 | 2.83±0.15$^{e-q}$ |
| NSLR-22 | 51±23$^{uvw}$ | 27.78±5.05$^{b-n}$ | 0.326±0.021$^{efgh}$ | 67 | 3.22±0.07$^{abcd}$ |
| NSLR-23 | 103±19$^{d-u}$ | 24.06±4.88$^{f-s}$ | 0.305±0.015$^{e-k}$ | 63 | 2.73±0.25$^{i-s}$ |
| NSLR-24 | 113±49$^{a-s}$ | 22.56±8.60$^{h-s}$ | 0.293±0.019$^{g-m}$ | 61 | 2.59±0.22$^{n-w}$ |
| NSLR-25 | 117±30$^{a-r}$ | 34.56±7.46$^{a-e}$ | 0.268±0.01$^{j-q}$ | 52 | 2.90±0.55$^{b-o}$ |
| NSLR-26 | 96±72$^{g-v}$ | 21.11±9.72$^{i-s}$ | 0.293±0.01$^{g-n}$ | 51 | 2.91±0.42$^{b-o}$ |
| NSLR-27 | 101±20$^{f-u}$ | 34.61±7.93$^{a-e}$ | 0.442±0.04$^{ab}$ | 91 | 3.26±0.18$^{ab}$ |
| NSLR-28 | 101±50$^{f-u}$ | 29.00±5.75$^{b-m}$ | 0.188±0.02$^{vwx}$ | 39 | 2.61±0.07$^{m-w}$ |
| NSLR-29 | 92±19$^{i-v}$ | 30.11±4.96$^{a-i}$ | 0.192±0.01$^{vwx}$ | 40 | 2.29±0.07$^{vwx}$ |
| NSLR-30 | 84±12$^{k-v}$ | 21.5±1.32$^{i-s}$ | 0.290±0.037$^{g-o}$ | 60 | 2.96±0.33$^{a-n}$ |
| NSLR-31 | 120±41$^{a-p}$ | 19.94±6.55$^{l-s}$ | 0.129±0.013$^{yz}$ | 27 | 1.92±0.16$^{x}$ |
| NSLR-33 | 66±28$^{q-v}$ | 22.78±7.03$^{g-s}$ | 0.252±0.026$^{m-r}$ | 52 | 2.64±0.13$^{l-w}$ |
| NSLR-34 | 75±13$^{o-v}$ | 26.89±5.23$^{d-o}$ | 0.224±0.013$^{q-v}$ | 46 | 2.36±0.14$^{s-w}$ |
| NSLR-36 | 70±40$^{p-v}$ | 20.39±0.85$^{k-s}$ | 0.188±0.011$^{vwx}$ | 39 | 2.66±0.24$^{l-v}$ |
| NSLR-37 | 116±66$^{a-r}$ | 17.67±3.58$^{pqrs}$ | 0.184±0.009$^{vwx}$ | 38 | 2.55±0.10$^{o-w}$ |
| NSLR-38 | 109±1$^{c-t}$ | 21.11±1.63$^{i-s}$ | 0.309±0.047$^{e-j}$ | 64 | 3.23±0.10$^{abc}$ |
| NSLR-39 | 120±45$^{a-q}$ | 18.11±5.29$^{o-s}$ | 0.198±0.022$^{uvwx}$ | 41 | 2.46±0.35$^{q-w}$ |
| NSLR-40 | 140±69$^{a-j}$ | 26.56±15.52$^{d-p}$ | 0.244±0.019$^{n-s}$ | 50 | 2.90±0.10$^{b-o}$ |
| NSLR-41 | 108±24$^{c-t}$ | 21.50±1.16$^{i-s}$ | 0.192±0.021$^{vwx}$ | 40 | 2.75±0.12$^{h-r}$ |
| NSLR-44 | 115±23$^{a-r}$ | 28.17±1.31$^{b-n}$ | 0.248±0.022$^{n-s}$ | 51 | 2.86±0.21$^{c-p}$ |
| NSLR-45 | 115±61$^{a-r}$ | 24.94±5.48$^{f-r}$ | 0.253±0.021$^{m-r}$ | 52 | 2.95±0.20$^{a-n}$ |
| NSLR-46 | 73±26$^{o-v}$ | 17.28±3.71$^{qrs}$ | 0.154±0.005$^{xyz}$ | 32 | 2.31±0.27$^{uvw}$ |
| NSLR-48 | 107±37$^{c-t}$ | 27.61±2.76$^{b-n}$ | 0.173±0.007$^{wxy}$ | 36 | 2.27±0.11$^{wx}$ |
| NSLR-49 | 94±6$^{h-v}$ | 31.55±2.43$^{a-h}$ | 0.303±0.015$^{e-l}$ | 63 | 3.15±0.23$^{a-g}$ |
| NSLR-51 | 61±8$^{stuv}$ | 33.22±12.31$^{a-f}$ | 0.260±0.035$^{l-q}$ | 54 | 2.54±0.10$^{o-w}$ |
| NSLR-52 | 129±20$^{a-n}$ | 24.33±6.02$^{f-s}$ | 0.327±0.041$^{efg}$ | 68 | 3.29±0.15$^{a}$ |
| NSLR-53 | 110±23$^{c-t}$ | 26.05±0.25$^{d-q}$ | 0.249±0.028$^{m-s}$ | 51 | 2.72±0.24$^{i-s}$ |
| NSLR-54 | 65±6$^{r-v}$ | 36.11±5.68$^{abc}$ | 0.343±0.044$^{def}$ | 71 | 2.98±0.07$^{a-m}$ |
| NSLR-55 | 106±39$^{e-t}$ | 22.17±8.35$^{i-s}$ | 0.249±0.041$^{m-s}$ | 51 | 2.76±0.09$^{h-r}$ |
| NSLR-56 | 86±29$^{j-v}$ | 27.00±3.97$^{c-o}$ | 0.260±0.017$^{l-q}$ | 54 | 2.94±0.51$^{a-n}$ |
| NSLR-57 | 148±9$^{a-g}$ | 22.33±1.09$^{i-s}$ | 0.248±0.017$^{o-s}$ | 51 | 2.79±0.05$^{g-r}$ |
| NSLR-58 | 102±21$^{e-t}$ | 25.50±5.20$^{e-q}$ | 0.301±0.019$^{f-l}$ | 62 | 3.07±0.10$^{a-j}$ |
| NSLR-60 | 46±12$^{vw}$ | 15.89±2.22$^{rs}$ | 0.259±0.022$^{l-r}$ | 54 | 3.08±0.19$^{a-i}$ |
| NSLR-61 | 148±34$^{a-h}$ | 26.28±7.42$^{d-q}$ | 0.313±0.010$^{e-i}$ | 65 | 3.11±0.20$^{a-h}$ |
| NSLR-62 | 144±18$^{a-i}$ | 29.22±4.01$^{c-k}$ | 0.242±0.042$^{p-u}$ | 50 | 2.70±0.38$^{j-t}$ |
| NSLR-63 | 84±27$^{k-v}$ | 26.67±0.84$^{d-p}$ | 0.199±0.022$^{t-x}$ | 41 | 2.58±0.32$^{n-w}$ |
| NSLR-64 | 103±25$^{d-u}$ | 28.44±6.57$^{b-n}$ | 0.281±0.028$^{h-p}$ | 58 | 2.88±0.07$^{b-p}$ |
| NSLR-65 | 156±33$^{abcd}$ | 27.11±10.90$^{c-o}$ | 0.348±0.042$^{de}$ | 72 | 3.17±0.41$^{a-f}$ |
| NSLR-66 | 104±12$^{d-u}$ | 15.39±3.51$^{s}$ | 0.199±0.012$^{tuvw}$ | 41 | 2.33±0.32$^{tuvw}$ |
| NSLR-67 | 134±16$^{a-l}$ | 19.72±5.29$^{n-s}$ | 0.190±0.032$^{vwx}$ | 39 | 2.60±0.28$^{m-w}$ |
| NSLR-68 | 79±15$^{m-v}$ | 22.89±6.86$^{g-s}$ | 0.215±0.033$^{r-w}$ | 44 | 2.77±0.19$^{h-r}$ |
| NSLR-69 | 57±34$^{tuv}$ | 38.44±8.63$^{a}$ | 0.406±0.011$^{bc}$ | 84 | 3.16±0.41$^{a-g}$ |
| NSLR-71 | 124±48$^{a-o}$ | 24.33±9.94$^{f-s}$ | 0.205±0.015$^{s-w}$ | 42 | 2.42±0.36$^{r-w}$ |
| NSLR-72 | 137±55$^{a-k}$ | 22.89±2.04$^{g-s}$ | 0.214±0.045$^{r-w}$ | 44 | 2.26±0.31$^{wx}$ |
| NSLR-73 | 82±20$^{l-v}$ | 29.78±2.95$^{a-j}$ | 0.199±0.033$^{t-x}$ | 41 | 2.30±0.42$^{vw}$ |
| EAL-600 | 121±3$^{a-p}$ | 24.44±0.67$^{f-s}$ | 0.306±0.026$^{e-j}$ | 63 | 2.98±0.10$^{a-m}$ |
| positive control | - | - | 0.484±0.033$^{a}$ | - | 3.06±0.24$^{a-k}$ |
| negative control | - | - | 0.112±0.010$^{yz}$ | - | 0.69±0.15$^{y}$ |
| CV% | 31.804 | 22.887 | 10.758 | - | 8.541 |
| $LSD_{0.05}$ | 53.834 | 9.201 | 0.045 | - | 0.379 |

NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, %SE= % of symbiotic effectiveness, TN= total nitrogen, CV= coefficient of variation, LSD= least significant difference.
Numbers in the same column followed by the same letter(s) are not significantly different at p≤0.05.

**Physiological and biochemical characteristics**

Biochemical and physiological characterization of isolates revealed the existence of versatile and tolerant lentil nodulating rhizobial isolates (Table 2). Many of the isolates were versatile in utilization of different carbon and nitrogen sources. The highly effective isolate NSLR-69 utilized all the tested nitrogen sources. It showed tolerance to 2% of NaCl and a pH range of 5.5-10.5. In addition, it showed tolerance to 78% of the tested antibiotics and to 70%of the tested heavy metals. The isolates NSLR-54, NSLR-51 and NSLR-64 have also utilized all tested nitrogen sources. However, these isolates had a weak tolerance to salt, pH and antibiotic. Aregu Amsalu (2006) also isolated field pea nodulating rhizobial isolates that were versatile in utilization of different carbohydrate substrates and resistant to different antibiotics and heavy metals at different concentrations as well as tolerant to a pH range of 4.5 -9.5, incubation temperature ranging from 5-40$^{o}$C and NaCl concentrations ranging from 0.1-6%.

**Table 2:** Summary of biochemical and physiological properties of highly effective and effective isolates of lentil nodulating rhizobia from different sampling sites.

| Isolates | site | Eff | Carbon source (14)* | Nitrogen source (14)* | NaCl (%) | pH range | Antibiotics (14)* | Heavy metals( 10)* | T ($^{0}$C) |
|---|---|---|---|---|---|---|---|---|---|
| NSLR-17 | E/Shewa | HE | 12 | 12 | 0.5 | 6.5-10.5 | 7 | 5 | 20-35 |
| NSLR-27 | S/W/Shewa | HE | 12 | 13 | 0.5 | 6.5-10.5 | 2 | 3 | 20-40 |
| NSLR-69 | W/Shewa | HE | 6 | 14 | 2.0 | 5.5-10.5 | 11 | 7 | 20-35 |
| NSLR-12 | E/Shewa | E | 12 | 12 | 0.1 | 6.5-10.5 | 6 | 5 | 20-35 |
| NSLR-6 | E/Shewa | E | 12 | 12 | 0.5 | 5.5-10.5 | 5 | 5 | 20-35 |
| NSLR-65 | W/Shewa | E | 12 | 12 | 0.1 | 6.0-10.0 | 6 | 6 | 20-30 |
| NSLR-8 | E/Shewa | E | 11 | 13 | 4.0 | 5.5-10.5 | 11 | 5 | 20-35 |
| NSLR-54 | N/Shewa | E | 11 | 14 | 1.0 | 5.5-10.0 | 9 | 8 | 20-35 |
| NSLR-52 | N/Shewa | E | 12 | 12 | 1.0 | 6.0-10.5 | 7 | 6 | 20-35 |
| NSLR-22 | S/W/Shewa | E | 12 | 12 | 0.5 | 6.5-10.5 | 6 | 4 | 20-30 |
| NSLR-61 | W/Shewa | E | 12 | 12 | 0.1 | 5.5-10.5 | 7 | 5 | 20-30 |
| NSLR-38 | N/E/Shewa | E | 9 | 11 | 0.5 | 6.5-10.5 | 2 | 2 | 20-30 |
| NSLR-23 | S/W/Shewa | E | 12 | 11 | 0.5 | 6.5-10.5 | 6 | 5 | 20-35 |
| NSLR-49 | N/Shewa | E | 8 | 13 | 5 | 6.5-10.0 | 11 | 5 | 20-30 |
| EAL-600 | - | E | 11 | 12 | 0.1 | 6.5-10.0 | 5 | 5 | 20-30 |
| NSLR-58 | W/Shewa | E | 12 | 12 | 0.1 | 5.5-10.0 | 4 | 5 | 20-30 |
| NSLR-24 | S/W/Shewa | E | 12 | 12 | 0.1 | 6.5-10.5 | 6 | 5 | 20-35 |
| NSLR-30 | N/E/Shewa | E | 10 | 9 | 0.1 | 6.0-10.0 | 3 | 5 | 20-30 |
| NSLR-64 | W/Shewa | E | 12 | 14 | 0.1 | 5.5-10.0 | 3 | 5 | 20-35 |
| NSLR-7 | E/Shewa | E | 9 | 13 | 1 | 5.5-10.5 | 8 | 4 | 20-35 |
| NSLR-20 | S/W/Shewa | E | 6 | 9 | 0.1 | 6.5-10.0 | 3 | 6 | 20-30 |
| NSLR-51 | N/Shewa | E | 10 | 14 | 1 | 6.5-10.5 | 4 | 4 | 20-35 |
| NSLR-56 | W/Shewa | E | 12 | 13 | 0.5 | 6.0-10.5 | 4 | 4 | 20-35 |
| NSLR-60 | W/Shewa | E | 12 | 12 | 0.1 | 6.0-10.0 | 3 | 6 | 20-35 |
| NSLR-1 | E/Shewa | E | 12 | 12 | 0.1 | 6.5-10.5 | 5 | 5 | 20-35 |
| NSLR-4 | E/Shewa | E | 8 | 10 | 0.5 | 6.5-10.0 | 6 | 4 | 20-30 |
| NSLR-16 | E/Shewa | E | 12 | 12 | 0.1 | 6.5-10.5 | 9 | 6 | 20-35 |
| NSLR-25 | S/W/Shewa | E | 4 | 7 | 2 | 6.5-10.5 | 6 | 3 | 20-35 |
| NSLR-33 | N/E/Shewa | E | 12 | 12 | 0.5 | 6.5-10.5 | 8 | 5 | 20-35 |
| NSLR-45 | N/Shewa | E | 12 | 12 | 1 | 6.5-10.0 | 4 | 5 | 20-35 |
| NSLR-13 | E/Shewa | E | 12 | 12 | 1 | 6.5-10.5 | 7 | 5 | 20-35 |
| NSLR-18 | S/W/Shewa | E | 12 | 12 | 0.5 | 6.5-10.0 | 5 | 5 | 20-35 |
| NSLR-26 | S/W/Shewa | E | 12 | 13 | 0.5 | 6.5-10.5 | 6 | 5 | 20-35 |
| NSLR-44 | N/Shewa | E | 7 | 11 | 0.1 | 7.0-10.0 | 1 | 4 | 20-30 |
| NSLR-53 | N/Shewa | E | 12 | 12 | 0.1 | 6.0-10.5 | 7 | 6 | 20-35 |
| NSLR-55 | N/Shewa | E | 11 | 13 | 0.1 | 6.0-10.0 | 5 | 6 | 20-40 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| NSLR-57 | W/Shewa | E | 12 | 13 | 0.1 | 5.5-10.0 | 3 | 5 | 20-35 |
| NSLR-2 | E/Shewa | E | 12 | 12 | 0.5 | 6.5-10.0 | 4 | 5 | 20-30 |
| NSLR-10 | E/Shewa | E | 12 | 12 | 0.1 | 5.5-10.5 | 8 | 6 | 20-35 |
| NSLR-40 | N/E/Shewa | E | 10 | 12 | 0.5 | 6.5-10.5 | 5 | 6 | 20-30 |
| NSLR-62 | W/Shewa | E | 12 | 12 | 1 | 5.5-10.5 | 11 | 5 | 20-35 |

Eff = effectiveness

* = the number in brackets indicates the different sources or concentrations used to test utilization or tolerance of the isolates.

**Carbohydrate sources utilization**

Most of the isolates utilized a large number (> 80%) of the tested carbohydrates (data not shown). Stowers (1985) has reported that rhizobia have the ability to utilize a wide variety of carbon sources for growth and energy with several pathways available for carbon catabolism.

However, only three isolates were able to catabolize citrate and none of the isolates managed to utilize starch. In the case of starch, this result is contrary to what Shewakena Belayneh (2009) reported, in that all isolates of lentil nodulating rhizobia from Western and Northern Shewa utilized starch as carbon source. In case of citrate, our results agree with the report of Jordan (1984) that citrate can be utilized by limited number of rhizobia. Lindstrom and Lehtomaki (1988) and Gebremeskel Gebremariam (2007) also reported three out of thirteen and two out of twenty isolates were able to utilize citrate, respectively.

**Nitrogen sources utilization**

Almost all isolates utilized isoleucine, phenyl alanine, valine and leucine. More than 95% of the tested isolates were able to utilize the tested nitrogen sources except urea, glycine and alanine indicating that the isolates have a wide range of nitrogen sources (data not shown). Shewakena Belayneh (2009) also observed lentil nodulating rhizobial isolates that can catabolize a wide range of amino acids.

**Salt tolerance**

All of the tested isolates tolerated 0.1% NaCl concentration on YEMA plates. Only 54.7, 25 and 7.8% of the tested isolates tolerated salt concentrations of 0.5, 1 and 2%, respectively. Graham and Parker (1964) reported that all strains of *R. leguminosarum* failed to tolerate 2 – 3% of NaCl concentrations. A few isolates (4.7%) were able to tolerate 3 - 4% of NaCl concentrations. Only two isolates (NSLR-41 and NSLR-49) showed the highest salt tolerance (5% NaCl). Gebremeskel Gebremariam (2007) also isolated faba bean rhizobial isolates that could tolerate up to 6% NaCl from Northern Ethiopia.

**pH tolerance**

Like other physiological tests, the isolates showed significant variation in response to tolerance to different pH levels. Almost all isolates showed tolerance to a pH range of 6.5-10.0. Few isolates (25%) showed tolerance to a pH level of 5.5 although, more than half (57.8%) of the isolates were found to tolerate high pH (10.5) indicating that the isolates are sensitive to lower pH values. Jordan (1984) also reported that fast growing rhizobia are more sensitive to lower pH than slow growing rhizobia. Shewakena Belayneh (2009) also isolated lentil nodulating rhizobial isolates that tolerated pH level of 10.5.

**Temperature tolerance**

The growth response of the isolates to various incubation temperatures greater than $20^0$C revealed the existence of thermo tolerance variation among the isolates. All the isolates grew well at incubation of temperatures 20, 25 and $30^0$C. Only 68.8% of the isolates were able to grow at $35^0$C and two isolates (NSLR-27 and NSLR-55) grew at temperature of $40^0$C. None of the isolates grew at incubation temperature of $45^0$C. Zerihun Belay (2006) and Getaneh Tesfaye (2008) reported faba bean nodulating rhizobial isolates survive up to $40^0$C incubation temperatures. Moawad and Beck (1991) also reported that lentil nodulating rhizobial isolates that were capable of growing at incubation temperatures of 35 to $40^0$C.

**Intrinsic antibiotics resistance**

The tested isolates showed variations in growth response to different types of antibiotics and concentrations (data not shown). The isolates were generally resistant to neomycin and erythromycin and sensitive to neomycin, tetracycline, streptomycin, ampicillin and kanamycin at concentrations of 5 and 10µgml$^{-1}$. Shewakena Belayneh (2009) also reported that 95% of lentil nodulating rhizobia from Northern and Western Shewa tolerated erythromycin at concentration of 2.5µgml$^{-1}$ and 28 and 35% of the isolates were resistant to kanamycine and streptomycin, respectively, at concentration of 10µgml$^{-1}$. Only 6.3% of the isolates were resistant to neomycin at concentration of 10µgml$^{-1}$ whereas 48.4% at concentration of 5µgml$^{-1}$.

**Intrinsic heavy metals resistance**

Based on intrinsic heavy metals resistance, 100% of the isolates were resistant to $MnCl_2.H_2O$ at concentrations of 250µgml$^{-1}$ and 500µgml$^{-1}$. 87.5% and 71.9% of the isolates were also resistant to $CoCl_2$ at concentrations of 10µgml$^{-1}$ and 20µgml$^{-1}$, respectively. None of the isolates were able to resist $HgCl_2$ at concentration of 10µgml$^{-1}$ and 78.1% at concentration of 5µgml$^{-1}$. Daniel Muleta (2009) has reported that isolates of chickpea nodulating rhizobia were sensitive to Hg at concentration of 10µgml$^{-1}$ whereas all of the isolates were resistant to Mn at concentration of 500µgml$^{-1}$. Only four isolates (NSLR-41, NSLR-54, NSLR-69 and NSLR-71) and five isolates (NSLR-41, NSLR-54, NSLR-63, NSLR-69 and NSLR-71) were capable of resisting $ZnCl_2$ at concentrations of 100µgml$^{-1}$ and 50µgml$^{-1}$, respectively. The isolates NSLR-8 and NSLR-63 grew on medium containing $CuCl_2$ at concentration of 100µgml$^{-1}$ whereas 60.9% of the isolates were resistant at concentration of 50µgml$^{-1}$. Daniel Muleta (2009) also reported that 16.2% of the isolates of chickpea nodulating rhizobia were resistant to Cu at concentration of 50µgml$^{-1}$.

**Total nitrogen content**

Compared to the positive and negative controls, 17.2% of the inoculated plants had higher total nitrogen content. Plants inoculated with isolate NSLR-52 (from Girar Jarso Wereda of North Shewa) and NSLR-17 (from Gimbichu Wereda of East Shewa) had the highest total nitrogen content of 3.29% and 3.26%, respectively. Plants inoculated with isolate NSLR-31 (from Alelitu Wereda of North East Shewa) had the lowest total nitrogen content of 1.92%. Our results are comparable to that reported for field pea (Aregu Amsalu, 2006) in a sand culture experiment.

In conclusion, this particular study showed the existence of variability among lentil nodulating rhizobial isolates from the highlands of Shewa. Majority of the isolates were able to utilize several carbon and nitrogen sources. Some of the isolates were resistant to different types of antibiotics and heavy metals at different concentrations suggesting that the isolates are competitive enough to effectively colonize the rhizosphere. Based on cultural, physiological and biochemical characteristics and symbiotic effectiveness, the isolates such as NSLR-17, NSLR-27 and NSLR-69 were found to be superior and we recommend them to be one of the candidates for commercial production of lentil nodulating rhizobial inoculant. However, field evaluations are suggested before commercial use of the inoculants.

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