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Evaluation of symbiotic effectiveness and size of resident *Rhizobium leguminosarum* var. *viciae* nodulating lentil (*Lens culinaris* medic) in some Ethiopian soils

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Abstract

This study was initiated to isolate, characterize and select symbiotically effective rhizobia nodulating lentil (*Lens culinaris* medic) and to enumerate of indigenous rhizobia nodulating lentil in some Ethiopian soils. More than 84 nodule and soil samples were collected. In sand culture, only 62 isolates were authenticated as rhizobia nodulating lentil. Analyses of variance indicated that most of the parameters measured were significantly ($p< 0.05$) improved by inoculation except root length. Inoculation increased the shoot length, shoot dry weight, and plant total nitrogen as 82.3%, 196.7% and 452%, respectively, over negative control. Tested isolates was found to be very effective (20.9%), effective (77.4%), and only one ineffective isolate. Indigenous rhizobia in investigated soils ranged from 30 to $5.8x10^3$ cell g^{-1} of dry soil. A pot experiment with selected rhizobia and nitrogen fertilizer on Chefedonsa and Debrezeit soils did not show any significant difference in shoot dry weight at $P < 0.05$. From the study it was observed that most Ethiopian soils were inhabited with moderate to high number of indigenous rhizobia and also rhizobia inoculation did not improve lentil productivity in investigated soils.

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Introduction

The lentil (*Lens culinaris* Medikus subsp. *culinaris*) is a lens-shaped grain legume well known for its nutritional value. India is the major lentil producing countries, followed by Canada and Turkey, which collectively accounts for 68% of global production (FAO, 2008). Ethiopia is also the leading producer of lentil in Africa and accounts for 68% of lentil produced (CSA, 2007). Lentil stands fifth both in acreage and production after fababean, field pea, chickpea and common bean and occupies about 95,000 hectares of area with a gross annual production of about 94,773 metric tonnes (CSA, 2009).

The productivity of lentil in Ethiopia is 0.7 ton ha^{-1} which is very low compared to other neighboring countries such as Egypt where productivity is about 1.7 ton ha^{-1} (FAO, 2008; CSA, 2009). This is mainly due to low soil fertility (Angaw Tsigie and Asnakew Woldeab, 1994). Nitrogen (N) deficiency is one of the most important factors of limiting soil fertility in most Ethiopian soils (Desta Beyene and Angaw Tsigie, 1986). Stoorvogel and Smaling (1990) estimated soil nutrient losses from the highlands of Ethiopia to be in excess of 80 kg of N per cultivated hectare. Application of chemical fertilizers have played a significant role to increase the productivity of soils, however, unbalanced use of fertilizers has led to reduction in soil fertility and environmental degradation. Moreover, the cost of chemical fertilizers have been increased many folds and made unaffordable in developing countries such as Ethiopia. Therefore, the need to take advantage of inexpensive means of soil fertility enhancement through biological nitrogen fixation is becoming vital for increasing crop productivity and ensuring food security in the region.

According to Sariolu *et al.* (1993), *Rhizobium* symbiosis with legume species is of key importance, contributing 50% of 175 million tonnes of total biological N fixation annually worldwide. In Ethiopia, the research work on the nodulation status and nitrogen fixation potential of lentil is very limited; however, Angaw Tsigie and Asnakew Woldeab (1994) demonstrated that rhizobial isolates inoculation in Ethiopian soils have showed significant improvement of lentil yield and yield components at preliminary screening under controlled condition.

Generally the success of inocula highly depends on soil chemical properties, mainly soil pH, salinity, moisture content, and some soil physical properties, particularly soil texture. Moreover, often low recovery of inoculated rhizobia have been recorded where soil harbored with sufficient number of indigenous rhizobia (Anteneh Argaw, 2007; Slattery *et al.,* 2004). Thus, a thorough understanding of the size and effectiveness of background population of *rhizobia* will enable recommendations to be made regarding the need for re-inoculation of pulse legumes. Therefore, the objectives of this study were to isolate, characterize and select effective indigenous rhizobia nodulating lentil and to determine the population sizes of indigenous *Rhizobium leguminosarum* bv. *Viciae* in some major lentil growing area of Ethiopian soils.

Materials and method

Soil sampling

The soil samples were collected from about 100 different locations using aseptic technique in August 2007 at the planting time of lentil, which covered some parts of Gojam, Gonder, Tigray Wello and Central Ethiopia from as many sampling sites where lentil has been grown for a long time and the climate and soil types favoured the growth of lentil with no history of inoculation with lentil *Rhizobium* biofertilizer. The number of soil sampled with their regional location are indicated as follows: Central Ethiopia (34 sites), Gojam (7 sites), Gonder (10 sites), Wello (12 sites) and Tigray (9 sites) (Figure 1).

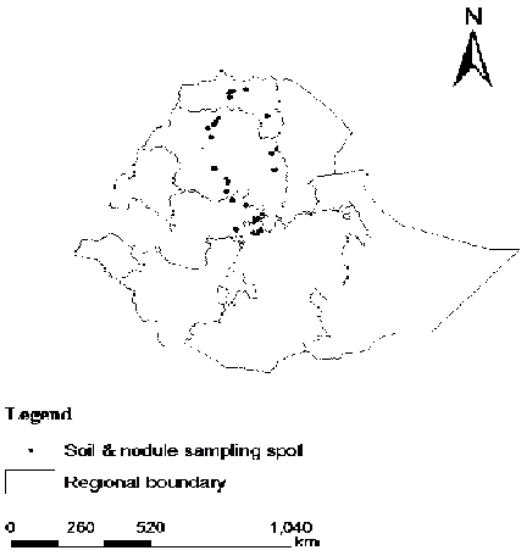

Fig. 1. Location of nodule and soil sampling sites.

Induction of nodulation and isolation of root nodule bacteria

Nodulation was induced by 'plant trap' method using growth pouches (Vincent, 1970). Five pre-germinated seedlings were transplanted and thinned to three after one week. Separate suspensions of collected soils were made in a sterilized test tube by adding 10 g of soil to 100 ml of distilled water and mixing for 2 min. The transplanted lentils were separately inoculated with 5 ml of the appropriate soil suspension. The growth pouches were kept in the green house and watered when it required with distilled sterilized water every day for two months. At the end, fresh, red and large nodules were carefully and separately collected from one healthy plant from each soil suspension treatment pouch.

Nodules were sterilized (95% ethanol (v/v) 10 s, 1% NaOCl 3 min, and six washes in water), crushed, and streaked on Yeast Extract Mannitol (YEM) agar (Vincent, 1970). Single colonies were marked and checked for purity by repeated streaking on YEM agar medium and verifying a single type of colony morphology, absorption of Congo red (0.00125 mg kg-1) and a uniform Gram-stain reaction. Colony morphology (color, mucosity (mucoid), borders, transparency and elevation) and acid/ alkaline reaction were evaluated on YEMA containing bromothymol blue (0.00125 mg kg-1) as indicator. All the strains were tested for its purity by streaking on Glucose-Peptone agar medium supplemented with Bromocresol Purple (GPA-BCP) (Lupwayi and Haque, 1994). The collected and purified rhizobial cultures were coded as $NSRLN_1$, $NSRLN_2$.....$NSRLN_{84}$. All strains were incubated at 28°C and stored at -20°C in 25% glycerol-YEM broth.

Authentication and symbiotic effectiveness of collected isolates

Each of the selected strains was authenticated as root nodulating bacteria by re-inoculating them on the host as described by Somasegaran and Hoben (1994) in controlled environment using growth pouch. Treatments without inoculation and chemical nitrogen fertilizer (negative control) and treatment without inoculation and with nitrogen fertilizer at a rate of 70 µg N ml^{-1} applied as KNO_3 solution (positive control) at least once a week were included as controls. This experiment was made in triplicates. Nitrogen-free nutrient solution (Broughton and Dilworth N-free medium) alternated with distilled sterilized water was used to water the plants.

After sixty days of sowing, root and shoot fractions were separated and nodules were severed from the roots to count, then dried at 70°C for 24h. Root length, shoot length, shoot dry weight, nodule number per plant and nodule dry weight per plant were also measured from each pouch and recorded separately. Shoot nitrogen concentration was analyzed using the Kjeldahl method and N content (shoot dry weight x N concentration) per plant and the N fixed (plant N content in inoculated pouch - plant N content in uninoculated pouchs) were calculated (Yaman and Cinsoy, 1996).

Symbiotic efficiency percentage (SE%) was calculated by comparing each inoculated plants with N applied control (plant N content in inoculated plant/plant N content in N applied plant) x 100 (Beck *et al.,* 1993). Accordingly, the isolates were

arbitrarily rated very effective (VE) when the shoot dry matter yield of the associated host was higher than the total mean of all isolates plus the standard deviation (greater than 0.1805±0.084); effective (E) when its yield was between that of the mean ± the standard deviation (between 0.1805±0.084); and ineffective (I) when its yield was smaller than the mean minus the standard deviation (less than 0.1805-0.084) as described by Lalande *et al.* (1990).

Enumeration of indigenous Rhizobium leguminosarum var. viciae and symbiotic effectiveness of selected isolates on soil culture

Nine unfertilized soil samples (1kg) were obtained aseptically in the laboratory for enumeration of rhizobia nodulating lentil and for soil chemical and physical properties analysis. Out of which, bulk samples of 250 kg from Chefedonsa and Debrezeit Agricultural Research Station for screening of symbiotic effectiveness of selected isolates of *Rhizobium leguminosarum* var. *viciae* were collected from the upper 30 cm soil surface. They were ground and sorted by hand to remove stones and plant residues and were sent for analysis to the National Soil Research Center, Addis Ababa, in sealed polyethylene bags. Soil samples were collected from lentil field with no history of inoculation with rhizobial biofertilizer.

Nine soil samples from major lentil growing areas of Ethiopia were randomly selected for detailed soil chemical and particle size analysis and their number of indigenous rhizobia nodulating lentil (Table 1). The most probable number (MPN) plant infection test was used to enumerate rhizobia capable of nodulating lentil in the collected soil following the procedure stated by Somasegaran and Hoben (1994).

Based on the total shoot dry weight, plant total nitrogen (mg plant^{-1}) and net nitrogen fixed, the top eight isolates were selected and used as inoculants to study on the symbiotic effectiveness on lentil in unsterillized Debrezeit and Chefedonsa soils. The results of chemical, physical and biological analyses of the investigated soils are given in Table 1. This experiment (temperatures averaged 28/10°C, day/night) was conducted under greenhouse condition using plastic pot to evaluate the N_2 fixation rates of selected isolates following Somasegaran and Hoben (1994).

Statistical analysis

Data collected was statistically analyzed by subjecting to analysis of variance (ANOVA) using General Linear Models Procedure of SAS software ver. 10. Means of all treatments were calculated and the differences tested for significance using the least significant differences (LSD) test at 0.05 probability (P) level. Correlation coefficients were calculated to study the associative relations among the measurement traits using Pearson correlations.

Result and Discussion

Authentication and Pre-screening the Symbiotic Effectiveness of Isolates

The results of Gram staining and growth of the root nodulating bacteria in YEMA-CR medium conformed to the standard cultural and morphological characteristics of *Rhizobium* sp. as stated by Somasegaran and Hoben (1985) and Vincent (1970). All isolates were found to be gram negative rods and did not absorb Congo red except some isolates which absorbed it very slightly on YEMA-CR media. Almost all tested isolates changed the BTB color into yellowish when culturing on YEMA-BTB medium. This could possibly be considered as an indicator of production of acidic substances and verifying the common characteristics of fast growing *Rhizobium* sp. (Somasegaran and Hoben, 1985). This result confirmed previous works by Alemayehu Workalemahu (2009). These tests helped in the preliminary screening of rhizobial isolates from contamination and enabled the rejection of impure isolates (Vincent, 1970).

Most of the authenticated isolates formed dome-shaped colonies with shiny appearance and buttery texture. All retrieved isolates produced gelatinous

colonies due to the production of much exopolysaccharides with the colony diameter ranging from 3.5 - 4.5 within five days indicating colony morphology of rhizobia (Alemayehu Workalemahu, 2009; Anteneh Argaw, 2007). Jordan (2005) also found that *Rhizobium leguminosarum* var. *Viciae* displays highly mucoid and larger colony diameter up to 5mm within 4-5 days on YEMA medium.

Table 1. The legume cropping history, most probable number of *Rhizobium leguminosarum* bv. *Viciae*, soil texture and chemical characteristics of the soils used in this study.

	pH(H_2O)	EC	Soil texture	Na (Cmol(+)/kg	K (Cmol(+)/kg	Ca (Cmol(+)/kg	Mg (Cmol(+)/kg	CEC (Cmol(+)/kg	Total nitrogen	Organic carbon	C/N ratio	Available phosphorus	Cropping history	MPN (No. of rhizobia gm^{-1} of soil)
71	7.8	0.200	C	0.41	2.87	61.29	10.44	68.38	0.070	0.915	13	2.28	CL	5.8×10^3
35	8.1	0.252	C	0.46	2.98	63.89	11.00	67.76	0.070	0.836	12	8.30	CL	5.8×10^3
89	7.0	0.095	C	0.35	2.78	41.72	9.62	56.14	0.081	0.915	11	28.54	CL	3.1×10^3
58	7.4	0.200	C	0.09	2.11	15.12	6.83	37.92	0.079	0.711	9	6.84	LCL	3.1×10^2
47	6.9	0.115	C	0.09	1.04	15.12	6.41	36.38	0.070	0.770	11	8.56	ML	3.1×10
64	7.9	0.298	C	0.04	0.88	20.11	28.62	76.94	0.065	0.650	10	2.52	ML	1×10^2
00	8.1	0.232	CL	1.04	0.42	18.11	15.87	53.28	0.071	0.781	11	3.80	LCL	1×10^3
40	7.3	0.119	C	0.02	0.17	13.84	4.49	27.62	0.085	0.765	9	9.70	LCL	3.1×10^3
40	7.2	0.025	C	0.30	3.78	20.06	4.03	33.44	0.111	0.999	9	14.54	LCL	3.1×10^2

C-Clay; CL-Clay loam; CL-Cultivated land; ML-Mashy land; LCL-Lentil cultivated land

Eighty-four isolates obtained from lentil nodules were assessed for their infectiveness and effectiveness of N_2-fixation on lentil using growth pouch under control environment, at the National Soil Research Center Greenhouse. Out of these, only sixty two isolates formed nodules on the test plant root authenticating as rhizobia. However, authenticated isolates were not grown or growing on PGA medium, confirming the characteristics of rhizobia (Lupwayi and Haque, 1994). The remaining isolates failed to nodulate the parent hosts that were grown on PGA medium during preliminary screening of root nodulation bacteria, confirming the characteristics of contaminants (Lupwayi and Haque 1994).

The first criteria for a *Rhizobium* used as biofertilizer or nitrogen inoculum is that it must be superior and highly effective in nitrogen fixing ability forming symbiotic association with the host legume (Graham O'Hara *et al.,* 2002). Symbiotic effectiveness efficiency of isolates was found to be very effective (20.9%) in 13 authenticated isolates and effective (77.4%) with 48 rhizobia and only one ineffective strain (NSLNR-24) was isolated from Alem-gena area (Table 2). Similar observations have been reported by Ayneabeba Adamu *et al.* (2001) that most of *Rhizobium leguminosarum* bv. *Viciae* nodulating faba bean isolated from Central Ethiopia have been characterized as symbiotically effective isolates while tested in a controlled environment using sterilized sand culture. The highest symbiotic efficiency (SE%) was noted with isolate NSLNR-69 isolated from Wegera wereda soil gave 275%. This could be due to the fact that some rhizobia isolate produced plant growth promoting hormone (Erum and Bano, 2008).

The results of analyses of variance showed that bacterial inoculation significantly ($P<0.05$) increased all investigated parameters except root length as compared to negative control (Table 1). This promotive effect of inoculation treatments is on the same line with those obtained by El-Wakeil

and El-Sebai, (2007), who stated that vegetative growth parameters of faba bean were significantly increased in the inoculation treatments compared to control. Rhizobial inoculation increased shoot length up to 82.3% compared to negative control. A similar result was obtained on Common Vetch (*Vicia sativa* L.) (Albayrak *et al.,* 2004) that inoculation had increased shoot length over uninoculated plants. Hoque and Haq (1994) reported that inoculation of seed with *Rhizobium* significantly increased plant height of lentil. Most rhizobia inoculation resulted in a significant ($p<0.05$) increase in shoot dry weight as compared to negative control. Inoculation with rhizobial cultures increased shoot dry weight ranging from 8% to 196.7% over negative control**.** Some inoculated plant produced higher shoot length and shoot dry weight over nitrogen treated plant. This could be due to the fact that some rhizobia isolate produced plant growth promoting hormone in addition to fixing nitrogen (Erum and Bano, 2008). On the other hand, none of the tested isolates significantly increased the root length over the negative control. Similarly, Diouf *et al.* (2005) stated that inoculation on *Acacia* spp. improved the shoot growth rather than root growth.

Table 2. The effect of inoculation of rhizobia nodulating lentil on shoot length, root length, nodule number, nodule dry weight, shoot dry weight, plant total nitrogen and net nitrogen fixed of lentil under control condition using growth pouch experiment.

RL (cm)	NN	NDW (gm Plant^{-1})	SDW (gm Plant^{-1})	PTN (%)	PTN (mg plant^{-1})	NF (mg plant^{-1})	SE (%)
28.2±1.3a-o	48.3±12.9a-m	0.008±0.006a-m	0.240±0.10a-p	2.72±0.53e-p	6.809±3.836b-	4.786	138
26.2±2.5e-t	46.3±3.5a-m	0.005±0.001g-o	0.203±0.07b-s	2.24±0.25m-	4.452±0.973g-	2.429	117
30.0+2.2a-h	27.3+2.5j-o	0.0047+0.0005g-o	0.254+0.06a-l	2.61+0.30f-q	6.524+0.787b-	4.501	146
22.5+1.7q-	50.3+17.0a-l	0.0065+0.0026d-o	0.328+0.11abc	2.23+0.20n-v	7.326+2.667a-i	5.303	189
24.7±2.9j-v	54.0±17.6a-i	0.0033±0.0024j-o	0.131±0.06k-u	2.14±0.15q-v	2.745±1.146k-	0.722	75
24.1+1.2n-	62.7+14.2a-f	0.005+0.0009g-o	0.163+0.01g-t	2.21+0.23o-v	2.133+1.010l-p	0.11	94
28.1±2.5a-o	59.0±16.7a-g	0.008±0.005a-n	0.290±0.12a-g	2.74±0.20e-p	8.041±3.595a-g	6.018	167
27.3+1.7b-	39.0+24.2e-o	0.007+0.009b-n	0.154+0.18g-t	2.14+0.45q-v	3.820+5.075g-	1.797	89
29.7+3.5a-h	54.0+25.6a-i	0.008+0.007a-m	0.279+0.13a-i	2.79+0.37c-n	7.524+3.053a-i	5.501	160
25.3±0.8h-	25.3±4.5l-o	0.0045±0.0027g-o	0.217±0.01a-q	2.25±0.45m-	4.888±1.075f-p	2.865	125
27.0+3.6b-r	49.7+4.2a-m	0.0027+0.000521-o	0.147+0.12h-t	2.19+0.28p-v	3.007+2.105j-p	0.984	84
29.8±2.8a-h	20.7±4.0n-p	0.0037±0.0018i-o	0.259±0.02a-l	2.53±0.28g-t	6.568±1.166b-	4.545	149
32.5+0.7a	66.3+9.7ab	0.0085+0.0053a-n	0.272+0.11a-k	2.91+0.14b-i	7.838+2.797a-h	5.815	156
26.7+2.2d-r	27.3+3.8j-o	0.0041+0.00063g-o	0.143+0.06h-u	2.04+0.30q-x	3.026+1.529j-p	1.003	82
24.7±1.8j-v	16.7±2.9j-p	0.0032±0.0032j-o	0.205±0.05b-s	1.87±0.27v-x	3.926±1.483g-	1.903	118
28.8+3.6a-	32.7+2.9h-o	0.0029+0.00078k-o	0.168+0.02f-t	3.08+0.32a-g	5.238+1.263e-o	3.215	97
29.2±2.2a-l	37.3±16.9g-o	0.0075±0.00095b-n	0.079±0.06q-u	3.48±0.44a	2.740±2.312k-	0.717	45
24.5+2.3l-v	46.7+14.4a-m	0.0083+0.0011a-n	0.176+0.11e-t	2.61+0.43f-q	4.888+3.341f-p	2.865	101
27.2+0.8b-r	63.7+34.8a-e	0.0138+0.0032abc	0.315+0.21a-l	2.18+0.19p-v	7.111+4.984a-j	5.088	181
28.8±3.1a-	33.7±14.4h-o	0.0055±0.0025f-o	0.092±0.07q-u	2.18±0.44p-v	2.145±2.089k-	0.122	53
26.1+2.5f-t	33.7+20.2h-o	0.0092+0.0065a-l	0.162+0.16g-t	2.90+0.16b-j	4.852+4.769f-p	2.829	93
25.8±0.5g-t	33.7±10.5h-o	0.0042±0.0040g-o	0.202±0.08b-s	2.82±0.17c-l	5.783±2.519d-	3.76	116
20.9+1.0t-v	45.3+21.1a-n	0.007+0.009c-o	0.205+0.15b-s	2.89+0.11b-j	6.023+4.521d-	4	118
29.9+2.6a-h	39.7+28.5e-o	0.0058+0.0083e-o	0.167+0.07f-u	2.78+0.33c-n	4.676+2.253f-p	2.653	96
30.7±4.1a-f	37.7±7.1f-o	0.0128±0.0089a-e	0.253±0.11a-m	2.93±0.31a-i	7.658±4.040a-i	5.635	145
24.7+1.5j-v	44.3+10.5a-n	0.0016+0.0002	0.166+0.14f-t	2.64+0.12f-q	4.411+3.763g-	2.388	95
25.7±0.6g-	39.7±31.5e-o	0.0106±0.0077a-i	0.279±0.12a-h	2.52±0.42g-t	7.404±4.494a-i	5.381	160
30.3+3.0a-g	35.7+23.2g-o	0.0053+0.0065g-o	0.138+0.11h-u	2.81+0.48c-	4.164+4.089g-	2.141	79

19.9+3.1v	68.0+11.5a	0.011+0.0024 a-h	0.247+0.06a-n	2.84+0.17b-k	7.069+2.006a-j	5.046	142
30.1±0.9a-h	40.0±9.2d-o	0.008±0.0025 a-n	0.144±0.04h-t	3.29±0.88a-d	4.805±1.976f-p	2.782	83
23.5+3.5o-	38.7+14.4e-o	0.005+0.0053 g-o	0.185+0.07d-s	2.87+0.12b-k	5.344+2.330e-n	3.321	106
23.1±1.7o-	25.7±4.9k-o	0.004±0.0024 h-o	0.144±0.05h-t	3.02±0.24a-h	4.435±1.874g-	2.412	83
26.7+2.9d-r	45.7+11.4a-n	0.010+0.0024 a-l	0.210+0.10b-r	2.0+1.44s-x	5.189+4.466f-o	3.166	121
26.2±3.6n-	46.7±8.6a-m	0.014±0.0016 a-e	0.262±0.04a-g	2.87±0.08a-f	9.325±2.155a-e	7.302	151
32.2±3.6a	50.7±7.1a-k	0.0104±0.003a-k	0.273±0.02a-k	3.25±0.30a-l	8.852+0.859a-f	6.829	157
21.4+3.5t-v	34.0+16.4g-o	0.005+0.005g-o	0.156+0.13g-t	2.12+0.22q-v	3.505+3.122i-p	1.482	90
26.0±1.0f-t	31.0±15.7h-o	0.005±0.005g-o	0.138±0.11h-u	2.8±0.25c-m	4.046±3.591g-	2.023	79
22.7+4.2q-	40.3+23.3c-o	0.0051+0.0057g-o	0.215+0.07b-r	2.64+0.17f-q	5.691+2.033d-	3.668	124
30.0±7.7a-h	65.0±10.4a-d	0.015±0.003a	0.359±0.09a	2.93±0.11a-h	10.574±2.991a	8.551	206
25.6±3.3g-	41.3±9.1b-o	0.008±0.001a-n	0.188±0.04c-s	2.50±0.18h-u	4.741±1.382f-p	2.718	108
24.8+1.7i-u	47.3+7.0a-m	0.0127+0.001a-f	0.276+0.03a-k	2.71+0.17e-p	7.517+1.188a-i	5.494	159
31.3±4.0a-d	35.0±6.0g-o	0.0064±0.0033d-o	0.191±0.04c-s	2.36±0.41l-v	4.642±1.748f-p	2.619	110
25.4+0.8j-v	29.3+12.9i-o	0.004+0.003g-o	0.220+0.03a-q	2.93+0.13a-i	6.479+1.080b-	4.456	126
26.8±3.8c-r	47.7±10.3a-m	0.009±0.006a-l	0.198±0.05b-s	2.59±0.26g-r	5.205±1.924e-o	3.182	114
24.7±1.4j-v	25.0±21.7m-p	0.007±0.009c-n	0.193±0.13b-s	2.20±0.67o-v	4.852±4.592f-p	2.829	111
24.6+2.0k-	30.7+11.0h-o	0.009+0.0019a-l	0.208+0.02b-s	2.77+0.09c-o	5.783+0.643d-	3.76	120
29.5±3.0a-j	53.7±14.1a-k	0.011±0.007a-g	0.306±0.11a-f	3.24±0.25a-e	9.762±2.642a-d	7.739	176
25.7+3.3g-	39.3+26.3e-o	0.008+0.006a-n	0.155+0.09g-t	2.11+0.29q-x	3.466+2.612i-p	1.443	89
26.0±1.8f-t	17.3±4.6op	0.003±0.0011j-o	0.101±0.06o-u	1.97±0.17s-x	2.058±1.437l-p	0.035	58
28.4±7.8a-l	55.0±30.0abc	0.012±0.0007ab	0.333±0.04ab	2.39±0.25abc	11.167±2.278a	9.144	275
25.9+4.2f-t	38.3+21.1f-o	0.007+0.005c-o	0.239+0.10a-p	2.56+0.24g-s	6.250+2.895c-l	4.227	137
28.7±3.4a-	37.0±8.0g-o	0.005±0.002g-o	0.189±0.03c-s	2.31±0.27k-v	4.421±1.106g-	2.398	109
31.6+1.9ab	37.0+16.7g-o	0.007+0.007c-o	0.242+0.08a-o	2.74+0.21 6d-	6.727+2.789b-	4.704	139
23.7±1.4n-	26.3±12.1j-o	0.003±0.001l-o	0.111±0.05m-u	2.53±0.22g-s	2.879±1.660j-p	0.856	64
29.9±6.2a-h	40.3±18.6c-o	0.006±0.003c-o	0.154±0.11h-t	2.44±0.34i-u	3.951±3.281g-	1.928	89
22.4+1.2r-v	51.0+27.6a-k	0.010+0.008a-k	0.325+0.14a-d	3.05+0.49a-h	10.375+5.864a	8.352	187
26.9±3.1c-r	27.0±13.9j-o	0.006±0.006d-o	0.136±0.06i-u	2.11±0.30q-v	2.849±1.191j-p	0.826	78
26.3+4.3e-t	35.7+6.7g-o	0.003+0.002j-o	0.182+0.08e-s	1.93+0.24s-x	3.650+2.072h-	1.627	105
21.7±1.2s-v	45.7±18.0a-n	0.013±0.007a-d	0.269±0.08a-k	1.99±0.04s-x	5.357±1.569e-n	3.334	155
32.5+6.3a	32.3+4.6h-o	0.004+0.003g-o	0.107+0.10n-u	1.83+0.15v-x	2.026+1.912l-p	0.003	61
30.7±4.8a-f	35.7±12.5g-o	0.0046±0.0058g-o	0.235±0.07a-p	3.04±0.16a-h	6.709±2.551b-	4.686	135
29.6+1.5a-i	35.0+9.5g-o	0.006+0.002f-o	0.135+0.11j-u	2.39+0.34i-v	4.000+3.938g-	1.977	78
31.1±2.6a-e	0p	0o	0.174±0.10f-t	3.41±0.38ab	5.790±3.462d-	-	-
31.7+4.6ab	0p	0o	0.121+0.051-u	1.55+0.38x	2.023+1.439l-p	-	-
11.2	39.5	67.3	47.0	13.7	53.2	-	-
3.0	15.5	0.0045	0.089	0.349	2.661	-	-
4.9	25.1	0.0072	0.143	0.563	4.295	-	-

Data are means of three replicates ± SE.

Means within a column of the same factor followed by the same letter(s) are not significant at $p<0.05$.

SL= shoot length (cm); RL= root length (cm); NN= nodule number; NDW= nodule dry weight (mg); PTN(%)= plant total nitrogen in %; PTC(mg/pl)= plant total nitrogen in mg/plant; NNF (mg/pl)= net nitrogen fixed in mg/plant and SE (%)= symbiotic effectiveness efficiency.

Shoot length was found to be positively correlated with nodule number (r=0.39 P<0.001) and with plant total nitrogen (%) (r = 0.40 P <0,001); and strongly correlated with nodule dry weight (r = 0.46 P <0.001); plant total nitrogen (mg/plant) (r = 0.58 P <0.001) and SE% (r = 0.52 P <0.001) (Table 3). Shoot length was also strongly correlated with net nitrogen fixed (mg/plant) (r = 0.57 P <0.001). Shoot dry weight was found to be positively correlated with plant total nitrogen (%) (r = 0.24 P <0.05). There was strong correlation among plant total nitrogen (mg plant^{-1}) (r = 0.94 P <0.0001); net nitrogen fixed (r = 0.94 P <0.0001) and SE% (r = 0.99 P <0.0001) (Table 3). A similar result was reported on lentil, using growth pouch experiment by Zafar-ul-Hye *et al.,* (2007) and groundnut (Nguyen Thi Lien Hoa *et al.,* 2002). Much of the research output indicated that nodulation status positively correlated with plant tissue nitrogen and shoot biomass (Mnalku *et al.,* 2009; Atici *et al.,* 2005).

Table 3. Correlation coefficients among investigated parameter in lentil, shoot length, root length, nodule number, nodule dry weight, shoot dry weight, plant total nitrogen (%), plant total nitrogen (mg/plant), net nitrogen fixed and symbiotic effectiveness.

	SL	RL	NN	NDW	SDW	PTN (%)	PTN (mg/Pl)	NNF (mg/pl)	SE (%)
L	1.00000								
L	0.22803ns	1.00000							
N	0.39398***	-0.01029ns	1.00000						
DW	0.45843***	0.01822ns	0.60067***	1.00000					
DW	0.51654***	-0.03337ns	0.46042***	0.58934***	1.00000				
TN (%)	0.39525***	0.20135ns	0.22057ns	0.23888*	0.28576*	1.00000			
TN (mg/Pl)	0.58474***	0.05231ns	0.44812***	0.62655***	0.94083***	0.53226***	1.00000		
NF (mg/pl)	0.57235***	0.03554ns	0.42989***	0.59369***	0.94147***	0.52746***	0.99085***	1.00000	
E (%)	0.51664***	-0.03461ns	0.46030***	0.58933***	0.99998***	0.28475***	0.94046***	0.94111***	1.00000

*, ** and *** = Significant at p<0.05, p<0.01 and p<0.001, respectively; ns= Not significant at p<0.05
SL= shoot length (cm); RL= root length (cm); NN= nodule number; NDW= nodule dry weight (mg); PTN(%)= plant total nitrogen in %; PTC(mg/pl)= plant total nitrogen in mg/plant; NNF (mg/pl)= net nitrogen fixed in mg/plant and SE (%)= symbiotic effectiveness efficiency

Table 4. Symbiotic effectiveness of rhizobia nodulating Lentil on Debrezeit soil under greenhouse condition

Isolates	Nodule number plant^{-1}	Nodule dry weight (mg plant^{-1})	Shoot dry weight (gm plant^{-1})	Plant total nitrogen (%)
NSLNR-10	53 ± 8.1c	0.188 ± 0.039cd	0.726 ± 0.036bc	3.760 ± 0.073bc
NSLNR-16	55 ± 9.0c	0.199 ± 0.047bcd	0.704 ± 0.121bcd	4.121 ± 0.332abc
NSLNR-48	70 ± 10.0abc	0.217 ± 0.034a-d	0.618 ± 0.206cd	4.111 ± 0.671abc
NSLNR-51	77 ± 23.0ab	0.279 ± 0.122abc	0.727 ± 0.049bc	4.307 ± 0.450ab
NSLNR-56	52 ± 3.1c	0.220 ± 0.014a-d	0.806 ± 0.039ab	4.153 ± 0.288abc
NSLNR-64	64 ± 11.3abc	0.252 ± 0.041a-d	0.719 ± 0.072bcd	4.700 ± 0.348a
NSLNR-69	69 ± 8.4abc	0.277 ± 0.005abc	0.541 ± 0.199d	4.167 ± 0.073abc
NSLNR-77	83 ± 14.7a	0.304 ± 0.028a	0.804 ± 0.039ab	3.656 ± 0.393c
+ve control	56 ± 7.9bc	0.171 ± 0.011d	0.825 ± 0.093ab	4.230 ± 0.101abc
-ve control	70 ± 19.0abc	0.278 ± 0.079abc	0.941 ± 0.018a	3.780 ± 0.123bc
MSE	12.9	0.052	0.107	0.350
CV (%)	19.8	22.0	14.4	8.5
LSD	22.2	0.090	0.183	0.600

Data are means of three replicates ± SE; Same letters are not significantly different at LSD P<0.05 level.
-ve (negative control)-without chemical and biological fertilizers; +ve (positive control)-With optimum amount of nitrogen fertilizer.

Screening of symbiotic effectiveness of Rhizobia on soil culture

Results of soil analysis (Table 1) depict that Debrezeit soils are characterized with low nitrogen, neutral pH, and high available phosphorus. However, soil collected from Chefedonsa area analyzed had low nitrogen and slightly basic pH, indicating low available phosphorus for legume production according to Desta Beyene and Angaw Tsige (1989). Due to phosphorus fertilizer on Debrezeit soil during past three decades, it was analyzed as high as 28.54 ppm available phosphorus (Tekaligne Mamo *et al.,* 1996).

In general, inoculation in Debrezeit soils did not significantly improve the investigated parameters except plant total nitrogen as compared to negative control plants (Table 4). However, greater number

of nodules per plant (83) and nodule dry weight per plant (0.304 mg plant^{-1}) was noted in NSLNR-77 inoculation treatment. The nitrogen application significantly decreased nodule dry weight (0.171 mg plant^{-1}) and this indicates the negative effect of nitrogen fertilizer application on nodulation of the legume plants (Crews *et al.,* 2004). Inoculation did not improve the shoot dry weight. This might be due to native rhizobia that are sufficient in number and more effective than the inoculums used (Raza and Jornsgard, 2005). Moreover, this could also be attributed to the low competitive ability of inoculated strains (Raza and Jornsgard, 2005). According to Amos *et al.* (2001), competition between rhizobia strains in the soil is a common phenomenon as the introduced inoculum strains compete with indigenous rhizobia for nodule sites.

The data (Table 5) revealed that some inoculated plants produced significantly ($P<0.05$) higher nodule number, nodule dry weight and total plant nitrogen in Chefedonsa soil, but did not reflect increased shoot dry weight. Rhizobial inoculation with NSLNR-10 and NSLNR-48 isolates resulted in significantly ($P<0.05$) higher nodule number per plant and nodule dry weight per plant (mg plant^{-1}) respectively. This indicates that the soils contained indigenous rhizobia that nodulated the grain legume species (Chemining'wa *et al.,* 2004). These results are in accordance with the previous reports on Brazilian soil by Mostasso *et al.* (2002) in which inoculation of bean rhizobia resulted in nodulation increase even in soil harbored with high number of residential rhizobia. However, higher number of nodules produced does not reflect effectiveness of inoculated *Rhizobium* isolate instead it is evidenced for higher infectiveness of that particular *Rhizobium* strain (Shisanya, 2002). There was no statistically significant difference ($P< 0.05$) among treatments in shoot dry weight. Similar results are reported by Raza and Jornsgard, (2005) and Hungria *et al.* (2000) who also found that inoculation significantly affected plant total nitrogen (mg plant^{-1}), nodule number and nodule dry weight but did not indicate on shoot dry weight. Poor nodulation and failure to respond to inoculation have often been reported for the pulse crop (Thies *et al.,* 1991; Hungria *et al.,* 2000). They also suggested that resident rhizobia of $>10^3$ cells gm^{-1} of soil compete with inoculated isolate which in turn failed to respond and improve the plant biomass.

In both soil inoculation of NSLNR-64, isolate significantly increased plant total nitrogen (%). Moreover, NSLNR-64 inoculated plants accumulated statistically higher amount of plant total nitrogen than those of mineral nitrogen treated plants. It was determined that the maximum plant total nitrogen (4.70% and 5.19%) was in Debrezeit and Chefedonsa soils, respectively. However, NSLNR-64 treated plants did not record significant amount of shoot dry weight as compared to negative control on both investigated soils. This finding is in line with the report of Diriba Temesgen (2007) that higher shoot dry weight has not always been associated with the accumulation of higher total plant nitrogen.

The study found that external mineral nitrogen fertilizer application did not improve growth and development plants; instead it delayed nodulation and inhibited the symbiotic nitrogen fixation potential of indigenous rhizobia. Inhibitory effects of added nitrogen fertilizer to nodulation and nitrogen fixation have been reported by Chemining'wa et al. (2004). This could be due to the fact that lentils can fix adequate amounts of N for growth (McNeil and Materne, 2007). Similarly, external nitrogen fertilizer has not shown any significant yield difference on lentil (Angaw Tsigie and Asnakew Woldeab, 1994).

In general, shoot dry matter yield on Debrezeit soil was much higher than Chefedonsa soils within the same treatments. This might be due to the fact that Chefedonsa soil had low and insufficient available phosphorus (Table 1) which in turn affects the symbiotic nitrogen fixation and developing effective nodules which finally affect yield and yield components of legume plants (Desta Beyene and

Angaw Tsige, 1989). A similar result was reported by Philpotts, (1975) who found that low phosphorus availability was responsible for poor nodulation which led to low plant yield.

Table 5. Symbiotic effectiveness of rhizobia nodulating Lentil on Chefedonsa soil under greenhouse condition.

Isolates	Nodule number plant^{-1}	Nodule dry weight (mg plant^{-1})	Shoot dry weight (gm plant^{-1})	Plant total nitrogen (%)
NSLNR-10	87 ± 17.5a	0.281 ± 0.126ab	0.435 ± 0.003a	4.570 ± 0.434bc
NSLNR-16	58 ± 4.6cd	0.214 ± 0.052bc	0.649 ± 0.174a	4.965 ± 0.536ab
NSLNR-48	80 ± 14.6ab	0.319 ± 0.024a	0.556 ± 0.123a	4.408 ± 0.390bc
NSLNR-51	39 ± 5.1e	0.107 ± 0.015e	0.457 ± 0.059a	4.013 ± 0.389c
NSLNR-56	49 ± 13.7de	0.165 ± 0.070cd	0.477 ± 0.035a	4.517 ± 0.282bc
NSLNR-64	67 ± 2.5bc	0.204 ± 0.055bcd	0.591 ± 0.038a	5.187 ± 0.148a
NSLNR-69	71 ± 11.5abc	0.153 ± 0.030cde	0.669 ± 0.231a	4.379 ± 0.178bc
NSLNR-77	49 ± 8.3de	0.119 ± 0.032de	0.444 ± 0.105a	4.616 ± 0.441abc
-ve control	68 ± 4.5bc	0.186 ± 0.026de	0.569 ± 0.097a	4.046 ± 0.128c
+ve control	60 ± 1.0cd	0.191 ± 0.029cde	0.442 ± 0.243a	4.116 ± 0.310c
SEM	9.99	0.053	0.141	0.352
CV (%)	15.9	27.3	26.6	7.9
LSD	17.1	0.091	0.242	0.605

Data are means of three replicates ± SE; same letters are not significantly different at LSD P<0.05 level.
-ve (negative control)-without chemical and biological fertilizers; +ve (positive control)-With optimum amount of nitrogen fertilizer.

Enumerating indigenous Rhizobia nodulating lentil

The soil physico-chemical and number of indigenous rhizobia nodulating lentils of the nine selected soils are stated in Table 1. According to the farmer's response in the interview, all the investigated soils had a cropping history with lentil for many years without inoculation. The rhizobial counts were generally ranging from 1 x 10^2 to 5.8 x 10^3 cells g^{-1} of soils of major lentil growing areas of Ethiopia. It is possible to conclude that Ethiopian soils are harbored with sufficient number of lentil rhizobia (Slattery *et al.,* 2004). However, the number of rhizobia in Tulubolo was detected to be less than 100 cells g^{-1} of soils. Soil pH was ranging from 6.9 to 8.1 in all investigated soils; therefore, it is unlikely to have markedly affected the survival and multiplication of rhizobia. It is known that the survival of rhizobia can be affected at soil pH <5.5, with severe reductions in numbers at pH < 4.5 (Martyniuk and Oron, 2008).

There was no significant correlation between soil factors (pH, CEC, Available phosphorus, EC, Total nitrogen, Organic carbon) and number of rhizobia harbor in tested soil at P >0.30 except it was found to be positively correlated with Ca (cmol(+))/kg (r=0.82 P<0.01, data not presented). A similar result was reported by Musiyiwa *et* al. (2005) who found that population sizes were poorly correlated with soil physico-chemical properties. This might be considered as a result of the fact that all soils have been analyzed with similar soil physico-chemical properties and favorable for rhizobia persistence and growth (Giller, 2001). The least number of indigenous rhizobia nodulating lentils was obtained from Tulubolo (3.1x10), followed by Sheno (1x10^2) soils which was collected from marshy lands. This result confirmed previous results in Mhondoro district of Zimbabwean soils, where no rhizobial cell was detected using the MPN method when soil was collected from the marshy land (Musiyiwa *et al.,* 2005).

Conclusion

Inoculation significantly increased all investigated parameters such as shoot length; shoot dry weight plant total nitrogen and net nitrogen fixed through forming nodules on its own host. About 20.9% of isolates collected from major lentil growing areas of Ethiopia were harbored with very effective isolates, 77.3% tested isolates as effective rhizobia and only one isolate from Alem gena area grouped as ineffective strain. In general, inoculation selected rhizobia and nitrogen application did not improve shoot dry weight of lentil in soil culture, mainly due to the reason that both soils had sufficient number of background rhizobia nodulating lentil harbored with $>10^3$ rhizobia g^{-1} of soils. Finally, further investigations of very effective isolates need to be tested under greenhouse and field condition on soil culture to assess their competitiveness ability, adaptability to the wide edaphic condition and survival and colonization within soil.

Enumeration of background rhizobia nodulating lentil indicated that, except soil sampled from Tulubolo area, all investigated soils have had higher indigenous rhizobia of $\geq 10^2$ cell g^{-1} of soil. This variation was not correlated with the investigated soil physico-chemical properties. Therefore, it may be concluded that, the major lentil growing area of the country except soils collected from Tulubolo area, have shown enough background rhizobia nodulating lentil.

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