Ethiop. J. Biol. Sci., 6(2): 97-114, 2007
 ISSN: 1819-8678

PHENOTYPIC CHARACTERISTICS OF COMMON BEAN (*PHASEOLUS VULGARIS*)-NODULATING RHIZOBIA FROM SOME PARTS OF SOUTHERN ETHIOPIA

Alemayehu Workalemahu[1,*] and Fassil Assefa[2]

ABSTRACT: Eighteen strains of common bean rhizobia from Konso (GI), Arbaminch (GII), Sodo (GIII) and Ziway-Awassa-Dilla (GIV) areas in southern Ethiopia were isolated. The strains were characterized by sixty-four phenotypic traits. These traits were tested for cluster analysis using unweighted pair group method with average (UPGMA) with NTSYS version 2.1. pH tolerance/sensitivity, antibiotic tolerance/sensitivity together with the numerical analysis results clearly showed diversity among the strains. The cluster analysis grouped the strains into two clusters that included 78% of the strains at 85% similarity level, except four strains which were not clustered. These strains were less related to the clustered strains and to each other. The clustered stains were with the presumed similar characters of *Rhizobium leguminosarun/Rhizoium etli* group except AUPR10. Two isolates, AUPR9 (unclustered) and AUPR10 (cluster I), exhibited rough colony appearance on Peptone Yeast Extract Agar medium (PY) that resemble the characteristics of *Rhizobium gallicum*. Strain AUPR8 (from unclustered strains) displayed creamy colony appearance on PY, tolerance to extreme pH, salt, and temperature and inability to utilize dulcitol and utilization of glycine that resembled with characters of *Rhizobium tropici*. This result, therefore, indicates the possibility of obtaining inoculant strains with tolerance to environmental conditions of common bean-producing areas of Ethiopia.

Key words/phrases: Common bean; Diversity; Phenotype; Rhizobia; Southern Ethiopia.

INTRODUCTION

Common bean is capable of fixing nitrogen to the tune of 50-180 kg N ha^{-1} (Nipe-Nolt and Pindeda, 1988), in association with several groups of root nodule bacteria known as rhizobia. Consequently, it is an important component of low input agriculture to maintain the fertility of soil. The efficiency of symbiotic nitrogen fixation in common bean varies depending on the diversity of host cultivar, bacteria strains, and different environmental factors (Hardarson *et al*., 1993; Zaharan, 1999; Rengel, 2002).

[1]Department of Biology, Mekelle University, P.O. Box 1176, Mekelle, Ethiopia.
E-mail:alemayehuwork@yahoo.co.uk
[2]Department of Biology, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia.
*Author to whom all correspondence should be addressed.

The root nodule bacteria that nodulate common bean were initially grouped under the species *Rhizobium leguminosarum* with a broader host range including beans, peas, and clover (Jordan, 1984). It was later classified into *Rhizobium leguminosarum* biovar phaseoli (Jordan, 1984). Later on, several species of *R. etli*, *R.* tropici, *R. giardnii* and *R. gallicum* were named based on their genetic, ecological and geographical affiliations (Martinez-Romero *et al*., 1991; Anyango *et al*., 1995; Amarger *et al*., 1997; Mhamdi *et al*., 1999).

Common bean is one of the major tropical leguminous crops in Ethiopia where more than 10 million people use it as a major source of protein and cash crop (EIAR, 2000). Its production is expanding to areas with altitudes between 2000-2200 m a.s.l, where horse bean has failed to fulfill the protein requirements of the people growing starchy foods like *enset* (Getachew Kassaye, 1990). Common bean is also the main component of the cropping system of lowlands of Ethiopia because of its double cropping, shade tolerance and early maturity (Tenaw Workayehu and Yeshi Chiche, 1990). It also has drought tolerant properties. Consequently, it is considered as a risk aversion crop in drought-prone areas (Shimelis Woldehawariat *et al*., 1990).

The crop covers large area among pulses. However, the average national yield per hectare is low compared to other pulses and yield at experimental stations. Even though different factors may be attributed to yield reduction, one of the most important factors could be N-deficiency of most Ethiopian soils (Desta Beyene and Angaw Tsigie, 1987). Under such conditions, the cheaper and environmentally friendly option is using the crop's symbiotic association with rhizobia since commercial N-fertilizer is neither available nor affordable to resource-poor farmers. To exploit the symbiosis in enriching nitrogen to the soil, effective rhizobial strains adaptable to different environmental conditions should be isolated from indigenous soils.

In Ethiopia, many researches have been undertaken to improve common bean cultivars (EIAR, 2000). But, there are very few studies on the characterization of common bean nodulating rhizobia from Ethiopian soils (EIAR, 2000; Desta Beyene *et al*., 2004). Although Desta Beyene *et al*. (2004) recently reported the limited diversity of the common bean nodulating rhizobia from their study areas, mainly central Ethiopia, the possibility of finding other groups still remains unexploited. These necessitate the need for screening more rhizobial isolates to define their diversity, characterize and study their adaptability to different

environmental conditions. Such study is important to fully realize the biological nitrogen fixing symbiotic association in low input agricultural production systems in Ethiopia. This study was, therefore, initiated to isolate and characterize common bean rhizobia for phenotypic traits from some parts of southern Ethiopia.

MATERIALS AND METHODS

Study sites and soil sampling

The selected study sites are important common bean-producing areas of southern Ethiopia (Table 1) and are grouped into four reference areas. The areas are distributed on altitudes between 1,210-2,200 m a.s.l., soil pH ranging from 5.5 moderately acidic to pH 8.7 alkaline and grouped into GI (Konso), GII (Arbaminch), GIII (Soda), and GIV (Zeway-Awassa-Dila) regions, based on their physical location (Fig. 1).

Table 1 Soil sampling sites of authenticated strains of root nodule bacteria from some common bean-growing areas of southern Ethiopia.

No.	Name of the site	Altitude	Soil pH	Code
Group I (Konso area)				
1	Gaho	1840	6.6	AUPR2
2	Mechela	1720	6.7	AUPR5
3	Tishale	1210	6.9	AUPR6
4	Gato	1295	8.7	AUPR7
Group II (Arba Minch area)				
5	Genetameche	2200	5.5	AUPR1
6	Pura	1230	6.7	AUPR8
7	Marka	1310	7.0	AUPR9
8	Ambokesha	1780	5.8	AUPR10
Group III (Sodo area)				
9	Selam Ber	1380	6.5	AUPR3
10	Gogara	1300	6.6	AUPR11
11	Wachigo Esho	1730	5.6	AUPR12
12	Shoya	1860	5.7	AUPR13
13	Gedeba	1800	6.8	AUPR14
14	Awergama (Siraro)	1700	6.4	AUPR15
Group IV (Zeway-Awassa-Dilla areas)				
15	Leku	1850	6.8	AUPR16
16	Tugaweransa	1760	6.7	AUPR17
17	Adello	1900	6.1	AUPR18
18	Kemo Gerbi	1630	7.9	AUPR4

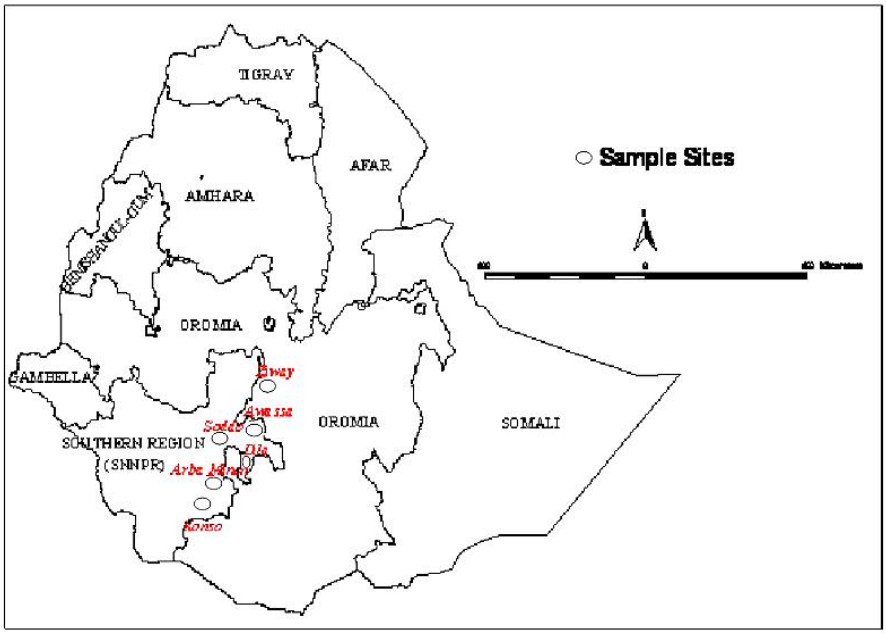

Fig.1. Location of sampling sites

Eighteen soil samples were collected in November 2005 from farmers' fields where common bean has been grown and with no previous history of inoculation with rhizobia. Three kg of soil samples were collected from each site. Five farmers' fields were selected from which soils were excavated from the upper 10-15 cm depth, pooled and composite samples were collected in ethanol (70%) sterilized plastic bags.

Isolation of rhizobia

Rhizobia were isolated from the soil samples by inducing nodulation on Red Wolaita (RW) cultivar of *Phaseolus vulgaris* using 'plant infection' method (Vincent, 1970). Each soil sample was thoroughly mixed, sieved using 2 mm mesh-size sieve, and filled into 3 kg capacity surface-sterilized (70% ethanol) plastic pots. Five selected seeds were surface-sterilized with 95% ethanol and 0.1% acidified mercuric chloride (5 ml conc. HCl with 1L distilled water) for 4 min. They were repeatedly washed with sterilized distilled water and allowed to germinate on sterile water agar plates (7.5g of agar in 1L water) for 2 days. Four pre-germinated seeds were transplanted to each pot, which were, then, thinned down to 3 after a week. The pots were watered at field capacity every three days for 30 days (Vincent, 1970).

Thirty days after planting (DAP), the plants were gently uprooted from the pots and immersed several times in a container containing water to remove soil particles. Nodules were collected from roots and surface sterilized and transferred into sterilized Petri dishes to be crushed with flamed glass rod. The extract from nodules was streaked on to Yeast Extract Mannitol Agar-Congo red (YMA-CR) plates with pH adjusted to 6.8 by using 1N HCl or 1N NaOH and incubated for 3-5 days at 28°C ± 2°C. YMA-CR contains g 1^{-1} (Vincent, 1970): Mannitol, 10; K_2HPO_4, 0.5; $MgSO_4$; $7H_2O$, 0.2; NaCl, 0.1; Yeast Extract, 0.5; Agar, 15.

Plates were periodically examined for growth. Single colonies were picked and purified by re-streaking on new YMA plates for growth. Pure isolates were then preserved on YMA slants containing 0.3% (w/v) $CaCO_3$ and stored at 4°C (Vincent, 1970).

Authentication of isolates

Authentication of isolates as root nodule bacteria was undertaken by re-inoculating them on to the host. Three-kilogram capacity pots were filled with acid washed and heat sterilized (121°C, 151b/in) river sand. Each isolate was grown on Yeast Extract Broth (YMB) for three days and adjusted to a concentration of 10^9 cells ml^{-1}. Seeds of Red Wolaita (RW) were surface-fertilized and planted on 3 kg capacity pots as before (Vincent, 1970). Pots were once fertilized with 20 ppm nitrogen as a starter nitrogen source just before planting (Gibson, 1980) and grown under greenhouse conditions. Four seeds were planted per pot and individually treated with 1 ml of inoculum ($10^9 ml^{-1}$) and thinned to three plants per pot after 5 days of emergence (DAE). All pots received sterilized quarter strength Broughton and Dilworth nitrogen-free nutrient solution once a week, and distilled water twice a week according to Lupwayi and Haque (1994). Plant roots were checked 30 days after emergence (DAE) for the presence or absence of nodules form each pot.

Characterization of isolates

All isolates were characterized by their morphological, biochemical and physiological features. All inoculations were standardized by growing the isolates in shaker at 125 rev/min at room temperature with an inoculum size of approximately 10^4 cells/10 μl, unless otherwise specified.

All tests were carried out in triplicates on Tryptone Yeast Extract medium (TY), except tests for morphology (colony diameter and texture), acid-alkaline production, and growth on 2% (W/V) urea, which were made on

YMA medium. In addition, Peptone Yeast Extract Agar medium (PY) was used for further morphological characteristics of bean isolates. Each plate was spot inoculated eight times with 10 µl (10^4 cells) of the isolate (Amarger *et al*., 1997). All inoculated plates were incubated at 28 $\pm$ 2°C except cultures used to determine minimum and maximum growth temperature. Monitoring was made after 5 days. Result for growth tests was determined qualitatively and presented as '+' = growth and '-' = no growth.

Colony morphology and growth

Colony morphology and acid/base reaction were evaluated on YMA containing, 25 $\mu l ml^{-1}$ Congo red and 0.5% bromothymol blue (BTB) (Vincent, 1970). Growth and colony appearance were characterized as small dry (SD), large mucoid (LM) and large watery (LW) on YMA according to Ahmed *et al*. (1984) and on TY, Tryptone Yeast-extract medium lacking Calcium medium (TY-Ca), PY, and Luria-Bretani (LB) medium according to Amarger *et al*. (1997).

Mean generation time or doubling time of isolates was evaluated by growing them on YMB. Turbidity was measured every 6 hours at 540 nm. The experiment was conducted in triplicates. Mean generation times were then determined from logarithmic growth according to Somasegaran and Hoben (1994). Growth of isolates on urea and melanin production were evaluated on YMA medium containing 2% (W/V) urea, and TY medium containing tyrosine and $CuSO_4$, respectively (Hungria *et al*., 2000).

Tolerance to acidity, alkalinity, salinity and temperature

Tolerance to acidity and alkalinity of each isolate was evaluated on TY medium with pH previously adjusted to 4.0, 4.5, 5.0, 8.5 and 9.0 with 1N HCl or 1N NaOH. For salt tolerance, the isolates were transferred to TY plates supplemented with NaCl at concentrations of 0.5, 1.0, 1.5 and 2% (W/V). The ability of bacterial strains to grow at high and low temperature was monitored at incubation temperatures of 4°C, 10°C, 15°C, 30°C, 35°C, 40°C and 45°C on YMA medium pH adjusted at 6.8 (Jordan, 1984). They were also incubated at 37°C, and 40°C on TY medium pH adjusted at 6.8 (Hungria *et al*., 2000). Controls consisting of bacteria grown on YMA and TY at pH of 6.8 were included.

Utilization of carbohydrates and nitrogen

The different carbon sources were added as described by Amarger *et al*. (1997) at a final concentration of 1g/L to a basal medium containing (g/ L): K_2HPO_4, 1; KH_2PO_4, 1; $FeCl_3$ $6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $(NH_4)_2SO_4$, 1; and 15 g of agar. The following filter sterilized (0.22μm millipore) sole carbon sources were added after autoclaving: L- arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D- mannose, raffinose, L-rhamnose, D-sorbitol, xylose, dulcitol, inositol, citrate, tartarate, cellobiose, glycerol, anoditol and gluconate. Filter sterilized L- tryptophan, L- tyrosine and glycine were used as a sole nitrogen source for isolates by adding a final concentration of 0.5g L^{-1} to the above basal medium from which ammonium sulfate had been omitted and to which mannitol had been added at a concentration of 1g L^{-1} (Amarger *et al*., 1997).

Intrinsic antibiotic resistance

The intrinsic antibiotic resistance of isolates was determined on seven antibiotics and fourteen concentrations (Amarger *et al*., 1997). The antibiotics were prepared μl ml^{-1} as indicated in brackets below; Ampicilline (5, 10) and Kanamycine (10, 15) were dissolved in distilled water. Nalidixic acid (40, 60), and Chloramphenicol (5, 10), Streptomycine (3, 10) and Erythromycin (5, 10) were dissolved in NaOH and ethanol, respectively. Methanol was used to dissolve Rifampincin (5, 10). They were then filter-sterilized (0.22 μl pore size) and added to autoclaved TY medium.

Phosphate solubilizing ability

This was determined by inoculating the isolates on Pikovskaya Agar medium (PA) containing (g/L): Glucose (10), Tricalcium phosphate (5), Ammonium sulphate (0.5), Yeast extract (0.5), Magnesium sulphate heptahydrate (0.1), Sodium chloride (0.2), Manganese sulphate (0.002), Ferrous sulphate (0.002) and Agar (15). The pH of the medium was adjusted to 7.00. This ability was detected based on growth and the presence of clear zone around the colonies (Nautiyal, 1999).

Numerical analysis

Sixty-four phenotypic traits of the 18 isolates were used. Traits were coded '2' for growth and '1'for no growth. A computer cluster analysis of the 64 phenotypic traits was carried out using similarity coefficient and a dendrogram was constructed by the unweighted pair group method with average (UPGMA) clustering method using NTSYS-pc version 2.1.

RESULTS

Authentication of isolates

Eighteen rhizobial strains were collected from many sites of some common bean-growing areas of southern Ethiopia. All isolates obtained from different geographical locations which ranged from lowlands (1,210 m a.s.l) to highlands (2,200 m a.s.l), and soil with contrasting pH (5.5-8.7), induced nodule formation.

Colony morphology and mean generation time (MGT)

Almost all strains displayed large mucoid (LM), except large watery (LW) colonies by AUPR8 (2-4 mm), and small dry (SD) colonies by AUPR18, displayed fast growth (2-4 h doubling time), and acidified the YMA-BTB medium (Table 2). All strains, except AUPR8, AUPR9 and AUPR10, appeared smooth and gummy (SG). Strain AUPR8 displayed creamy colonies (C) while that of AUPR9 and AUPR10 displayed rough colonies (R) on PY medium.

Physiological and biochemical characteristics

Almost all strains did not grow on LB, TY-Ca and 2% urea (Table 3), 1-2% NaCl, pH 4.0, and at 10 and 40°C on TY medium. Melanin production and carbohydrate utilization was a common feature of most strains. However, AUPR3, AUPR4, AUPR8, AUPR9 and AUPR10 were exceptions to one or more of the above features. Two third of the strains and almost all strains were found to grow at pH 4.5, and pH 5.0-8.5, respectively. Although L-tryptophan and L-tyrosine were used as nitrogen source for almost all strains, glycine was only utilized by AUPR8 (Table 3).

Almost all strains grew on all concentrations of Chloramphenicol, Ampicillin and Nalidixic acid. AUPR7 was found to grow on all tested antibiotics, followed by AUPR3, AUPR9, and AUPR16. The most sensitive strains that failed to grow on several of the tested antibiotics were AUPR8, AUPR12 and AUPR13. Eighty percent and ninety percent of the strains were found to be sensitive to Erythromycin (10), and Rifampincin (10), respectively (Table 3).

Table 2 Morphological and cultural characteristics of strains.

Morphological characteristics	AUPR1	AUPR2	AUPR3	AUPR4	AUPR5	AUPR6	AUPR7	AUPR8	AUPR9	AUPR10	AUPR11	AUPR12	AUPR13	AUPR14	AUPR15	AUPR16	AUPR17	AUPR18
Colony size (mm)	2.4	3.5	2.5	2.3	2.5	2.0	2.0	4.0	2.3	2.4	2.0	2.0	2.0	3.0	2.6	2.2	2.2	1.0
Colony morphology on YMA	LM	LM	LM	LM	LM	LM	LM	LW	LM	LM	LM	LM	LM	LM	LM	LM	LM	SD
Colony morphology on PY	SG	SG	SG	SG	SG	SG	SG	C	R	R	SG	SG	SG	SG	SG	SG	SG	SG
Growth rate (hr)	2.0	1.5	2.0	2.08	1.94	2.14	3.81	1.22	1.90	2.0	3.0	2.74	3.0	2..33	2.0	2.70	2..50	4.0

Note: LM= large mucoid, LW= large watery, SD= small dry, SG= smooth gummy, C= creamy, R= rough

Table 3 Physiological and biochemical characteristics of the isolates.

Characteristics	AUPR1	AUPR2	AUPR3	AUPR4	AUPR5	AUPR6	AUPR7	AUPR8	AUPR9	AUPR10	AUPR11	AUPR12	AUPR13	AUPR14	AUPR15	AUPR16	AUPR17	AUPR18
Salt tolerance																		
0.5% (w/v) NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1% (w/v) NaCl	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
1.5% (w/v) NaCl	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
2% (w/v) NaCl	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Temperature tolerance TY																		
37°C	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
40°C	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
YMA																		
10°C	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
40°C	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
pH tolerance																		
4	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
4.5	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	+	-	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
9	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Antibiotics Resistance(mg/ml)																		
Nalidixic acid																		
40	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Streptomycin																		
3	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
10	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-

Continued from Table 3

Kanamycin																		
10	+	-	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	+
15	+	-	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	+
Erythromycin																		
5	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+
10	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-
Rifampcin																		
5	-	+	+	-	-	-	+	+	+	-	+	+	-	+	+	+	-	+
10	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Chloramphenicol																		
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Ampicillin																		
5	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
10	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Growth on Dulcitol	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Nitrogen utilization																		
Glycine	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
L- tryptophan	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Tyrosine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on LB	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Growth on TY-Ca	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Phosphate solubilizing ability	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on 2% urea	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Melanin production	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+

Note: '+'growth and '-' no growth

The result of cluster analysis performed on the 18 strains for 64 phenotypic traits is shown in Fig. 2. The result grouped the strains into two distinct clusters which contain 78% of the strains at 85% similarity level, except four strains (AUPR8, AUPR9, AUPR12, and AUPR18) which were not clustered. These strains are less related to the strains in cluster I and cluster II, and to each other. Particularly, AUPR8 is more divergent from the other strains. In the dendrogram, 100% similarity was observed only between AUPR5 and AUPR6, and AUPR2 and AUPR15 from different sub-clusters. Even though most of the strains are similar at 83% level, this analysis generally showed the diversity of strains among the isolates.

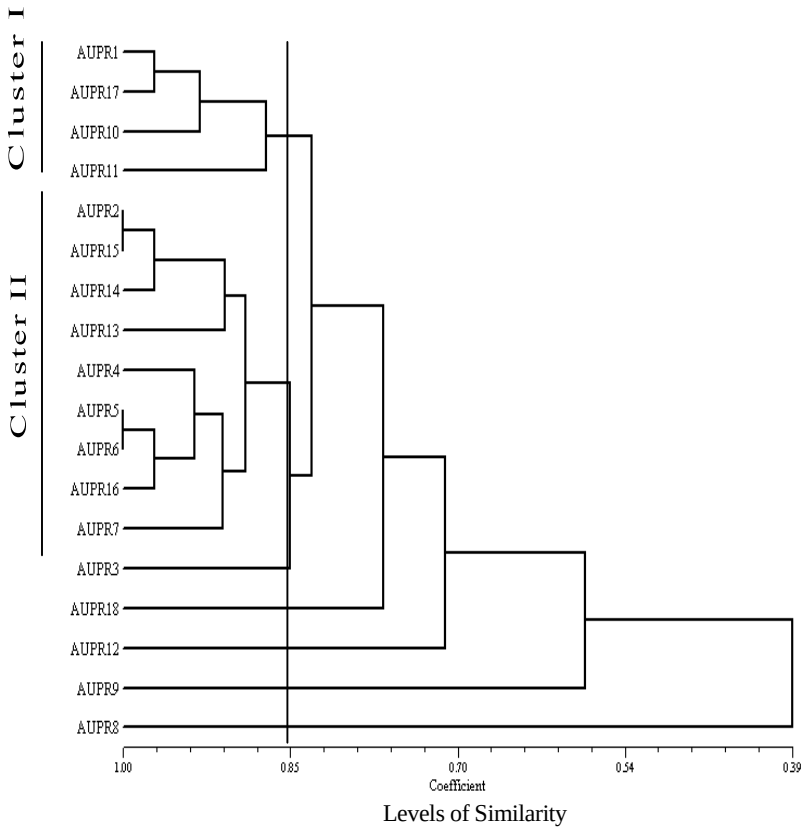

Fig. 2. Dendrogram highlighting the phenotypic similarities among the strains.

DISCUSSION

According to the classification of Rhizobiaceae in Bergey's Manual (Jordan, 1984), the strains fall into fast growing rhizobia, based on their generation time (< 4 hr), acid production and large growth with production of copious exopolysaccharide at optimum temperature range (25-30°C) and pH of the medium (6-7). These characters are widespread among common bean-nodulating rhizobia (Amarger *et al*., 1997; Aguilar *et al*., 1998; Diouf *et al*., 2000; Andrade *et al*., 2002; Desta Beyene *et al*., 2004).

Most strains displayed LM colonies except LW and SD colonies by AUPR8, and AUPR18, respectively. Morphological characteristics of common bean strains on PY medium differentiated them into respective type groups. AUPR8 were found to be creamy colonies, which is a characteristic of *R. tropici* strains (Martinez-Romero *et al*., 1991). AUPR9 and AUPR10 were found to be rough colonies, which is a characteristic of *R. gallicum* (Silva *et al*., 2003). All the other strains were found to be smooth gummy colonies, which could be *R. leguminosarum* or *R. etli* (Martinez-Romero *et al*., 1991; Silva *et al*., 2003).

Strains were generally found to be salt-sensitive. All except AUPR8, AUPR9 and AUPR18 were unable to grow above 0.5% (w/v) NaCl. Amarger *et al*. (1997) found no common bean strains which were tolerant at 1% (w/v) NaCl, except *R. giardinii* and *R. tropici* strains. Molecular tools have also identified six osmotolerant strains that could grow at 2% (w/v) NaCl as *R. tropici* in Morocco (Bouhmouch *et al*., 2001). Similarly, Boncompagni *et al*. (1999) reported reference strains of *R. leguminosarum* bv. phaseoli and *R. etli* which could not tolerate 100mM NaCl whereas *R. tropici* is inhibited at 200mM NaCl. Bouhmouch *et al*. (2001) also found strains from saline soils which were more tolerant than others. These results revealed that in addition to their genotype, the tolerant strains were naturally selected by saline soils.

All strains except AUPR8 were unable to grow at low (10°C) and high (40°C) temperature. Thus, our result suggests that most strains were sensitive at 10°C (YMA) and 40^{o}C on TY and YMA media. Raposerias *et al*. (2002) indicated that *R. tropici* strains revealed less alteration in phenotypes than *R. leguminosarum* bv. phaseoli strains after their exposure to thermal shock at 45°C. Hungria *et al*. (2000) also found that *R. tropici* was tolerant at 40^{o}C on TY medium.

All strains could grow at pH 5 and 8.5 except AUPR12 which failed to grow at 8.5. In contrast to high pH, all strains except AUPR8 were unable to grow at low pH. This suggests that most strains are sensitive to lower pH and tolerant to higher pH. Similar explanation was made by Jordan (1984), fast- and slow-growing rhizobia were more tolerant at alkaline and lower pH, respectively. However, some fast growing strains such as *R. tropici* and *Mesorhizobium loti* could grow at a pH of 4 (Cooper, 1982; Cunningham and Munns, 1984; Graham, 1992; Gao *et al*., 1994). Amarger *et al*. (1997) found all strains of *R. tropici* were able to grow at pH of 4. However, the fact that only some strains of *R. giardinii* and *R. leguminosarum* bv. phaseoli showed tolerance to pH is not only related to their growth rate but also to the type of strains and probably their adaptation to acidic soils.

We generally found no difference in carbohydrate utilization among strains. Amarger *et al*. (1997) found most carbohydrates could be utilized by all tested rhizobia. However, most of bean nodulating bacteria could grow on dulcitol except *R. giardinii* and all strains of *R. tropici*. Similar finding was also shown by Andrade *et al*. (2002) that the *R. tropici* strains were unable to grow on dulcitol. Since similar observation was made in our study, this carbohydrate test was used as one of the traits for tentative classification of strains. All strains except AUPR8 were unable to grow on glycine. Amarger *et al*. (1997) and Andrade *et al*. (2002) also reported that most of bean strains could grow on L- tryptophan and L- tyrosine whereas glycine was utilized only by strains of *R. tropici*.

All strains but AUPR8 and AUPR9 could not grow on LB, TY- Ca and 2% urea media. Amarger *et al*. (1997) and Andrade *et al*. (2002) showed that most common bean rhizobia were unable to grow on LB and TY- Ca media, except *R. tropici* strains. Similarly, Martinez-Romero *et al*. (1991) found that all *R. tropici* strains were able to grow on LB and TY-Ca media, except *R. leguminosarum*. In our result, except AUPR8 and AUPR9, all bean strains were unable to grow, which seems that this character was correlated with salt tolerance.

In the present study, no strain was found to exhibit the ability to solubilize phosphate. On the other hand, melanin production was a common feature of all strains, except AUPR3, AUPR8 and AUPR9. Andrade *et al*. (2002) found that melanin production was observed in *R. tropici* strains. Similarly, melanin production was observed in *R. leguminosarum* bv. phaseoli by Cubo *et al*. (1988).

Most strains were found to grow in all concentrations of Chloramphenicol, Ampicillin, Nalidixic acid, and failed to do so on Rifampicin (10), and Erythromycin (10). Majority of the strains that failed to grow on one or the other concentrations of these antibiotics were in unclustered strains (AUPR8, AUPR9, and AUPR12). The pattern of resistance to many of these antibiotics has been used to identify diversity among strains of root nodule bacteria. Because of this, antibiotics resistance could be used as supplementary diagnostic character to show diversity among strains (Amarger *et al*., 1997).

Even though common bean rhizobia are diverse and hence difficult to assign to their respective species without genetic study, it is possible to use standard phenotypic features to tentatively group these bacteria into their respective species types. Phenotypic features such as growth rate and colony morphology described by Jordan (1984) and other phenotypic features that differentiate common bean nodulating rhizobia were used to make preliminary classification of the strains (Martinez-Romero *et al*., 1991; Amarger *et al*., 1997; Silva *et al*., 2003).

Based on the tested phenotypic traits, the strains can be tentatively placed into three groups. AUPR8 was classified as *R. tropici*-like strain mainly by its growth on glycine and failure to grow on dulcitol, AUPR9 and AUPR10 as *R. gallicium*-like strains because of their rough appearance on PY medium and the rest of the strains into *R. leguminosarum*- or *R. etli*-like strains because of the lack of clear demarcation in phenotypic features between them.

Generally inter-strain differences on several of the tested phenotypic characters were not distinctly observed among strains. However, pH tolerance/sensitivity, antibiotic tolerance/sensitivity together with the numerical analysis result clearly showed the diversity among the strains. Clusters I and II contain strains that grew on most of the tested carbohydrates including dulcitol, pH 4.5-8.5, and at 37°C on TY medium. Most of the strains in this group appeared to be smooth and gummy (SG). They were resistant to many of the tested antibiotics and unable to grow on glycine. They were also sensitive to 1-2% NaCl. Most of these characteristics corroborate the important features of *R. leguminosarum* var phaseoli and *R. etli* (Martinez-Romero *et al*., 1991; Amarger *et al*., 1997; Andrade *et al*., 2002).

Unclustered strains like AUPR8 and AUPR9 had quite distinct characters in terms of growth on LB, TY-Ca and 2% urea and failure to grow on Nalidixic acid and production of melanin. The appearance of creamy colonies (C) on PY medium of AUPR8 was different from rough colonies (R) of AUPR9 and AUPR10, which are the characteristics of non-*R. leguminosarum* isolates such as *R. tropici* strains (Martinez-Romero *et al*., 1991) and *R. gallicum* (Silva *et al*., 2003), respectively.

The result of cluster analysis and placement of AUPR10 that has 92% similarity with other isolates rather than AUPR9 is conflicting. This might be due to use of a single trait to place them in to *R. gallicum*-like strains and most traits were strain specific (Amarger *et al*., 1997). Even though the trait was used in the previous works and it was similar with genetic classification, this work should be supported by other phenotypic and genotypic studies from Ethiopia.

Desta Beyene *et al*. (2004) reported that *R. leguminosarum* is the dominant symbiont type of common bean rhizobia in their study areas in Ethiopia, and ruled out the presence of American type such as *R. etli*. Our result is most likely in agreement with their generalization, and it is unlikely that Ethiopian soils are extensively colonized by rhizobia of American and European origin. But in some African countries such as Kenya, Senegal and Tunisia, rhizobia of American and Europe origin were common and abundant (Anyango *et al*., 1995; Mhamdi *et al*., 1999; Diouf *et al*., 2000). Our result indicates it is highly likely that different groups of bean nodulating rhizobia may exist in Ethiopia. This can be proved through extensive genetic studies covering most of the bean-growing areas. The result also indicates the possibility of obtaining inoculant strains with tolerance to different environmental conditions in the bean-growing areas of Ethiopia.

ACKNOWLEDGEMENTS

The authors are very grateful to Mekelle University for sponsoring Alemayehu Workalemahu to work on this topic as part of his M.Sc. dissertation. The financial support of the project “Microbial Inputs into Agroforesty” sponsored by SIDA/SAREC, and the supply of seeds by Melkasa Agricultural Research Centre are highly appreciated.

REFERENCES

Aguilar, O., Lopez, M., Riccillo, P., Gonzalez, R., Pagano, M., Grasso, D., Puhler, A., and Favelures, G. (1998). Prevalence of *Rhiboium etli*-like allele in genes coding for I6S rRNA among the indigenous rhizobial populations found associated with wild

beans from the southern Andes in Argentina. *Appl. Environ. Microbiol.* **64**: 3520-3524.

Ahmed, M., Rafique Uddin, M. and McLaughlin, W. (1984). Characterization of indigenous rhizobia from wild legumes. *FEMS Microbiol.Lett.* **24**: 197-203.

Amarger, N., Macheret, V. and Laguerre, G. (1997). *Rhizobium gallicum* sp. Nov. and *Rhizobium giardinii* sp. Nov from *Phaseolus vulgaris* nodules. *Int. J. Syst. Bacteriol.* **47**: 996-1006.

Andrade, D., Murphy, P. and Giller, K. (2002). The diversity of *Phaseolus* nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. *Appl. Environ. Microbiol.* **68**: 4025-4034.

Anyango, B., Wilson, K., Beynon, J. and Giller, K. (1995). Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Appl. Environ. Microbiol.* **61**: 4016-4021.

Boncompagni, E., Osteras, M., Poggi, M.C. and Lerudulier, D. (1999). Occurrence of choline and glycine betaine uptake and metabolism in the family of Rhizobiaceae and their roles in osmoprotection. *Appl. Environ. Microbiol.* **65**: 2072-2077.

Bouhmouch, I., Brhaoda, F., Filali-Maltouf, A. and Aurag, J. (2001). Selection of osmotolerant and effective strains of Rhizobiaceae for inoculation of common bean (*Phaseolus vulgaris*) in Moroccan saline soils. *Agron.* **21**: 591-599.

Cooper, J. (1982). Acid production, acid tolerance and growth rate of lotus rhizobia in laboratory media. *Soil Biol.Biochem.* **14**: 127-131.

Cubo, T., Buendia-Claveria, A., Beringer, J. and Ruiz-Sainz, J. (1988). Melanin production by *Rhizobium* strains. *Appl.Environ.Microbiol.* **54**(7): 1812-1817.

Cunnigham, S. and Munns, D. (1984). The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. *Soil Biol.Biochem.* **14**: 127-131.

Desta Beyene and Angaw Tsigie (1987). The response of pulse crops to N and P fertilizers. In: **Soil Science Research - Proceedings of the first soil science research review workshop,** 11-14 Feb., IAR. Institute of Agricultural Research, Addis Ababa, Ethiopia.

Desta Beyene, Serawit Kassa, Franklin, A.M., Amha Assefa, Tadesse Gebrmedhin, and Van Berkum, P. (2004). Ethiopian soils harbor natural population of rhizobia that form symbiosis with common bean (*P. vulgaris*). *Arch. Microbiol.* **181**: 129-132.

Diouf, A., Lajudie, P., Neyra, M., Kersters, K., Gillis, M., Martinez-Romero, E. and Gueye, M. (2000). Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia). *Int. J. Syst. Evol. Microbiol.* **50**: 159-170.

EIAR (2000). Lowland pulses research strategy. Addis Ababa, Ethiopia, 39 pp.

Gao, J.L., Sun, J.G., Li, Y., Wang, E.T. and Chen, W.X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. *Int. J. Syst. Bacteriol.* **44**: 151-158.

Getachew Kassaye (1990). Research recommendations and adaptation of improved technology for haricot bean in the southern zones of Ethiopia. In: **Proceedings on research on haricot beans in Ethiopia**, pp. 88-94, 1-3 October, IAR. Institute of Agricultural Research, Addis Ababa, Ethiopia.

Gibson, A. (1980). Methods for legumes in glasshouse and controlled environmental cabinets. In: **Methods for Evaluating Biological Nitrogen Fixation**, pp. 139-184 (Bergersen, F., ed.). John Wiley and Sons, Chichester.

Graham, P. (1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium* and nodulation under adverse soil conditions. *Can. J. Microbiol.* **38**: 475-484.

Hardarson, G., Bliss, F., Cigales-Rivera, M., Henson, R., Kipe-Nolt, J., Longeri, L., Manrique, A., Pean-Cabrialeg, J., Pereira, P., Sanabria, C. and Tsai, S. (1993). Genotypic variation in biological nitrogen fixation by common bean. *Plant Soil* **152**: 59-70.

Hungria, M., Andrade, D., Chueire, L., Probanza, A., Guttierrez-Manero, F. and Megias, M. (2000). Isolation and characterization of new, efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. *Soil Biol.Biochem.* **32**: 1515-1525.

Jordan, D. (1984). Rhizobiaceae. In: **Bergey's Manual of Systematic Bacteriology.** Vol. II, pp. 234-256 (Hendricks, D.P., Sneath, H.A. and Halt, J.H., eds.). Williams and Wilkins, Baltinore, MD, USA.

Lupwayi, N. and Haque, I. (1994). **Legume-*Rhizobium* Technology Manual.** Environmental Sciences Division, International Livestock Center for Africa, Addis Ababa, Ethiopia, 93 pp.

Martinez-Romereo, E., Segovia, L., Mecante, F.M., Franco, A.A., Graham, P. and Pardo, M.A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol.* **41**: 417-426.

Mhamdi, R., Jebara, M. and Aouani, M. (1999). Genotypic diversity and symbiotic effectiveness of rhizobia isolated from root nodules of *Phaseolus vulgaris* L. grown in Tunisian soils. *Biol. Fertil. Soils* **28**: 313-320.

Nautiyal, C. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* **170**: 265-270.

Nipe-Nolt, J. and Pindeda, P. (1988). A strategy to improved nitrogen fixation: Examples from EI Salvador. *Bean Newslett.* **10**: 8-10.

Raposerias, R., Pinto, P., Passos, R., Seldin, L., Paiva, E. Scotti, M. and Sa, N. (2002). Variability of isolated colonies in bean nodulating *Rhizobium* strains before and after exposure to high temperature. *Braz. J. Microbiol.* **33**: 149-154.

Rengel, Z. (2002). Breeding for better symbioses. *Plant Soil* **245**: 147-162.

Shimelis Woldehawariat, Mitiku Haile and Wogayehu Bekele (1990). Haricot bean production in Harrarghe Highlands. In: **Proceedings of the workshop on research on haricot beans in Ethiopia**, pp.14-18, 1-3 October, IAR. Institute of Agricultural Research, Addis Ababa, Ethiopia.

Silva, C., Vinuesa, P., Eguiarte, L., Martinez-Romero, E. and Souza, V. (2003). *Rhizobium etli* and *Rhizobium gallicum* nodulate common bean (*Phaseolus vulgaris*) in a traditionally managed Milpa plot in Mexico: Population Genetics and Biogeographic Implications. *Appl. Environ. Microbiol.* **69**: 884-893.

Somasegaran, P. and Hoben, H. (1994). **Handbook for Rhizobia**. Springer-verlag, New York, 138 pp.

Tenaw Workayehu and Yeshi Chiche (1990). Importance, production system and problems of haricot bean in the southern Zone. In: **Proceedings on research on haricot beans in Ethiopia**, pp. 8-13, 1-3 October, IAR. Institute of Agricultural Research, Addis Ababa, Ethiopia

Vincent, J.M. (1970). **A Manual for the Practical Study of Root Nodule Bacteria**. Blackwell, Oxford, 166 pp.

Zaharan, H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Rev.* **63**: 968-989.