Phenotypic Characteristics and Preliminary Symbiotic Effectiveness of Rhizobia Associated with Haricot Bean Growing in Diverse Locations of Southern Ethiopia

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Abstract

*Phenotypic characteristics of one hundred thirteen rhizobia nodulating haricot bean (***Phaseolus vulgaris** *L.) growing on soils of nine different locations from southern Ethiopia were studied. Their tolerance to varying temperature, salinity, soil pH, heavy metals, antibiotics, their symbiotic effectiveness and cultural characteristics were determined. Eight reference species belonging to four different genera were also included in the analyses. The analyses allowed the description of a wide physiological diversity among tested isolates. Numerical analysis, based on numerical taxonomic approach, of the phenotypic characteristics, using Unweighted Pair Group Method with Average algorithm as implemented in NTSYspc21 software package, showed that, the tested isolates fell into five major diversity groups (designated as group I-V), when 82% level of relative similarity were used as cut-off point. Four strains were found to occupy a separate branch, thus designated as U (unclustered) group. While strains belonging to groups 1-IV were found to associate with recognized species belonging to Rhizobium, Ensifer and Mesorhizobium genus, the remaining test strains in cluster V and U were found to occupy distinct branches of their own on the dendrogram. Under laboratory condition, they were able to grow at pH ranging from 4 to 10.5; majority tolerated salt concentration (0.5-1%) and grew at a maximum temperature between 35 and 40 °C. The isolates were able to utilize a wide range of carbon and amino acid source and tolerated range of antibiotics and heavy metals. Based on the symbiotic effectiveness test, a number of potential isolates have been identified for inoculation trials.*

***Keywords*:** Haricot bean, numerical taxonomy, rhizobia, Southern Ethiopia

Introduction

Common bean (*Phaseolus vulgaris* L.) is a leguminous plant of worldwide with social and economic values, and is one of Africa's most essential pulses, cultivated on greater than 4 million ha (Broughton et al., 2003). It provides protein (20-25%) and carbohydrate in Ethiopia, common bean is one of the major pulse crops widely cultivated by smallholders, either as a sole crop or intercropped with sorghum, maize and other cereals (Ohlander, 1977), where in all cases soil fertility is depleted. Despite its high economic importance, the average yield of bean obtained by smallholder farmers in Ethiopia is low (1.5 tons/ha) (CSA, 2014), which is far below the potential yield from research managed experimental plots (4.6 tons/ha) (Assefa and Teshale, 2007), a reflection of, among other things, low soil fertility (Beyene and Tsigie, 1986).

The yield gap and soil N fertility issues could partly be alleviated by using soil bacteria generally known as rhizobia involved in biological nitrogen fixation (BNF) process with legumes including common bean. BNF is a key process for the conversion of nitrogen gas (N_2) into ammonia (NH_3) performed by bacteria belonging to the different genera within the family rhizobiaceae. Common bean, in most cases, substantially make use of the BNF process, thus able to fix up to 50 kg N ha−1(Bliss, 1993). With the respect to its symbiotic adaptability, haricot bean is generally regarded as promiscuous, non-selective host that is nodulated by several groups of root nodule bacteria (Hardarson et al., 1993) belonging to different genera including *Rhizobium* (Amarger et al., 1997; Martínez-Romero et al., 1991; Ramírez-Bahena et al., 2008; Segovia et al., 1993), *Ensifer* (Andrade et al., 2002) and *Mesorhizobium* (Grange and Hungria, 2004). As a reflection of its promiscuous nature, common bean is considered as a poor nitrogen fixing plant in comparison to other grain legumes. Furthermore, because of its inherent behavior as being nodulated with inefficient population of native common bean nodulating rhizobia in soil (Brockwell and Bottomley, 1995; Rodriguez-Navarro et al., 2000), it generally respond poorly to rhizobial inoculation in the field condition (Buttery et al., 1987; Graham, 1981; Hardarson et al., 1993). There are several reports, however, that indicated nitrogen fixation and the yield in common bean can be increased through inoculation of the crop with highly efficient rhizobial strains (Giller and Cadisch, 1995). For example, elite *Rhizobium* isolates were shown to improve the productivity of common bean (Hungria et al., 2003; Mostasso et al., 2002). In inoculation trials in Ethiopia, using *Rhizobium* species (strain HB 429, currently recognized as being national commercial inoculant) and eight *P. vulgaris*

cultivars, it was clearly demonstrated that rhizobial inoculation increased the total yield of the crop pod by 18% (Beshir et al., 2015). On the other hand, it was shown that effectiveness of the indigenous rhizobia that nodulate common bean vary depending upon location and host genotypes. This necessitates isolation and evaluation of more common bean nodulating rhizobia with regard to their diversity and effectiveness to fully realize the benefit of biological nitrogen fixation in this crop. Accordingly, many isolating works have been performed using soil from different sites in Ethiopia (Aserse et al., 2012; Ashenafi, 2017; Beyene et al., 2004; Workalemahu and Assefa, 2007). Therefore, this study was conducted with the objectives to isolate and characterize rhizobia from root nodules of common bean (*Phaseolus vulgaris* L.) plants grown at different locations in Southern Ethiopia, thus to determine their diversity on the basis of morphological, physiological and biochemical characteristics in reference to known species. Furthermore, preliminary screening of their symbiotic effectiveness on haricot bean was also assessed with the objective to identify the most efficient nitrogen fixing isolates for further field trials.

Materials and Methods

Collection of nodules

The nodules were collected from nine different locations in Southern Nation Nationalities and Peoples Regional State (SNNPRS) in Ethiopia (Table 1), where common bean has been grown with no previous history of inoculation with rhizobia. Characterization and symbiotic effectiveness of the test strains were conducted at Hawassa University, College of Agriculture in the soil microbiology laboratory.

Table 1. Geographical locations, and other associated parameters from which the test strains were isolated

Site	pH of the soil	Max and min Mean annual T(^{0}C)
Humbo	7.2	17.6-25
Areka	7.4	17.6-25
Bodity	6.6	17.6-22.5
Goffa	6.8	17-29
Hawassa	7.8	12-27
Badiwacho	7.1	17.6-22.5
Kachabira	6.7	15.1-22.5
Jinka	6.4	17-27.5
Durame	5.9	17.6-25

Source: SNNPRS finance and economy Bureau, metrological data, 2007

Rhizobial isolation

Nodules stored on silica gel were rehydrated in water and surface sterilized following the procedures as described elsewhere (Somasegaran and Hoben, 1994b). To verify whether the nodules were surface-sterilized or not, the last rinse of the nodules were streaked on a Yeast Extract Mannitol Agar (YMA) plates, incubated and observed for the growth of any microorganisms. The sterilized root nodules were then crushed aseptically in sterile petri-dish placed in laminar air flow hood by sterile crushing glass rods using a drop of sterilized normal saline (0.85% NaCl) solution. Loop-full of the suspensions of the crushed root nodules were streaked with sterile inoculating loop on Yeast Extract Mannitol Agar (YMA, pH=7)) plates containing 0.025% (w/v) Congo Red and incubated at 28^0C for 4 days (Vincent, 1970). After 4 days, a single colony from each isolate was selected and re-streaked on new YMA plates for further purification and characterization

Presumptive tests of the isolates

After isolation on YMA, the purity of the isolates were determined based on different morphological characteristics of colonies and absorption of Congo Red (Somasegaran and Hoben, 1994b). Gram staining and the growth of colonies on Peptone Glucose Agar (PGA) medium were also determined following procedures described elsewhere (Lupwayi and Haque, 1994).

Authentication and symbiotic effectiveness of the test isolates

All test isolates were evaluated for their ability to produce nodules on the homologous host species (*Phaseolus vulgaris* L.). Seedlings were grown in modified Leonard jars filled with washed and sterilized river sand. Four surface-sterilized and pre-germinated seedlings were planted aseptically into each of three replicate pots. The seedlings were later thinned to one by snipping on the top, as described previously (Chen et al., 2008). A single colony of each isolate was picked from yeast extract-mannitol agar (YMA) plates and multiplied in yeast extract-mannitol broth (YMB). Each seedling was inoculated with 1 ml bacterial culture at exponential growth phase (Somasegaran and Hoben, 1994a). Non-inoculated seedlings of the plant species, either supplied with mineral nitrogen (as 0.1% KNO3 in nutrient solution) or grown without nitrogen, served respectively as positive and negative controls. Seedlings were grown under natural sunlight and temperature and were fertilized with one-quarter-strength modified Jensen's N-free medium (Somasegaran and Hoben, 1994a). Nodule assessment and harvesting

took place 5 weeks after inoculation (Maâtallah et al., 2002; Somasegaran and Hoben, 1994a). At harvest, plants were scored for nodulation as effective, moderately effective and ineffective.

Morphological characteristics

Individual colonies were characterized based on their colony color, size, shape, capacity to produce extracellular polysaccharide (EPS) gum and colony texture following procedures described elsewhere (Somasegaran and Hoben, 1994a).

Acid and base production

The isolates were grown in YEMB and loop full of suspension 10^8/ml were streaked on to YEMA contain 0.5% BTB. The color change (yellow) was observed after 3 days for fast growing and blue remains for slow grower (Jordan, 1984).

Carbon source use (CSU), tolerance to salinity and temperature (TST) and pH assays

Assays for CSU, TST and pH were performed for all the test isolates and the reference strains. For CSU, the following carbon sources including L-arabinose, D-fructose, D-galactose, D-glucose, sucrose, maltose, D-mannose, raffinose, L-rhamnose, D-sorbitol, xylitol, trehalose, xylose, dulcitol, arabinose, myoinositol, inulin, ribose, starch, citric acid, and malonic acid were tested following methods described before (Somasegaran and Hoben, 1994a). Similarly the test isolates were assessed for TST and pH on YMA culture medium with NaCl concentration of (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 9.5, 10, 10.5% (W /V), temperature of 35, 40, and 45^0C following methods detailed earlier (Maâtallah *et al*., 2002), and pH of 4, 4.5, 5, 5.5, 6, 7, 8, 9, 9.5, 10 and 10.5 (Hungria *et al*., 2000).

Nitrogen sources utilization

Sixteen different amino acids utilization namely: D-serine, L-histidine, L-pyroglutamic acid, uredine, thymidine, inosine, L-arginine, L-alanine, L-aspargine, L-asparatic acid, L-glutamic acid, L-proline, L-theronine, L-leucine, L-phenylalanine, and Glycine, were investigated following methods as described before (Amarger *et al*., 1997).

Intrinsic antibiotic resistance

The intrinsic antibiotic resistance test was performed by using YMA (Rodriguez-Navarro *et al.*, 2000), to which filter sterilized (0.2µm) antibiotics (µg/ml) was added; streptomycin 10; kanamycin sulphate salt 5, 15; novobiocin 0.5, 1.5; spectinomycin dihydrochloride pentahydrate 2.5, 5; neomycin trisulphate salt 5, 20; chloramphenicol 5, 15; erthromycin

10, 20; lincomycin hydrochloride 100; and trimethoprim 200.

Intrinsic heavy metal resistance

The resistances of strains to heavy metals were determined on solid Tryptone Yeast Extract Agar following methods detailed elsewhere (Zhang *et al*., 1991).

Phosphate solubilizing ability

This was determined by inoculating and incubating the isolates on Pikovskaya Agar medium. This ability was detected based on growth and presence of clear zone around the colonies (Nautiyal, 1999).

Numerical taxonomy

Traits were coded 1 for positive and 0 for negative. A cluster analysis of 92 phenotypic variables for a total of 121 strains (113 new isolates and eight reference strains including *Azorhizobium caulinodans, Ensifer meliloti, Mesorhizobium meditteranum, Rhizobium etli, R. gallicum, R. giardinii, R. leguminosarum* and *R. tropici*) were carried out using similarity coefficient and dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA) clustering method using NTSYS-pc version 2.1.

Results

In this study, a total of 113 isolates were obtained from the root nodules of haricot bean (*Phaseolus vulgaris* L.) that were grown in soils from nine localities in southern Ethiopia including Jinka (12 isolates), Bodity (19 isolates), Gofa (19 isolates), Hawassa (11 isolates), Badiwacho (4 isolates), Durame (24 isolates), Kachabira (10 isolates), Humbo (3 isolates) and Areka (11 isolates) were isolated and characterized. All isolates were Gram negative and rod shaped bacteria with no ability to absorb Congo red, when grown on YEMA-CR medium. Furthermore, none of them grew on peptone glucose medium supplemented with bromocresol purple, indicating that all isolates were presumed to be rhizobia. Up on authentication, (i.e when re-inoculated into the seedlings of variety, Red Wolaita), all the isolates were shown to induce nodulation on the host (data not shown), confirming that they were root nodule bacteria.

Cultural and morphological characteristics

The result for cultural and morphological characteristics is presented on Table 2. Accordingly, the isolates showed different cultural characteristics indicating the existence of diversity among rhizobia nodulating haricot bean in the region. In this study 91% of the isolates showed colony diameter of $\geq$ 2mm, when incubated for three days. Likewise the isolates displayed various colony textures of white translucent (42%), watery translucent

(36%), milky (19%) and dull glistering color (3%). Seventy seven percent of the isolates also showed convex shape; whereas 21% of the isolates were found to be flat. Fifty four percent of the isolates have produced copious amount EPS (Extracellular polysaccharide) while 19% produced medium and 27% were low in EPS production. The production of exopolysaccharides (EPS) by the isolates could be an adaptive feature in providing protection to bacteria against environmental stresses including temperature, salinity, and pH fluctuations in the soil. Rhizobia with wider elasticity of withstanding environmental stresses could be suitable for the development of commercial inoculants. All of the test isolates changed the YEMA-BTB media in to yellow. Turning of YEMA-BTB media into moderately yellow to deep yellow color is a characteristic feature of acid producing and fast growing rhizobia (Somasegaran and Hoben, 1994b).

Table 2. Summarized cultural and morphological characteristics of the isolates.

Properties	Traits	No of isolates	Proportion (%)
Colony color	WHT	48	42
	WT	41	36
	MK	21	19
	DG	3	3
	Total	113	100
Colony size(diameter)	> 5mm	2	2
	4.00-5.00mm	19	17
	3.00-3.99mm	34	30
	2.00-2.99mm	48	42
	< 2.00mm	10	9
	Total	113	100
Colony shape	C	87	77
	F	26	23
	Total	113	100
Colony texture	E	49	43
	B	64	57
	Total	113	100
EPS	CO	61	54
	M	21	19
	L	31	27
	Total	113	100
YMA-BTB reaction	Ac	113	100
	Ba	0	0
	Total	113	100

Key: WT= watery translucent, WTH= white translucent, MK= milky, DG= dull glistering, F= flat, C= convex, L= low, M= medium, CO= copious amount, E=Elastic, B= buttery, Ac= acid, Ba= base

Physiological and biochemical characteristics

Biochemical and physiological characterization of isolates revealed the existence of versatile and tolerant haricot bean nodulating rhizobial isolates in our collection. The test isolates exhibited a wide range of variation in their NaCl tolerance (data not shown). More than 42% of the isolates grew at 1% NaCl while only 3% of the isolates continued to grow at up to 9% NaCl. The result indicated that most isolates were very sensitive to salt concentrations. However, strains from Gofa, Jinka and Hawassa were comparatively salt tolerant than other sites. Isolates HB19a and HB19b, both from Gofa were found to be the most salt tolerant that grew on the medium containing up to 10% NaCl. On the contrary, isolates from Durame (pH of the soils 5.9), Humbo and Badiwacho (pH of the soils 7.1) were highly salt sensitive, thus they were unable to grow at 1% NaCl.

With the respect to their pH profile, all the test isolates were able to grow on a wide pH values ranging between 6 and 9 while only 53% of the isolates were able to grow acidic pH (pH=4). On the other hand, 91% of the isolates were able to grow at pH value of 10. All isolates from Jinka and Kachabira were able to grow at pH value of 5. In addition, Kachabira isolates were able to grow in a wide range of pH values (5-10.5). With the respect to temperature requirement, fifty four of the isolates were able to grow at 40^0C, whereas only 12% of the isolates were able to grow at 45^oC **(**Table 3). At higher temperature extremes, the proportion of tolerant isolates decreased. Yet there is no critical boundary line to be drawn that discriminated between tolerance and intolerance, be it for thermal or pH regime. It might also be important weather the area of adaptation has role on tolerance level association mapping.

Table 3. pH and temperature tolerance of the rhizobia isolated from different locations. Numbers indicate the number of isolates that were able to grow

Site	strains from each site	pH levels								Temperature (^{O}C)		
		4.0	4.5	5	5.5	6-9	9.5	10	10.5	35	40	45
Jinka	12	9	10	12	12	12	11	10	10	12	8	1
Gofa	19	14	14	16	19	19	17	17	13	19	7	4
Bodity	19	9	10	14	17	19	17	16	10	19	12	4
Hawassa	11	8	8	8	9	11	10	10	9	11	8	1
Badiwacho	4	2	3	4	4	4	4	4	3	4	1	0
Durame	24	5	10	12	18	24	24	24	14	24	13	3
Kachabira	10	7	9	10	10	10	10	10	10	10	1	0
Humbo	3	2	2	3	3	3	3	3	3	3	1	0
Areka	11	4	5	7	8	11	11	9	6	11	9	0
Total	113	60	71	86	100	113	107	103	78	113	61	13
%		**53**	**63**	**76**	**88**	**100**	**95**	**91**	**69**	**100**	**54**	**12**

With the respect to carbon source utilization, many of the isolates were versatile in utilization of different carbon and nitrogen sources. Of the carbon sources tested, 12 sources (mannitol, glucose, xylitol, trehalose, sucrose, raffinose, maltose, arabinose, sorbitol, myinositol, ribose, rhaminose) were assimilated by all (113) isolates, whereas D-fructose, mannose, D-xylose, dextrin, inulin were assimilated by 96%-99% of the isolates. Dulcitol, galactose and starch were utilized by 86-89% of the isolates. Taken together, our results clearly revealed that monosaccharide and disaccharide supported maximum growth, followed by polysaccharides and none of strains were able to assimilate organic acids. On the other hand, of the total of 16 nitrogen sources tested, all isolates were able to metabolize 13 of them (data not shown). D-serine and L-proline were utilized by 84% and 99% of the isolates, respectively.

Intrinsic antibiotic resistance (IAR) and heavy metal resistance

The result of intrinsic antibiotic resistance of the isolates is summarized in Table 4**.** The data showed that most of the isolates were tolerant to novobiocin and lincomycin and sensitive to Streptomycin. The result showed that the strains exhibited a wide range of differences in their antibiotic resistance. The results in this experiment showed that at least 86% of the isolates resist heavy metals.

Table 4. Antibiotic and heavy metal resistance of the isolates.

Antibiotics & Metals	Concentration (µg/ml)	No of resistant strains	Proportion (%)
Neomycin	5	111	98
	20	91	81
Kanamycin	5	104	92
	15	93	82
Novobiocin	0.5	111	98
	15	111	98
Spectinomycin	2.5	101	89
	5	94	83
Trimethoprim	200	109	96
Chloramphenicol	5	113	100
	15	111	98
Erythromycin	10	104	92
	20	104	92
Lincomycin	100	111	98
Streptomycin	10	89	79
Cu	100	113	100
Al , Fe	500	113	100
Pb	500	108	96
Zn	100	97	86

Numerical taxonomy

In total 92 traits were examined, based on which the isolates and reference strains were characterized for cluster analysis. Using 82% similarity level as a cut point, a total of 113 isolates and eight reference strains of recognized species, were grouped into five major clusters (designated as I, II, III, IV, and V) and four un-clustered positions on the dendrogram (Figure 1). Out of the five clusters, four of them (I-IV) contained reference strains while one cluster (V) comprised only the test isolates. Of the five clusters, cluster I and II contained 87% of the studies isolates.

Cluster I included 55% of the isolates and clustered with the reference strain *Rhizobium leguminosarum* at 82% relative similarity. At 87% level of similarity this major cluster I further subdivided into four sub-clusters (designated IA, IB, IC and ID). Sub-cluster 1D contained *R. leguminosarum*, the other sub-clusters were loosely linked to this reference strain. Sub-cluster ID comprised the isolates from four specific locations; Kachabira (4), Durame (1), Humbo (2), and Bodity (1) while sub-cluster IC included isolates only from Areka and Durame. However, sub-cluster IA and IB contained the isolates from nine different locations.

Cluster II comprised 36 new isolates and reference strain, *Ensifer meliloti* at 84% similarity cut point, which

further divided into three distinct sub-clusters, designated IIA, IIB and IIC. Sub-cluster IIB closely related to reference strain *Ensifer meliloti*. Sub-cluster IIC contained strains from Bodity, Durame and Hawassa. None of the isolates from Humbo and Badiwacho were clustered with sub-cluster IIA and IIB.

Cluster III consisted two strains from Durame (HB 109, HB 108), one strain from Kachabira (HB 110) and one from Bodity (HB 92b). These isolates formed cluster with *Mesorhizobium mediterraneum* at 85% similarity cut point in the dendrogram. Cluster IV contains four isolates: HB 114 from Areka, HB 113 from Kachabira, HB 39b and HB 20c from Gofa which were clustered along with *Rhizobium tropici* and *Rhizobium etli* at about 84% and 89% relative similarity, respectively. The main distinguishing characteristics of these isolates were that all of them could not tolerated Zn concentration and grew on glycine. Cluster V consists of only three isolates (HB 19a, HB19b from Gofa and HB 62 from Hawassa), for which the dendrogram suggested distant relatedness to the rest of the isolates found in other clusters and reference strains. Generally, these isolates showed the lowest relatedness (64% similarity) with the rest of the test strains and to the reference strains used in this study. However, the strains should be tested in field conditions and their phylogenetic positions should be further explored. The test isolates which occupied unclustered positions include HB 20b from Gofa, HB 2a from Jinka, HB 57b from Hawassa, and HB 119 from Durame and were more divergent from the isolates that were grouped in each of the main cluster (I–V). None of these isolates were related to each other and other isolates including the reference strains included in this study.

Nodulation capacity and effectiveness of the isolates

Isolates of haricot bean nodulating rhizobia indigenous to Southern Ethiopia were evaluated for their symbiotic potential in the greenhouse, and the results of which are presented in Table 5. In this study haricot bean rhizobia exhibited a high degree of heterogeneity concerning their symbiotic performance on sand culture. Of the isolates tested 69.9% formed deep green leaf, pink/red nodule and strong stem and, therefore they were effective. All isolates from Bodity and Hawassa were effective indicating that these soils harbour effective strains as compared to other sites.

About 16% of the tested isolate were found to be moderately effective. All isolates from Humbo were moderately effective. Only 2.65% isolates formed white nodules, thus were ineffective (from Jinka), however, the rest isolates from

different locations formed pink and green nodules. It is to be noted that the pattern of nodulation also varied widely between isolates. Ineffective isolates (for example HB27) formed many small white nodules, while most of the effective isolates (for example HB 1a, HB 1b and HB 139) formed nodules which were mainly pink/dark pink in color.

Table 5. Summarized nodulation and symbiotic performance of rhizobia nodulating haricot bean isolated from different locations in southern Ethiopia.

Geographic location	strain number	Effective*	Moderately effective*	Ineffective*	Nodule size (mm)	Nodule No	Nodule dry Weight(gm)
Jinka	12	75	0	25	1.4-4.5	18 – 204	0.01-0.09
Bodity	19	100	0	0	2.0-4.5	39 – 150	0.04-0.11
Gofa	19	84	16	0	2.0-5.0	38 – 203	0.06-0.12
Hawassa	11	100	0	0	2.0-4.0	34 – 162	0.04-0.13
Badiwacho	4	75	25	0	2.5-3.0	71 – 103	0.05-0.09
Durame	24	70	30	0	1.75-4.5	40 – 109	0.05-0.10
Kachabira	10	80	20	0	2.5-3.7	47 – 132	0.04-0.10
Humbo	3	0	100	0	2.9-3.7	63 – 78	0.06
Areka	11	64	36	0	2.5-4.0	22 – 107	0.04-0.09
Total	113	80	18	2	-	-	-
%	100	70	16	3	-	-	-

* Percent of nodules scored as effective, moderately effective and ineffective qualitatively, Effective = Deep green leaf, strong stem and pink nodule, moderately effective = Pale green leaf, medium stem strength, Ineffective = Yellow leaf, week stem, white nodule.

Discussion

In earlier studies on rhizobia from common bean in Ethiopia, it was clearly demonstrated that indigenous rhizobia nodulating common bean were diverse (Abebe, 1987; Beyene *et al*., 2004; Mitiku; Workalemahu and Assefa, 2007). Similarly, this study showed that rhizobia capable of eliciting nodules on haricot bean ***(****Phaseolus vulgaris)* were present at all the locations investigated in Southern Ethiopia**.** The colony diameter (≥ 2mm) determined in 91% of the test isolates within 3 days of incubation clearly signifies the fast growing nature of common bean rhizobia, which is also the case in other studies of similar nature (Aguilar *et al*., 2004; Amarger *et al*., 1997; Andrade *et al*., 2002; Beyene *et al*., 2004; Küçük *et al*., 2006; Workalemahu and Assefa, 2007). In addition, the acid production behavior of the test isolates corroborates earlier findings on common bean rhizobia (Aguilar *et al*., 2004).

Rhizobium phaseoli is one of the most halotolerant rhizobia and several isolates have been reported to grow at high salt concentrations (4-5%) (Hungria et al., 2000). Similarly, two isolates of common bean from Southern Ethiopia tolerated 2%

concentration of salt (Workalemahu and Assefa, 2007). Isolates that were tolerant of up to 9% NaCl were also found to be highly resistant to alkaline pH of 10 except HB 19a. This result agreed with the results of other works from Egypt (Shamseldin and Werner, 2005). The isolates which were tolerant to high salt concentration and alkaline pH could be a candidate for field trials where salinity and alkalinity domain is increasingly a constraint, in lowland valies and western hemisphere. The presence of isolates that could resist higher NaCl concentration offers a possibility for choosing inoculant for production sites where salinity is a problem (Mensah *et al*., 2006). The same author concluded that the population count of rhizobia was inversely proportional to the salt concentration; with most growing at lower concentrations while only a few are able to grow at higher concentrations. In earlier investigations rhizobia of common bean tolerant to varying NaCl concentration levels have been reported, including 2% (Jordan, 1984), 3% (Odee *et al*., 1997), 5% (Maâtallah *et al*., 2002), 10% (Zahran, 1999) and 14% (Abdelmoumen *et al*., 1999).

With the respect to their pH profile, all isolates were able to grow on a pH range from 6 – 9 while 53% of the isolates were able to grow at pH 4. The results indicated that 100% of the isolates were tolerant to pH 9 which is similar to the study conducted in Ethiopia (Workalemahu and Assefa, 2007). On the contrary the same authors reported that almost all their haricot bean isolates from Southern Ethiopia were unable to tolerate pH lower than 5. It can be suggested that isolates, which were able to grow at acidic pH in the lab, could be taken further to the field condition (with acidic soil pH) for field trials to develop them into inoculant. Currently, it is estimated that about 40% of the total arable land of Ethiopia is affected by soil acidity (Taye, 2007). In earlier studies, it was found and reported that all strains of *R. tropici* were able to grow at pH 4, with few representatives of *R. giardinii* and *R. leguminosarum bv. phaseoli* (Amarger *et al*., 1997). Similarly, *R. tropici* affiliated rhizobia from common bean, and that tolerated pH of 4.2 were identified (Graham *et al*., 1994). On the other hand, 91% of the isolates were able to grow at pH value of 10 and in agreement with other findings on common bean isolates identified elsewhere (Küçük *et al*., 2006). All isolates from Jinka and Kachabira were able to grow at pH value 5. In addition, Kachabira isolates were able to grow in a wide range of pH value; from pH value 5-10.5. Strains grew in wide pH range can be taken as a promising alternatives for inoculant production in areas where acidity and alkalinity are problem for haricot bean production.

With the respect to the temperature profile, and in agreement to our findings, several research identified that rhizobia from common bean were found to resist an incubation temperature of 40^0C (Hungria *et al*., 2000), 42^0C (Küçük *et al*., 2006) and 44^0C (Diouf *et al*., 2000). Similar response of *R. tropici* to incubation of high temperature was recorded, tolerating 40^0C on different growing media (Martínez-Romero *et al*., 1991). Furthermore, rhizobia capable of growing at temperature values ranging between 32 and 47^0C (Hungria and Vargas, 2000) were identified, although tolerance varies among species. In our study, thus, it can generally be concluded that, most of the isolates can overcome high temperatures (35^0C and 40^0C) which are one of the major problems for biological nitrogen fixation and might be used as haricot bean inoculants in areas where high temperature is a problem for bean production especially in rift valley areas in Ethiopia. However, further work is needed to verify their performance in the field condition.

Regarding the carbon source utilization, most bean nodulating bacteria could utilize dulcitol except *R. tropici*. Similar finding was also shown by (Andrade *et al*., 2002) that the *R. tropici* strains were unable to grow on dulcitol. Fast growing rhizobia were reported to utilize a large variety of carbon sources than slow growing counterparts (Gao *et al*., 1994; Kalita and Małek, 2004). Our results also revealed that monosaccharide and disaccharide, support maximum growth, followed by polysaccharides and none of strains were able to assimilate organic acids. Similar findings have been reported (Kumari *et al*., 2009; Küçük *et al*., 2006). Nitrogen source utilization by the test isolates revealed similar pattern as that pattern generated from carbon source utilization, and this is supported by other studies. For example, (Amarger *et al*., 1997) and (Andrade *et al*., 2002) found that most of the bean isolates could utilize wide range of amino acids. The most resistant amino acid was glycine; where only 61% of the tested isolates were unable to utilize it. This is similar to glycine utilization of Brazilian isolates (Amarger *et al*., 1997), Ethiopian isolates (Workalemahu and Assefa, 2007) and fast growing rhizobia (Jordan, 1984). The ability to utilize a wide range of carbon and amino acid sources have significance for rhizobia strain classification and provide an ecological advantage to the rhizobia in colonizing the soil and competing with other microorganisms around the rhizosphere, where carbon and nitrogen sources are the limiting factors.

Conclusion

The combined results (from cultural, physiological and symbiotic

characteristics) of this study have revealed that there are indigenous rhizobia capable of eliciting nodules on the host legume, *Phaseolus vulgaris*, growing at different locations in Southern Ethiopia. The diversity of the isolates that were collected from different sites might be the result of the promiscuous nature of the host or the presence of compatible rhizobial population in soils. Characterization of 113 isolates based on cultural, physiological and biochemical characteristics indicates that there is a wide diversity in rhizobial strains nodulating haricot bean in Ethiopia. The ability to utilize a wide range of carbon and amino acid sources and ability to tolerate a wide range of antibiotics and heavy metals may give competitive advantage for the test isolates under field condition where such factors affect their performance during colonization of the rhizosphere. Isolates that tolerated high pH, low pH and high NaCl concentration could be best candidates for inoculum production for haricot bean in locations where salinity and acidity is a problem.

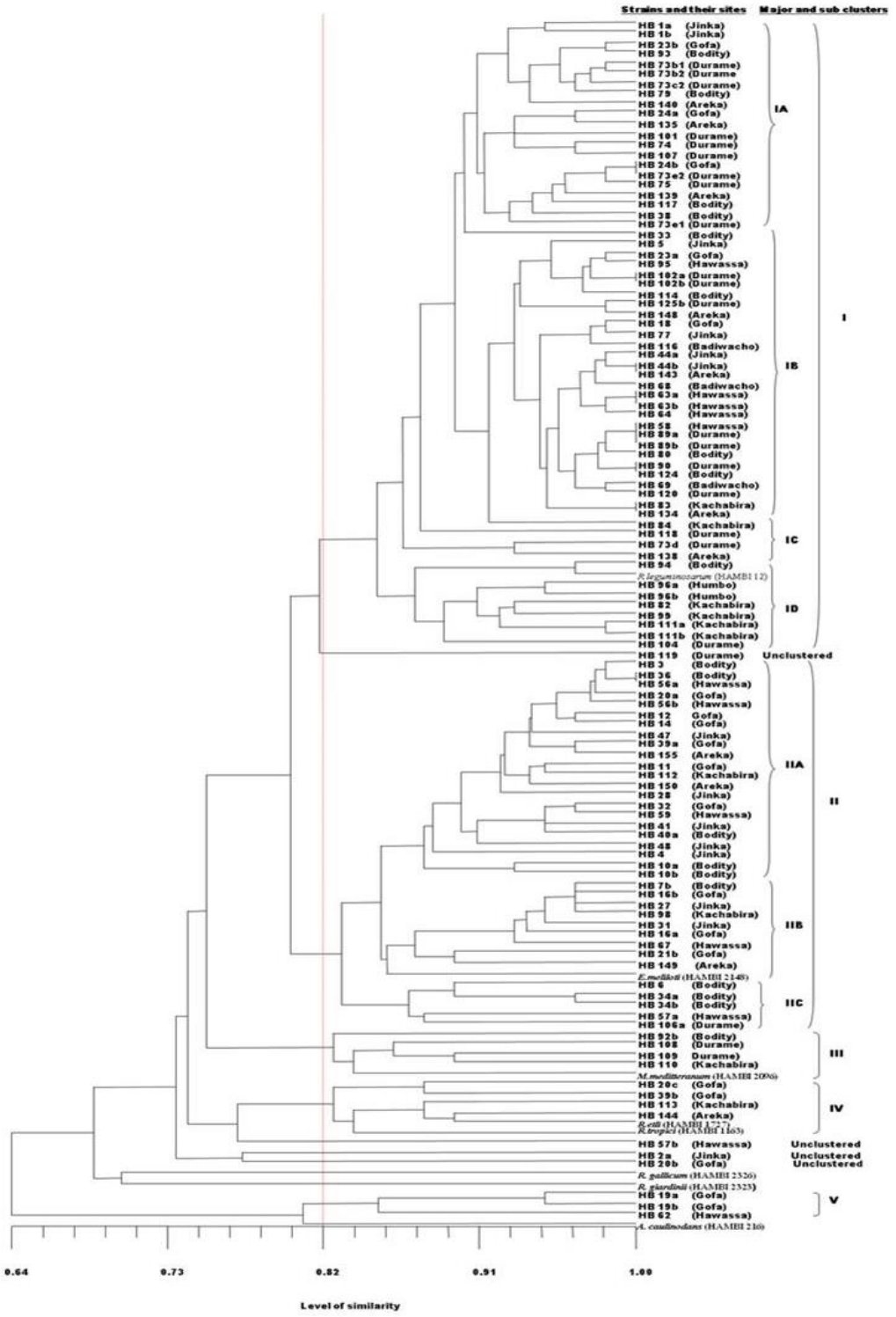
Strains and their sites
Major and sub clusters
HB 1a (Jinka)
HB 1b (Jinka)
HB 23b (Gofa)
HB 93 (Bodity)
HB 73b1 (Durame)
HB 73b2 (Durame
HB 73c2 (Durame)
HB 75 (Bodity)
HB 140 (Areka)
HB 24a (Gofa)
HB 135 (Areka)
HB 101 (Durame)
HB 74 (Durame)
HB 107 (Durame)
HB 24b (Gofa)
HB 73e2 (Durame)
HB 75 (Durame)
HB 139 (Areka)
HB 117 (Bodity)
HB 38 (Bodity)
HB 73e1 (Durame)
IA
HB 33 (Bodity)
HB 5 (Jinka)
HB 23a (Gofa)
HB 95 (Hawassa)
HB 102a (Durame)
HB 102b (Durame)
HB 114 (Bodity)
HB 125b (Durame)
HB 148 (Areka)
HB 18 (Gofa)
HB 77 (Jinka)
HB 116 (Badiwacho)
HB 44a (Jinka)
HB 44b (Jinka)
HB 143 (Areka)
HB 68 (Badiwacho)
HB 63a (Hawassa)
HB 63b (Hawassa)
HB 64 (Hawassa)
HB 58 (Hawassa)
HB 89a (Durame)
HB 89b (Durame)
HB 80 (Bodity)
HB 90 (Durame)
HB 124 (Bodity)
HB 69 (Badiwacho)
HB 120 (Durame)
HB 83 (Kachabira)
HB 134 (Areka)
IB
HB 84 (Kachabira)
HB 118 (Durame)
HB 73d (Durame)
IC
HB 138 (Areka)
HB 94 (Bodity)
R. leguminosarum (HAMBI 112)
HB 96a (Humbo)
HB 96b (Humbo)
HB 82 (Kachabira)
HB 99 (Kachabira)
HB 111a (Kachabira)
HB 111b (Kachabira)
HB 104 (Durame)
ID
I
HB 119 (Durame)
Unclustered
HB 3 (Bodity)
HB 36 (Bodity)
HB 56a (Hawassa)
HB 20a (Gofa)
HB 56b (Hawassa)
HB 12 Gofa)
HB 14 (Gofa)
HB 47 (Jinka)
HB 39a (Gofa)
HB 155 (Areka)
HB 11 (Gofa)
HB 112 (Kachabira)
HB 150 (Areka)
HB 28 (Jinka)
HB 32 (Gofa)
HB 59 (Hawassa)
HB 41 (Jinka)
HB 40a (Bodity)
HB 45 (Jinka)
HB 4 (Jinka)
HB 10a (Bodity)
HB 10b (Bodity)
IIA
HB 7b (Bodity)
HB 16b (Gofa)
HB 27 (Jinka)
HB 98 (Kachabira)
HB 31 (Jinka)
HB 16a (Gofa)
HB 67 (Hawassa)
HB 21b (Gofa)
HB 149 (Areka)
IIB
E. meliloti (HAMBI 2148)
HB 6 (Bodity)
HB 34a (Bodity)
HB 34b (Bodity)
HB 57a (Hawassa)
HB 106a (Durame)
IIC
II
HB 92b (Bodity)
HB 108 (Durame)
HB 109 Durame)
HB 110 (Kachabira)
M. mediterranum (HAMBI 2096)
III
HB 20c (Gofa)
HB 39b (Gofa)
HB 113 (Kachabira)
HB 144 (Areka)
R. etli (HAMBI 1727)
R. tropici (HAMBI 1165)
IV
HB 57b (Hawassa)
HB 2a (Jinka)
HB 20b (Gofa)
Unclustered
Unclustered
Unclustered
R. gallicum (HAMBI 2526)
R. giardinii (HAMBI 2323)
HB 19a (Gofa)
HB 19b (Gofa)
HB 62 (Hawassa)
A. caulinodans (HAMBI 216)
V
0.64
0.73
0.82
0.91
1.00
Level of similarity

Fig. 1. Dendrogram showing the grouping of the total study isolates and eight reference strains

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