Symbiotic Effectiveness and Phenotypic Characterization of Native Rhizobia Nodulating Lentil (*Lens culinaris Medik)* Collected from Highlands of Shewa, Ethiopia

Getahun Mitiku[1]*, Fassil Assefa[2] and Asfaw Hailemariam[3]
[1]Holeta Agricultural Research Center, P.O.Box 32, Holeta, Ethiopia;
[2]Addis Ababa University, P.O.Box 1176, Addis Ababa, Ethiopia
[3]Menagesha Biotech Industry PLC, P.O.Box 1453, Addis Ababa, Ethiopia

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. Sixty three lentil (Lens Culinaris Medik) nodulating rhizobial isolates were characterized for morphological and physiological properties under laboratory condition and evaluated for symbiotic effectiveness under greenhouse condition. In sand culture, the isolates showed significant (p=0.05) 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. The highest shoot dry weight (0.447 g per plant) was recorded from plants inoculated with the isolate NSLR-17 and the lowest (0.129 g per plant) from plants inoculated with isolate NSLR-31. The plants inoculated with the isolate NSLR-2 and NSLR-60 showed the highest (167) and the lowest (46) nodule number per plant, respectively. The maximum and minimum nodule dry weight per plant was obtained from the isolate NSLR-69 (38.4 mg) and isolate NSLR-66 (15.4 mg), respectively. A few (5%) of the isolates were highly effective while 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, some isolates are competent enough to colonize the rhizosphere under different edaphic and environmental conditions.

Key words: Lentil, Rhizobial inoculants, Symbiotic effectiveness

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 tons to 3.8 million tones mainly due to the adoption of improved varieties in combination with the 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 households making local consumption and price of this crop higher than most pulses.

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. Nitrogen is converted into utilizable form of ammonia and nitrate through industrial fertilizer production and biological nitrogen fixation (Postgate, 1998). However, the major and ecofreidly conversion of N_2 is brought through biological nitrogen fixation. 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 by annually converting approximately 120 million tones of atmospheric nitrogen in to ammonia (Freiberg, *et al.* 1997).

Lentil is 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).

Lentil has the potential to fix nitrogen by forming endosymbiotic association with *Rhizobium leguminosurum* (Zafar-ul-hye, 2008). 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 greenhouse condition. *Rhizobium* inoculation and nitrogen fertilizer application significantly affected nodule number, nodule dry weight, shoots dry weight, grain yield and yield component of lentil at Huletegna Choroko soil under field condition (Wondewosen Tana *et al*, 2016). Similar trend was also observed under control condition using growth pouch experiment (Anteneh Argaw, 2012). 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 was aimed at identifying the best lentil nodulating rhizobial isolates which enhance production and productivity of lentil from the major growing areas of Shewa.

Materials and Methods

Soil sampling 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 while the crop was growing in the field. Soil sample collection was made from in between lentil crops where no previous history of inoculation with the crop.

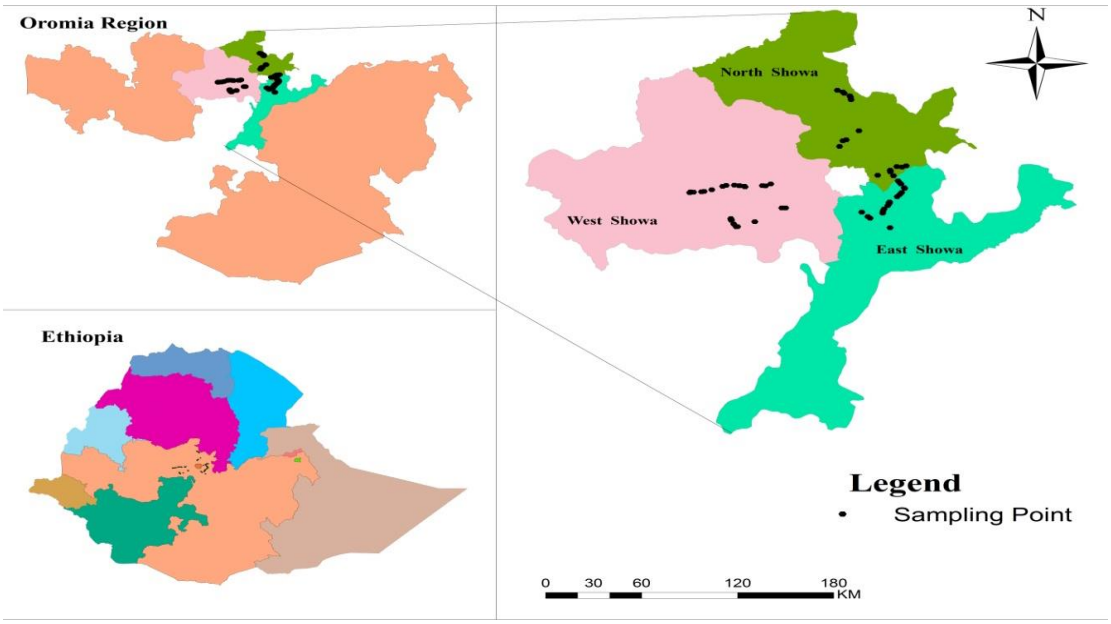

Figure: Map of soil sampling sites

Sample collection and isolation of Rhizobium

About 5 kg composite soil samples were collected from 0-20 cm of the top soil of the rhizosphere region of mature lentil crops from farmers' field and kept separately in surface sterilized (70% ethanol) plastic bags.

Nodulation was induced by plant trap method (Vincent, 1970). Three kg of soil from each site was weighed and added into 5 kg size surface sterilized (70% ethanol) plastic pots. Lentil seeds (variety: Alemeya) obtained from Debrezeit Agricultural Research Center. The seeds were surface sterilized with 70% ethanol for three minutes followed by 3% similar treatment with hydrogen per oxide for three minutes. 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 grow and later on two seedlings were thinned out and the rest three allowed to grow for 45 days with regular watering. Later on, plants were uprooted, the soil adhering to the roots removed and undamaged nodules were carefully detached and kept in 50 ml size beakers for immediate isolation.

Nodules were surface sterilized according to Somasegaran and Hoben (1994) procedure. The whole nodules from the three plants 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. To verify whether the nodules were surface-sterilized or not, the last rinse of the nodules were streaked on a Yeast Extract Mannitol Agar (YEMA) plates. The plates were incubated and observed for growth of microorganisms. The sterilized root nodules were crushed aseptically in 50 ml capacity beaker containing a drop of sterilized normal saline (0.85% NaCl) solution in laminar air flow hood by using sterile crushing glass rods. A loop-ful of the suspensions of bacteria from 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 28^0C 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 commercial inoculant (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 5 g of glucose, 10 g of peptone, 15 g of agar and 10 ml stock solution of bromocresol purple in a liter of distilled water were also examined for the presumptive test of the isolates as root nodulating rhizobia (Lupwayi and Haque, 1994).

Morphological characteristics

A loop-ful of each of the test isolates from 48 hours old Yeast Extract Mannitol Broth (YEMB) culture approximately containing 10^8 cells of bacteria per 1 ml of culture was inoculated into YEMA to examine the morphological 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 μgml^{-1} of bromothymol blue (BTB) indicator in YEMA plate at pH 6.8. A loop-ful of 48 hours old culture was streaked on the medium and incubated at 28^0C for 4 days. Color change to blue or yellow was recorded according to Jordan (1984).

A single colony of each of the isolate was transferred into test tube containing 10 ml YEMB vortexed and incubated on shaker incubator at 120 revolutions per minute (rpm) for 48 hours at 28^0C. Then, 1 ml of each broth culture of the isolate was transferred into 100 ml sterilized YEMB in 250 ml Erlenmeyer flask to allow growth for 48 hours on shaker incubator at 120 rpm at 28^0C. Immediately after the transfer of 1 ml 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 28^0C 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 5 L 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. The acid washed sand was then sterilized by autoclaving at 121^0C for two hours and 3 kg of the sterilized sand was added to 70% ethanol surface sterilized 5 kg size 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 was 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 28^0C. Six pre-germinated seeds were transferred into each pot by using sterile forceps. Each rhizobial isolate was grown in 50 ml size Erlenmeyer flasks containing 25 ml YEMB medium on shaker incubator adjusted at 28^0C with 120 rpm for 3 days. Finally, a one week 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 laid out in a complete random design (CRD) with three replications in the greenhouse, with approximately 12 hours illumination and average day and night temperature of 27^0C and 10^0C, respectively. Each replication contained a negative control (-N) in which nitrogen free chemical nutrient is applied without inoculation and a positive control (+N) in which 100 ml nitrogen in the form of KNO_3 0.05% (w/v) was applied per week without inoculation. In order to maintain the moisture, all pots were supplied with 400 ml water every day on the basement of the pots and fertilized with 100 ml 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 for nodule dry weight and shoot dry weight of the host plants were recorded after oven drying of the samples at 60^0C for 48 hours. In addition, the nodule number was also recorded.

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%.

$$\%\ \text{symbiotic effectiveness} = \frac{\text{shoot dry weight of plants inoculated with test strain}}{\text{shoot dry weight of plants supplied with nitrogen}} * 100$$

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 28^0C 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) 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.01 g of $FeCl_3.6H_2O$, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2$, 1 g of $(NH_4)_2SO_4$ and 15 g of agar. Then, 1 g of the carbon sources used for the 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 0.2 μm pore size disposable membrane filter and added to the medium after it was autoclaved and cooled to approximately 45^0C.

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 0.5 g/l to a similar basal medium used for carbon substrate utilization except omitting ammonium sulfate and supplementing mannitol at a concentration of 1 g/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 values was determined by inoculating a loop-ful of cultures on YEMA plates and incubating at the temperatures of 20, 30, 35, 40 and 45^0C (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 0.2 μm 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 45^0C (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 0.2 μm 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 $P \leq 0.05$.

Results and Discussion

Isolation of Rhizobium

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) (Table 1). The isolates were compared to the commercial inoculant for lentil (EAL-600) obtained from the National Soil Testing Center. Some of the isolates showed higher symbiotic effectiveness as compared to the commercial inoculant.

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.

Morphological characteristics

The isolates showed different morphological 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 2–7.5 mm at the fourth day of incubation. The largest colony diameter (7.5 mm) was for isolate NSLR-53 from North Shewa of Girar Jarso district and the smallest (2 mm) 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 ($p=0.05$) differences in nodule number, nodule dry weight, shoot dry weight and percentage of total nitrogen (Table 1). The highest (167) and the lowest (46) nodule number per plant was obtained from plants inoculated with isolates NSLR-2 of East Shewa and NSLR-60 of West Shewa, respectively. These values are higher than that of Shewakena Belayneh (2009), who reported the 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. This indicated the existence of ineffective nodules which could not contribute to the biological nitrogen fixation.

Nodule dry weight showed significant ($p=0.05$) differences between plants inoculated with different isolates (Table 1). The maximum and minimum nodule dry weight per plant was 38.4 mg for isolate NSLR-69 (from West Shewa) and 15.4 mg 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. Nodule dry weight is positively correlated with symbiotic nitrogen fixation. Hence, the isolates have higher nitrogen fixing potential as compared to the previous report.

The highest shoot dry weight (0.447 g per plant) was recorded from plants inoculated with isolate NSLR-17 from east Shewa and the lowest (0.129 g per plant) was from plants inoculated with isolate NSLR-31 from south west Shewa (Table 1). 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 that of Shewakena Belayneh (2009), who reported that shoot dry weight of 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 district of east Shewa followed by isolates NSLR-27 (91%) and NSLR-69 (84%) that were collected from Dawo district of south west Shewa and Ambo district of west Shewa, respectively (Table 1). Fifty nine percent of the isolates tested were found to be effective with symbiotic efficiency ranging between 50% and 80%, while 33% of the total isolates had low efficacy 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 are comparable to the results of Shewakena Belayneh (2009).

Physiological and biochemical characteristics

Biochemical and physiological characterization of the isolates revealed the existence of versatile and tolerant lentil nodulating rhizobial isolates (Table 3). 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 (Table 3). The isolates NSLR-54, NSLR-51 and NSLR-64 also utilized all the 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 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 (g 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

NSLR= National Soil Lentil Rhizobia, 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.
Shoot dry weight was found to be strongly correlated with symbiotic effectiveness (r=0.99560, p<0.001), total nitrogen (r=0.80629, p<0.001) and nodule dry weight (r=0.51972, p<0.001) (Table 2). Nodule dry weight was positively correlated with symbiotic effectiveness (r=0.52098, p<0.001) and total nitrogen (r=0.31622, p<0.001). There was also strong correlation between total nitrogen and symbiotic effectiveness (r=0.80115, p<0.001) (Table 2). A similar result was also reported on growth pouch experiment of rhizobial inoculation on lentil by Zafar-ul-hye, et al. (2007).

Table 2: Correlation coefficients among investigated parameters such as nodule number, nodule dry weight, shoot dry weight, symbiotic effectiveness and total nitrogen (%).

	NN	NDW	SDW	SE	TN
NN	1.000				
NDW	0.01590ns	1.000			
SDW	0.05993ns	0.51972**	1.000		
SE	0.06066ns	0.52098**	0.99560**	1.000	
TN	0.05331ns	0.31622*	0.80629**	0.80115**	1.000

* and ** = Significant at p<0.01 and p<0.0001, respectively; ns= Not significant at p<0.05
NN= nodule number; NDW= nodule dry weight in mg; SDW= shoot dry weight in g; SE= symbiotic effectiveness in % and TN= total nitrogen in %.

Table 3: 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	IAR (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, E= East, N= North, S= South, W= West

* = the number in brackets indicates the number of different sources or concentrations used.

Carbohydrate sources utilization

Most of the isolates utilized a large number (>80%) of the tested carbohydrates (Figure 1). Stowers (1985) has reported that rhizobia have the ability to utilize a wide variety of carbohydrate 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 the observation of Shewakena Belayneh (2009), who reported 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), who demonstrated 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.

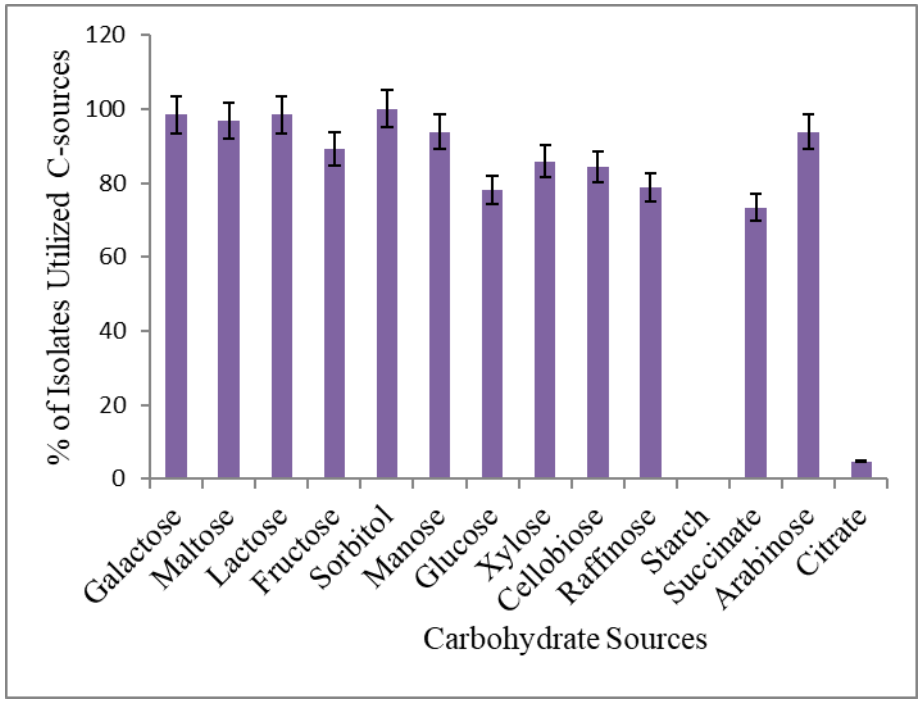

Figure 1: Percentage of growth response of isolates on different carbohydrate sources

Nitrogen sources utilization

Almost all isolates utilized isoleucine, phenyl alanine, valine and leucine (Figure 2). 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 (Figure 2). Shewakena Belayneh (2009) also observed lentil nodulating rhizobial isolates that can catabolize a wide range of amino acids.

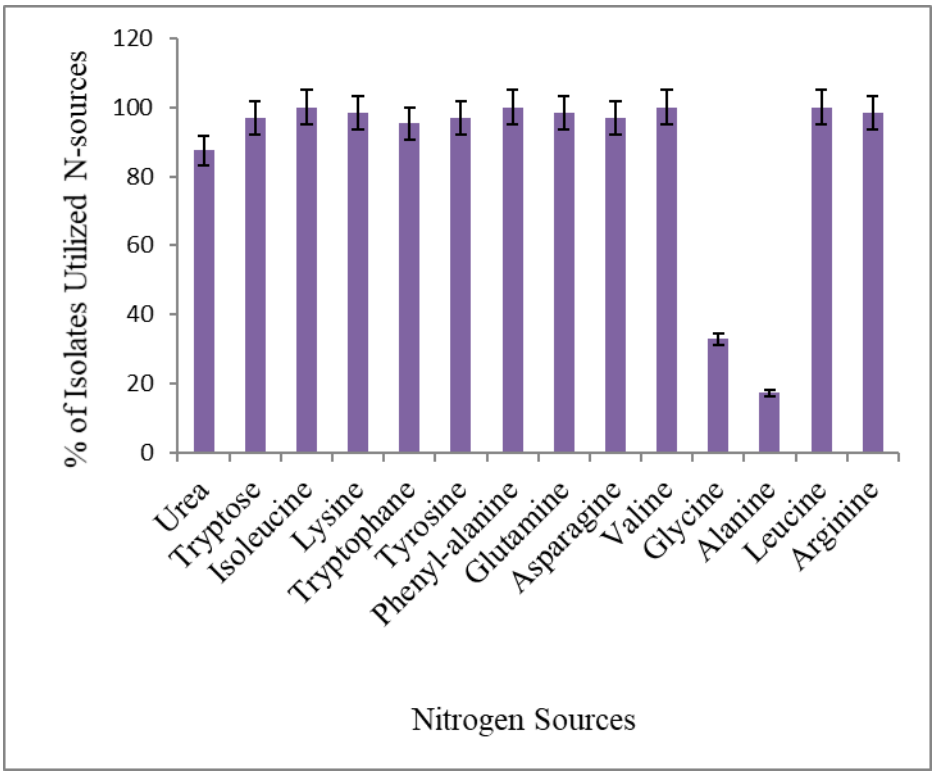

Figure 2: Percentage of growth response of isolates on different nitrogen sources

Salt tolerance

All of the tested isolates tolerated 0.1% NaCl concentration on YEMA plates (Figure 3). 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.

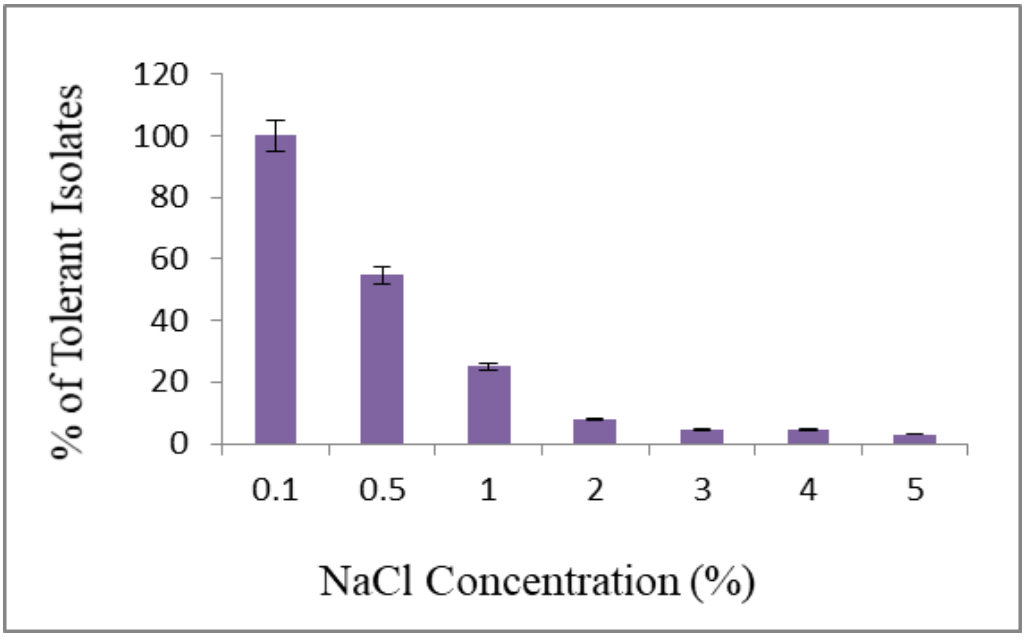

Figure 3: Percentage of tolerant isolates to different concentrations of salt (NaCl)

pH tolerance

Like other physiological tests, the isolates showed significant variation in response to tolerance to different pH levels (Figure 4). Almost all the isolates showed tolerance to a pH range of 6.5-10.0. Some 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.

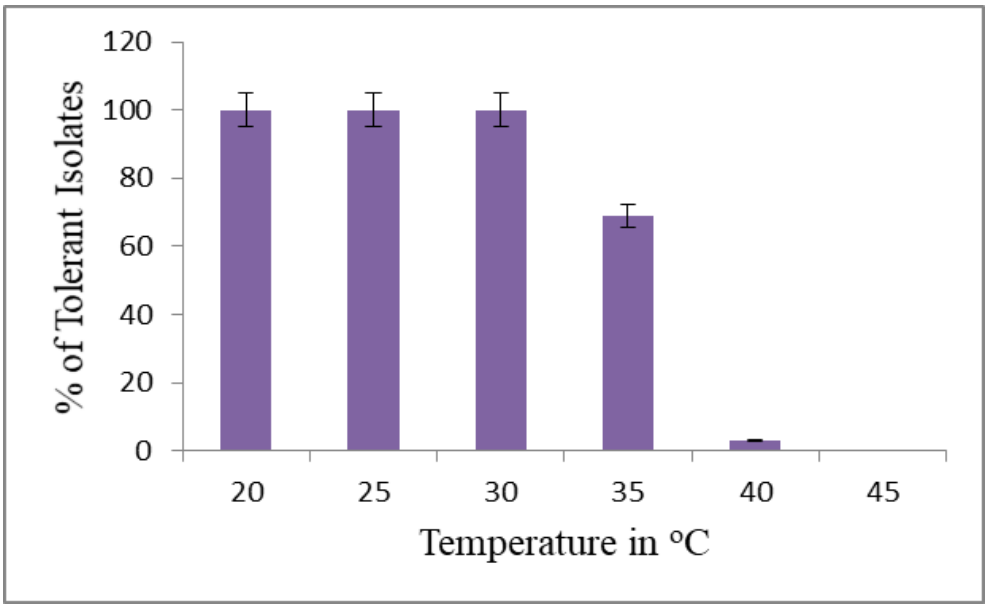

Figure 4: Percentage of tolerant isolates to different levels of pH values

Temperature tolerance

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

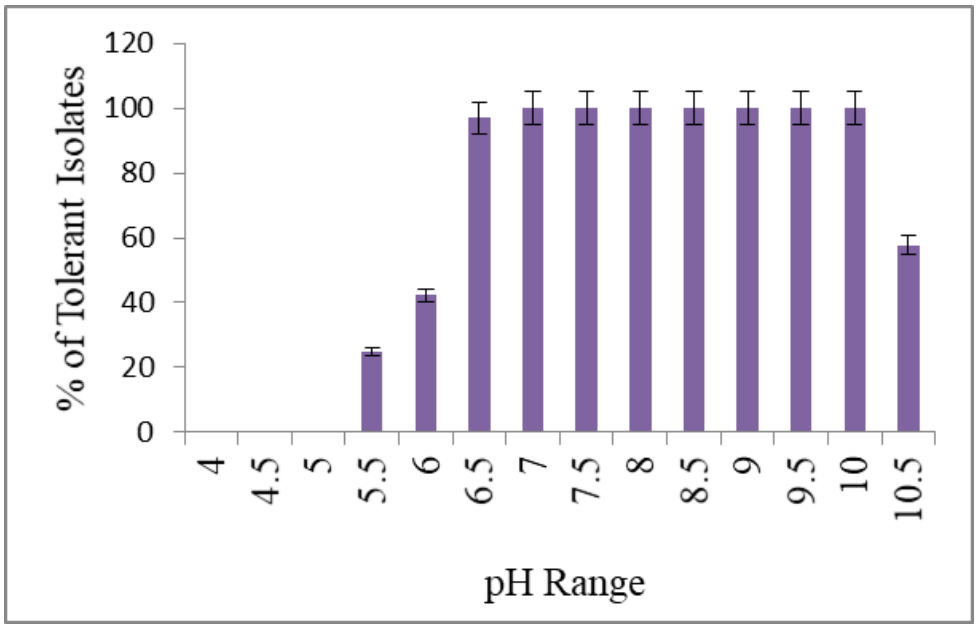

Figure 5: Percentage of tolerant isolates to different levels of temperature.

Intrinsic antibiotics resistance

The tested isolates showed variations in growth response to different types of antibiotics and concentrations (Figure 6). The isolates were generally resistant to neomycin and erythromycin and sensitive to neomycin, tetracycline, streptomycin, ampicillin and kanamycin at concentrations of 5 and 10 μgml^{-1}. Shewakena Belayneh (2009) also reported that 95% of lentil nodulating rhizobia from Northern and Western shewa tolerated erythromycin at concentration of 2.5 μgml^{-1} and 28 and 35% of the isolates were resistant to kanamycine and streptomycin, respectively, at concentration of 10 μgml^{-1}. Only 6.3% of the isolates were resistant to neomycin at concentration of 10 μgml^{-1} whereas 48.4% at concentration of 5 μgml^{-1}.

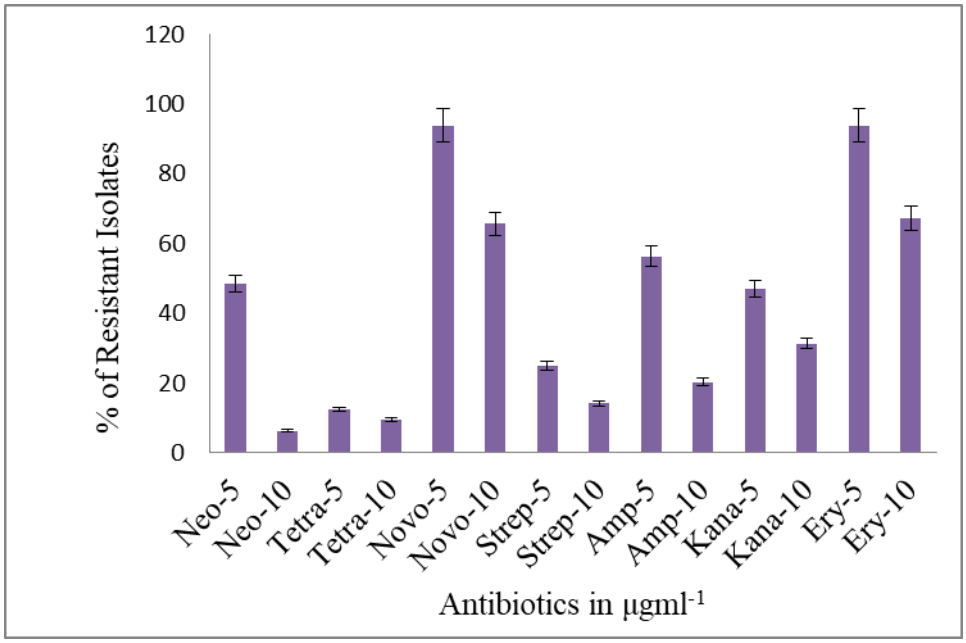

Figure 6: Percentage of resistant isolates to different types and concentrations of antibiotics

Intrinsic heavy metals resistance

Based on intrinsic heavy metals resistance, 100% of the isolates were resistant to $MnCl_2.H_2O$ at concentrations of 250 µgml^{-1} and 500 µgml^{-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 500 µgml^{-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 100 µgml^{-1} and 50 µgml^{-1}, respectively. The isolates NSLR-8 and NSLR-63 grew on medium containing $CuCl_2$ at concentration of 100 µgml^{-1} whereas 60.9% of the isolates were resistant at concentration of 50 µgml^{-1}. Daniel Muleta (2009) also reported that 16.2% of the isolates of chickpea nodulating rhizobia were resistant to Cu at concentration of 50 µgml^{-1}.

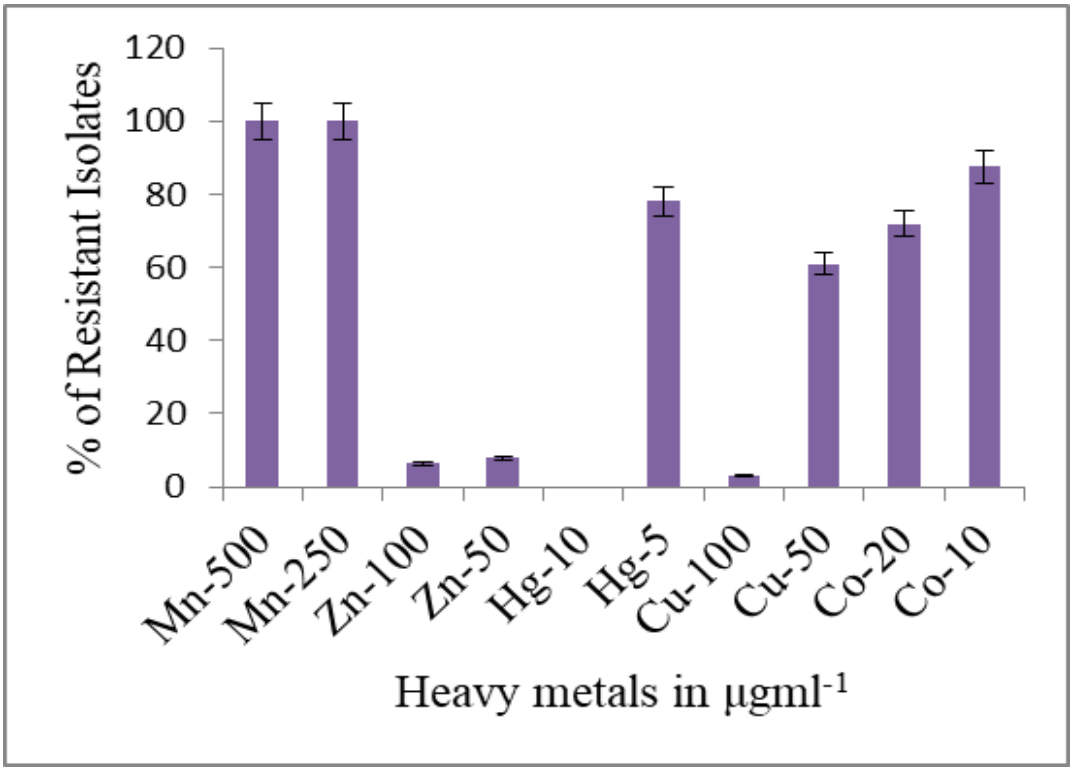

Figure 7: Percentage of resistant isolates to different types and concentrations of heavy metals

Total nitrogen content

Compared to the positive control, 18.2% of the inoculated plants had higher total nitrogen content. Plants inoculated with isolate NSLR-52 (from Girar Jarso district of North Shewa) and NSLR-17 (from Gimbichu district of East Shewa) had the highest total nitrogen content of 3.29% and 3.26%, respectively. Plants inoculated with isolate NSLR-31 (from Alelitu district 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.

Conclusion

This particular study showed the existence of phenotypic diversity 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 morphological, 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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