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Research Article

Isolation, Characterization and Evaluation of Symbiotic Effectiveness of Rhizobia Nodulating (*Phaseolus Vulgaris* L) Nodules Collected from Gurafarda, Southwest Ethiopia

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Abstract

Rhizobia are diazotrophic bacteria that fix nitrogen after becoming established inside the root nodules of legumes (*Fabaceae*). This study was conducted to isolate, characterize and evaluate the symbiotic effectiveness of rhizobia nodulating common bean. Nodule sample were collected from six randomly selected kebele of the study area for nodule induction under controlled condition. At the flowering stage after 45 days of plant growth, root nodules were collected and rhizobia bacteria isolated. The isolates were allowed for different biochemical and physiological characterization. Presumptive tests confirmed that isolates were root nodulating rhizobia. Rhizobial inoculants (Bio-fertilizer) effect on the host crop was tested under greenhouse condition on sterilized river sand culture. Plant treated with KNO_3 were set as positive and plants treated with sterile distilled water were used as negative control. Isolates treated plant group were experimental group. The experiment was laid in CRD design. After 45 days of inoculation different growth, related parameters were investigated. Collected data on growth related parameter after rhizobia inoculation were analyzed using SAS software version 9.1. Rhizobial treatment effect on plant growth related parameters were investigated. Effect of treatment was significant at ($P<0.05$) difference on SDW, NDW, SFW. SE. rhizobial treatment on host crop shown significant effect and rhizobia were capable to infect the host crop in sterile river sand culture under greenhouse condition. The isolates were highly effective on their symbiotic effectiveness rate. Therefore, the molecular characterization of the isolates of the area and effect of inoculant on yield related parameter of host crop (*Phaseolus Vulgaris* L.) study on field trial should be future perspective of the area.

Keywords

Biological Nitrogen Fixation, Common Bean, Nitrogen, Nodulation and Rhizobia

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1. Introduction

Common bean (*Phaseolus vulgaris*. L) is an important grain legume throughout the world [9]. Provides source of protein, dietary fiber, starch and minerals such as potassium, thiamine, vitamin B6 and folic acid in diets affordable by the poor [20]. Nitrogen and phosphorus deficiency are some of edaphic and environmental factors that constrain Common bean production in most areas where the crop is grown. Apart from that, soil acidity (including aluminum and manganese toxicity) and drought also affect bean production [4, 1]. The crop has the ability to enhance the plant growth and yield by fixing atmospheric nitrogen [12] Due to the abovementioned constraints production of Common bean in Ethiopia remains 1.4 t/ha below its potential 5 t/ha [11]. It is reported that common bean has the lowest N_2-fixation rate among the most widely grown grain legumes (Martinez-Romero, 2003). Thus, inoculation of common bean with rhizoidal strains is beneficial to increasing nodulation, enhancing biological nitrogen fixation, increased plant height, nodule dry weight, shown remarkable yield increase and ensured fertility of soils [10, 8, 25].

The inoculation of soils and legume seeds should be effective using compatible bacteria where the population of indigenous bacteria is low [1] In Previous studies, the proportion of screened isolates which has a wide biochemical and physiological properties with effective and highly effective symbiotic efficiency rate were not limited. Nevertheless, there is need to assess at Gurafarda woreda. However, there was no study at Gurafarda woreda inthis study physiologically diverse and symbiotically effective isolates will be screened that could help to develop inoculants. Therefore, this study aimed to isolate, characterize, and evaluate symbiotic effectiveness of rhizobia nodulating *Phaseolus Vulgaris* L (common bean) from some common bean growing areas of Gurafarda Woreda, South West Ethiopia.

2. Materials and Methodology

2.1. Description of the Study Area

Soil samples for nodule induction were collected from Common bean growing areas of Gurafarda woreda, Bench Maji Zone of the Southwest Ethiopia. The area situated between 6.30°-7.00°N latitude and between 34.45°-35.30°E longitude at an altitude of between 700 – 1800 m.a.s.l (Table 1). Soil textural class is loam to clay. The agro climatic zones of Gurafarda are low land (moist qolla) and medium (woynadega), which constitute 78.25% and 21.75% respectively. Annual rainfall of Gurafarda district varies from 1000-1300 mm. whereas the mean annual rainfall's 1132 mm. The mean annual minimum and maximum temperature of the area ranges between 20°C and 29°C respectively. Gurafarda woreda administration office data record department.

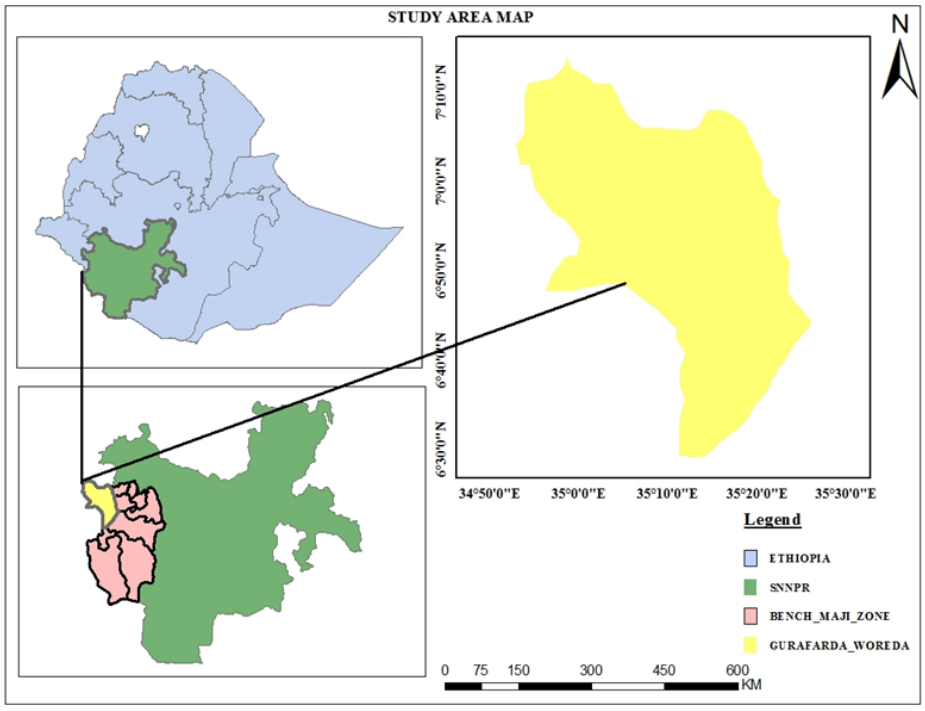

Figure 1. *Map of Study area.*

2.2. Description of Experimental Site

This investigation was conducted at laboratory of Bureau of SNNPRS Agriculture and Natural Resource Management, at Mizan-Aman Plant Health Protection and (Microbiology Department) regional laboratory. The experiment was undertaken under greenhouse with 12/12 hr light and dark condition from February 17/2019 to August 2019.

2.3. Experimental Design and Procedure for Induction of Nodulation

Thirty, soil samples were purposely taken using auger from six (6) Keble of Gurafarda woreda for nodule induction and soil chemical analysis. Soils were randomly collected five farmers each from six Keble using zigzag method. The Nodulation was induced by 'plant trap' method in greenhouse conditions as described by (Vincent, 1970). Healthy recently released Dicta-105 (Nassir locally named) common bean variety obtained from Bench Maji Zone Agriculture and Natural Resource Management Bureau were surface sterilized with 70% ethanol for 5 sec and 3% (v/v) of sodium hypo-chlorite for 3 minutes, and washed thoroughly with five changes of sterile distilled water. Five seeds were sown in each pot and the seedlings were thinned down to three. The plants were watered every two days for 45 days. After 45 days, the pink and undamaged nodules collected at flowering stage of the plants and stored in vial containing Silica gel covered with cotton for further work [22].

2.4. Isolation of Rhizobia from Root Nodules

The detached nodules were washed under running tap water to remove the adhering soil particles from nodule surfaces. Collected nodules were surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo- chlorite for 3 to 4 minutes. The surface sterilized nodules were rinsed in five changes of distilled water, to completely rinse the sterilizing chemicals [24]. Then the nodules were transferred into sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of sterile distilled water /in (0.85% NaCl) inside a laminar airflow hood [24]. Then 0.1 ml (loopful) of the suspension was streaked on plates containing yeast extract Mannitol agar (YEMA). P^H was adjusted to 7±0.1 media were autoclaved at 121°C for 15 minutes; plates were incubated at 28 ±2°C for 3-5 days.

Table 1. *GPS data of sampling sites.*

No	Designation	Name of site	kebele	GPS Location of collected soil sample			
				Altitude (m.a.s.l)	Latitude	Longitude	Soil pH
1	GCR1	Selamber	Otwa	1126	06°50.570' N	035°18.299' E	5.4
2	GCR2	Teramed	Otwa	1129	06°50.465 'N	035°18.068 'E	6.0
3	GCR3	Keramba	Otwa	1132	06°50.291 'N	035°18.213 'E	5.7
4	GCR4	Kembat camp	Otwa	1115	06°50.324 'N	035° 17.850 'E	6.0
5	GCR5	Chodit	Otwa	1114	06°50.203'N	035°18.005 'E	5.8
6	GCR6	Chirikori	Berji	1224	06°48.603 'N	035°15.585 'E	5.5
7	GCR7	Adis amba	Berji	1214	06°48.695' N	035°15.474 'E	5.7
8	GCR8	Almegena	Berji	1253	06°48.610 'N	035°15.097 'E	5.8
9	GCR9	Selam	Berji	1242	06°48.641 'N	035°15.322 'E	5.5
10	GCR10	Medanalem	Berji	1240	06°48.753 'N	035°15.217 'E	5.7
11	GCR11	Koy	Kuja	1384	06°46.372 'N	035°11.849 'E	5.7
12	GCR12	Mergin	Kuja	1388	06°46.199 'N	035°11.762 'E	5.9
13	GCR13	keydod	Kuja	1411	06°46.860 'N	035°11.341 'E	5.6
14	GCR14	Dimaber	Kuja	1403	06°46.782 'N	035°11.519 'E	5.8
15	GCR15	Berach	Kuja	1403	06°46.590 'N	035°11.632 'E	6.0
16	GCR16	Debark	Alenga	1421	06°48.137 'N	035°08.838 'E	5.5
17	GCR17	Tefasess	Alenga	1419	06°48.247 'N	035°08.666 'E	5.9

No	Desigination	Name of site	kebele	GPS Location of collected soil sample			
				Altitude (m.a.s.l)	Latitude	Longitude	Soil pH
18	GCR18	Megenag1	Alenga	1426	06°48.536' N	035°08.347 'E	5.7
19	GCR19	Meakutir2	Alenga	1370	06°47.891 'N	035°09.146 'E	5.7
20	GCR20	Bibita denber	Alenga	1429	06°47.315 'N	035°09.261 'E	5.8
21	GCR21	Shebela	Biftu	1166	06°51.310 'N	035°21.378 'E	5.7
22	GCR22	Michalchurch	Biftu	1230	06°51.271 'N	035°21.107 'E	5.5
23	GCR23	camp	Biftu	1132	06°51.402 'N	035°20.547 'E	5.6
24	GCR24	Zelekesfer	Biftu	1162	06°51.058 'N	035°20.615 'E	5.9
25	GCR25	Meha dankila	Biftu	1147	06°50.928 'N	035°20.609 'E	5.8
26	GCR26	Addismender	Dankila	1109	06°51.155 'N	035°19.936 'E	5.7
27	GCR27	Qutir7	Dankila	1081	06°51.691 'N	035°19.716 'E	6.0
28	GCR28	Biftu02	Dankila	1120	06°51.750 'N	035°19.092 'E	5.8
29	GCR29	peliya	Dankila	1109	06°51.788 'N	035°19.221 'E	5.7
30	GCR30	Juti	Dankila	1117	06°51.552 'N	035°20.048 'E	5.8

2.5. Presumptive Tests of the Isolates

Presumptive test performed according to [15] The isolated rhizobia strains then characterized on the basis of morphological, biochemical and physiological characters according to (Jordan, 1984). The following confirmatory tests made to confirm identity. According to [24] each isolates examined for presumptive purity using Growth on YEMA-BTB Medium, Peptone glucose agar test (PGAT) and Gram staining tests.

2.6. Cultural and Growth Characteristics of the Isolates

The Cultural characteristics of the isolates were determined according to [15] A loopful of 48 hrs old grown broth culture from each isolate inoculated onto YEMA and incubated at 28±2°C for 3-5 days. After 5 days, colony diameter, and colony texture recorded as Small dry, Large Mucoid, Large Watery, Smooth gummy and rough as indicated in [16].

Mean generation (doubling) time was calculated from the logarithmic phase of the optical density (OD) reading of Spectrophotometer [27]. The formula below used to calculate mean generation time:

$$G = \frac{log2(t)}{logx - logxo}$$

Where G is generation time, t is time elapsed, Xo is first OD reading in logarithmic phase, X is second OD reading in logarithmic phase.

2.7. Biochemical and Physiological Tests of the Isolates

Isolates characterized by different biochemical Tests, Carbohydrate utilization test, Amino acid utilization test, intrinsic antibiotic resistance test, Temperature tolerance test, Salt tolerance test and pH tolerance test [12].

2.8. Authentication of Symbiotic Effectiveness of Isolates on Sterilized Sand Culture Under Greenhouse

Fine graded river sand was well washed in tap water and immersed in 98% sulfuric acid for two days. Washed in several changes of tap and distilled water to get rid of the last traces of the acid and filled into surface sterilized plastic pots at 3 kg sand per pot [22]. Common bean variety Dicta-105 recommended for the study areas was selected surface sterilized in sodium hypo chlorate for 10 minutes to free from superficial microorganisms and washed several times with distilled and sterile water [19] The pot surfaces were sterilized with 95% ethanol as well. Five pre-germinated common bean seeds were soaked in 0.75 (w/v) distilled water and incubated in 25°C for 3 days, and then transplanted into each plastic pot. One milliliter of active culture of each isolate grown in YEMB inoculated by pipeting on to seedlings during planting date [12] After a week of growth, three

healthy plants maintained in each pot.

The experimental setup was replicated three times and laid out in CRD. There were two (negative and positive) control/standard treatments. The negative control was that lack both sources of N while the positive control supplied with KNO_3 at concentration of 0.05% (w/v) and 100 ml per pot was added week^{-1}. All pots were fertilized with N-free medium (Table 2).

Table 2. *N- free nutrient media composition.*

Stock Solution	Reagent groups	Quantity (g/l)
1	$CaCl_2.2H_2O$	294.0
2	KH_2PO_4	136.1
3	Fe $C_6H_5O_7$. $3H_2O$	6.700
	$MgSO_4$. $7H_2O$	123.3
	K_2SO_4. H_2O	87.00
	$MnSO_4$, H_2O	0.338
4	H_3BO_3	0.247
	$ZnSO_4$. $7H_2O$	0.228
	$CuSO_4$. $5H_2O$	0.100
	$CoSO_4$. $7H_2O$	0.056
	Na_2MoO_2. $2H_2O$	0.048

Source: Broughton and Dilworth, 1970).

After forty-five days of planting, plants were carefully uprooted to expose the whole root system. The adhering soils removed by washing the roots with water over sieve. The important parameters such plant height, nodule number, nodule dry weight, shoot dry weight, root length and relative symbiotic effectiveness were recorded.

Plant height and Root length measured and reported as cm plant^{-1}. Number of nodules averaged to per plant, and their dry weight measured by drying at 70°C to constant weight to measure dry weight gram plant^{-1}. Finally, the percent of symbiotic effectiveness (SE) of the isolates was computed.

$$SE = \frac{Inoculated\ plant SDW}{N-fertilized\ plant\ SDW}\ x100$$

Where the SE values were rated as: >80% = highly effective, 50-80% = effective, 35-50 = lowly effective, and <35% = ineffective [13].

2.9. Statistical Analysis

Growth related collected data were subjected to one way analysis of variance (ANOVA) using General Linear Models Procedure of SAS software version 9.1. Means of all treatments were calculated and the differences tested for Significance using the Least significance difference (LSD) test at probability ($P<0.05$) level. Correlation Coefficient calculated to study the associative relations among investigated parameters using Pearson correlations.

3. Results and Discussion

A total of 23 bacterial isolates were isolated from nodule sample of common bean collected from six kebele's of Gurafarda woreda South west Ethiopia (Table 3). All isolates did not absorb Congo red on YEMA-CR Media, (Figure 2). did not grow on PGA-BCP medium indicating the presumptive test for root nodulating bacteria (Figure 5).

All isolates changed YEMA-BTB media to yellow (Figure 4). This confirms that isolates were fast growing and acid producing [14] All isolates were Gram- negative and rod shaped under the microscope examination (Figure 3). This confirms that isolates were gram–negative [24].

Figure 2. *Growth of rhizobial isolate on YEMA-CR medium.*

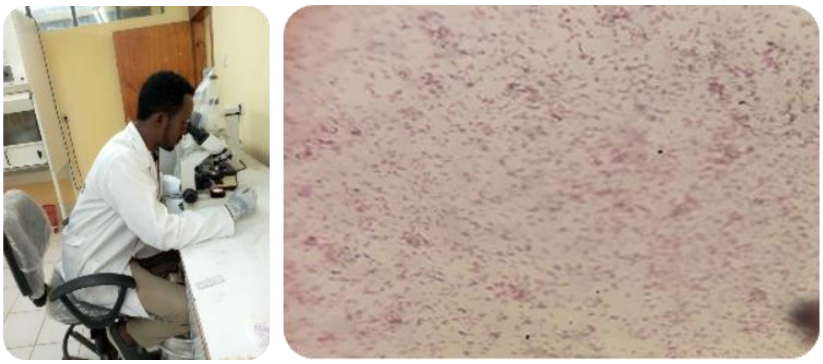

Figure 3. Gram staining reaction of isolates.

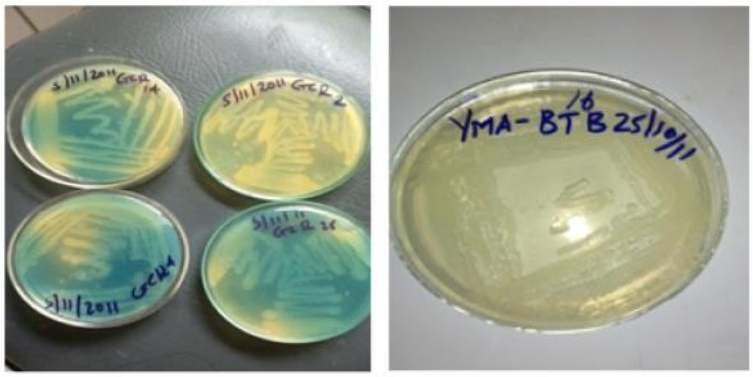

Figure 4. Growth of isolate on YEMA-BTB.

3.1. Cultural and Growth Characteristics of Isolates

Isolates were grown on YEMA medium to determine colony type, colony diameter and colony texture. With regard to colony texture, (22%) showed large watery colonies (LW) and (78%) of the isolates were characterized as large mucoid (LM) texture on YEMA media (Table 3). The colony diameter of all the isolates ranged between 2 mm and 6 mm.

All isolates displayed generation times between 2-4 hrs except, GCR6 and GCR9, GCR11, and GCR21 which showed less than 2 hrs to double its population (Table 3). Isolates GCR25 and GCR28 showed the fastest doubling time of 1.10 and 1.18 hrs.

Table 3. Cultural and growth characteristics of isolates on YEMA.

Isolates	Site name	Growth on YEMA	Colony texture	Colony diameter	M.G.T
GCR 1	Otuwa	LW	Smooth, gummy	6	4.2
GCR 2	Otuwa	LW	Smooth, gummy	6	3
GCR 4	Otuwa	LM	Rough	4.5	2
GCR 6	Berji	LM	Rough	4	1.20
GCR 7	Berji	LM	Smooth, gummy	6	3.22
GCR 8	Berji	LM	Smooth, gummy	5	2.10
GCR 9	Berji	LM	Smooth, gummy	6	1.20
GCR 11	Kuja	LM	Smooth, gummy	5	1.40
GCR 12	Kuja	LM	Smooth, gummy	5.5	3.12
GCR 13	Kuja	LM	Rough	3.5	3

Isolates	Site name	Growth on YEMA	Colony texture	Colony diameter	M.G.T
GCR 14	Kuja	LM	Smooth, gummy	4	3.3
GCR 16	Alenga	LM	Rough	4	4.17
GCR 17	Alenga	LM	Smooth, gummy	3.5	3.68
GCR 18	Alenga	LM	Smooth, gummy	2	4.2
GCR 19	Alenga	LM	Smooth, gummy	4	3.83
GCR 21	Biftu	LW	Smooth, gummy	5	1.35
GCR 22	Biftu	LM	Smooth, gummy	2	3.17
GCR 23	Biftu	LM	Smooth, gummy	4	2.46
GCR 24	Biftu	LM	Smooth, gummy	5.5	4.25
GCR 25	Biftu	LW	Smooth, gummy	4	1.10
GCR 27	Dankila	LM	Smooth, gummy	2.5	2.22
GCR 28	Dankila	LW	Smooth, gummy	6	1.18
GCR 29	Dankila	LM	Rough	5.5	2.34

Where LW = large watery, LM = large mucoid

In present study 18 (78%) isolates displayed Smooth gummy colonies. five isolates (22%) displayed Rough colonies on YEMA media growth. Smooth gummy colonies common bean rhizobia could be *R. Leguminosarium biv. phasoli* and *R. etli* (Martinez-Romero *et al.,* 1991). Rough colonies of common bean rhizobia could be *R. gallicum* [23] Similarly [8] reported almost all common bean rhizobia isolated from southern ethiopia displayed smooth gummy colonies. Morphological and cultural characterstics of isolates confirmed isolates as fast growers [17].

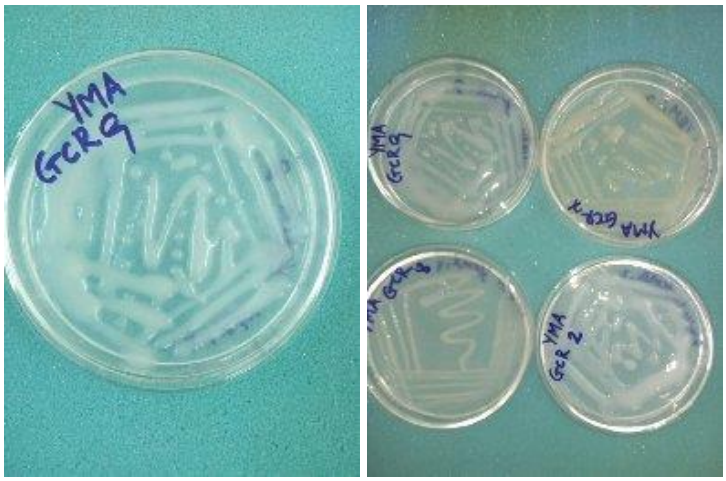

Figure 5. *Cultural and growth characteristics of isolates on YEMA.*

3.2. Biochemical and Physiological Test of Isolates

The isolates were capable of utilizing most of the carbohydrates provided as their sole carbon sources. In present study GCR6 (Berji Keble) was the only isolate utilizing narrow range of tested carbohydrate (Table 4).

The finding was in agreement to [2] reported that carbon sources were generally utilized by bacteria of the genus *Rhizobium*. [5] also observed that fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slow growing strains (Table 4). According to [14] catabolism of monosaccharides and disaccharides are widespread in fast growing

rhizobia including *Rhizobium leguminosarum* var *Phaseoli*. (Table 4).

Table 4. *Carbohydrate utilization test.*

Isolates	Carbohydrates /Carbon sources								
	Sucro	**Gluco**	**Lacto**	**Malto**	**Raffin**	**Arabino**	**Sorbit**	**Dextr**	**manit**
GCR-1	+	+	+	+	+	+	+	+	+
GCR-2	+	+	+	+	+	+	+	+	+
GCR-4	+	+	+	+	-	-	+	+	+
GCR-6	+	+	-	+	+	-	+	+	+
GCR-7	+	+	+	+	+	+	+	+	+
GCR-8	+	+	+	+	+	+	+	+	+
GCR-9	+	+	+	+	-	+	-	+	+
GCR-11	+	+	+	+	-	+	+	+	+
GCR-12	+	+	-	+	+	-	+	+	+
GCR-13	+	+	-	+	+	-	+	+	+
GCR-14	+	+	+	+	+	+	+	+	+
GCR-16	+	+	+	+	+	+	-	+	+
GCR-17	+	+	+	+	+	+	+	+	+
GCR-18	+	+	+	+	+	+	+	+	+
GCR-19	+	+	+	+	+	+	+	+	+
GCR-21	+	+	+	+	+	+	+	+	+
GCR-22	+	+	+	+	+	+	+	+	+
GCR-23	+	+	+	+	-	+	-	+	+
GCR-24	+	+	+	+	-	+	+	+	+
GCR-25	+	+	+	+	+	+	+	-	+
GCR-27	+	+	+	+	+	+	+	+	+
GCR-28	+	+	+	+	+	+	+	+	+
GCR-29	+	+	+	+	+	+	+	+	+
Total growth	23	23	20	23	18	19	20	22	23
% growth	100%	100%	86.95%	100%	78.26%	82.60%	86.95%	95.65%	100%

Where (-) no growth (+) growth

More than (90%) of the tested isolates were capable of catabblize L-Aspartic acid, L–Proline, L-Histadine, L-Isolucine, Arganine, and, L-Asparagine (Table 5). Isolate GCR9 (Berji) was the only isolate to utilize the least amino acid (5) five compared to other isolates. According to [14] glycine were utilized by some rhizobia isolated from common bean; Moreover, glycine rarely utilized by some species of rhizobia (Table 5). [4] reported most of the nitrogen sources were utilized by rhizobia nodulating common bean. Similarly, [2] reported all the tested isolates of common bean from south Ethiopia utilized L- arganine and L- asparagines.

Table 5. Amino acid utilization test.

Isolates	Amino acid listes							
	L- aspartic acid	L– proline	L-histadine	L-isolucine	L-Valine	L-Arganine	L-Glycine	L-Asparagine
GCR-1	+	+	+	+	+	+	-	+
GCR-2	+	+	+	-	-	+	+	+
GCR-4	+	+	+	+	+	+	+	+
GCR-6	+	+	+	+	+	+	-	+
GCR-7	+	+	+	+	+	+	+	+
GCR-8	-	+	+	+	-	+	+	+
GCR-9	+	+	+	+	-	-	-	+
GCR-11	-	+	+	+	-	+	+	+
GCR-12	+	+	+	+	+	+	+	+
GCR-13	+	+	+	+	+	+	+	+
GCR-14	+	+	+	+	-	+	+	+
GCR-16	+	+	+	+	-	+	+	+
GCR-17	+	+	+	-	+	+	+	+
GCR-18	+	+	+	+	+	+	+	+
GCR-19	+	+	-	+	+	+	-	+
GCR-21	+	+	+	+	+	+	+	+
GCR-22	+	+	+	+	+	+	+	+
GCR-23	+	+	+	+	+	+	-	+
GCR-24	+	+	+	+	+	+	+	+
GCR-25	+	+	+	+	+	+	+	+
GCR-27	+	+	+	+	+	+	+	+
GCR-28	+	+	+	+	+	+	+	+
GCR-29	+	+	+	+	+	+	+	+
Total	21	23	22	21	17	23	18	23
% growth	91.30%	100%	95.65%	91.30%	73.96%	100%	78.26%	100%

Where (-) no growth (+) growth

In present study the Highest (96%) and (91%) of the isolates were resistant to antibiotics Erythromycin (30 µg) and penicillin (10 µg) respectively (Table 6). whereas the highest sensitivity was recorded using antibiotics streptomycin (10 µg) and kanamycin (5 µg). 50% and more isolates were resistance to the remaining antibiotics tested. The result revealed that isolates from Kuja Keble were the only resistant to Streptomycin (10 µg). Generally the evaluation of intrinsic resistance to antibiotics of common bean rhizobia showed that most of the tested isolates exhibited highest resistant to Erythromycin > penicillin > Chloroamphinicol > Amphicillin>Kanamycin.

Table 6. Intrinsic antibiotic resistance test of isolates.

Isolates	Different antibiotics					
	Pen (10 µg)	Chlo (30 µg)	Kana (5 µg)	Eryth (30 µg)	Strepto (10 µg)	Ampic (10 µg)
GCR-1	R	20.1 mm	13.2 mm	R	23.1 mm	R
GCR-2	R	R	13 mm	R	21 mm	20 mm
GCR-4	R	R	14 mm	R	20.4 mm	R
GCR-6	R	22 mm	13.2 mm	R	18.6 mm	R
GCR-7	R	18 mm	R	R	18.6 mm	18.6 mm
GCR-8	R	R	R	R	21.3 mm	12.5 mm
GCR-9	10 mm	R	19 mm	R	20 mm	11.5 mm
GCR-11	R	R	R	R	R	R
GCR-12	R	R	18.5 mm	R	R	R
GCR-13	R	12.4 mm	R	R	R	R
GCR-14	R	R	R	R	R	R
GCR-16	R	R	R	R	20 mm	R
GCR-17	R	R	21 mm	R	26 mm	R
GCR-18	10 mm	R	29.2 mm	R	19 mm	10 mm
GCR-19	R	R	R	R	29 mm	R
GCR-21	R	R	15.4 mm	20.2 mm	25.4 mm	R
GCR-22	R	R	R	R	20 mm	R
GCR-23	R	R	R	R	16.8 mm	12.4 mm
GCR-24	R	R	13.6 mm	R	22 mm	R
GCR-25	R	R	R	R	21.2 mm	10.4 mm
GCR-27	R	R	R	R	20.4 mm	R
GCR-28	R	R	12.2 mm	R	22.1 mm	R
GCR-29	R	R	R	R	20 mm	16.3 mm
T growth	21	19	12	22	4	15
% growth	91.3%	82.60%	52.17%	95.65%	17.39%	65.21%

Where R= resistant

The ability of isolates to grow at different temperature level was seen. All of isolates were capable of growing between 15°C and 30°C temperature ranges. Eleven (48%) of the isolates were resistant to the highest 40°C temperature. The present finding showed temperature resistant of the isolates between of 26.03% and 100% of. This finding was in agreement to [7]. Similar response of *Rhizobium tropici* to incubation of high temperature was recorded at 40°C on different media [16].

All isolates showed inconsistency in tolerance to different salt concentrations. More than 69% of the isolates grown on YEMA medium containing, (0.5%-3.0%) salt concentration. On increasing the concentration of the salts, the growth of the isolates decreased. Present results was in line to the report of [20] on haricot bean reported that fast growing *rhizobium* generally grew well at 3% to 4% NaCl concentration.

All the tested isolates were able to grow at pH values between 5 to 8.5. Fastidious, growth of isolates was recorded at a pH value 4 with growth rate (17.3%) followed by 43.4% of growth of isolates at pH value 10. This indicates that these isolates have the potential to be used as inoculants in acidic soils. This isolates showed wide range pH valued tested. This indicated isolates capability to tolerate both acidic and basic media. The results were comparable with the reports of [21].

Table 7. Physiological tests of the isolates.

		Growth	Total growth	% of growth
Physiological tests of the isolates	Temperature (ºC)	4	7	30.43%
		10	11	47.80%
		15	23	100%
		20	23	100%
		30	23	100%
		40	11	47.82%
	Salt concentration (%)	0.5	23	100%
		1	21	91.30%
		2	19	82.60%
		3	16	69.50%
		4	8	34.70%
		5	2	8.69%
	pH	4	4	17.30%
		4.5	14	60.86%
		5	23	100%
		5.5	23	100%
		8	23	100%
		8.5	23	100%
		9	21	91.30%
		9.5	18	78.20%
		10	10	43.40%

3.3. Evaluating Symbiotic Effectiveness of Rhizobial Isolates on Sterile Sand Culture

Out of the twenty-three isolates, twelve were selected based on their morphological, biochemical, and physiological tests efficiency to evaluate the symbiotic effectiveness efficiency of isolates on sterilized sand culture under greenhouse condition.

Twelve (12) isolates inoculated for symbiotic effectiveness on (host) common bean (Dicta-105) showed that, all isolates were capable of forming nodules (Table 8). The internal color of all nodules was pinkish, which indicates the presence of rich leg-hemoglobin content and the effectiveness of the isolates. [24] suggested that nodules with a pink color indicate an effective nodule. The inoculated plants showed variations on their physical appearance, plant height, nodule number, nodule dry weight, and shoot dry weight (Table 8). The mean nodule number per plant record indicated that all isolates exhibited more than 50 nodules per plant. The highest mean nodule number (157.5 p^{-1}) was recorded for isolate GCR19 (Alenga kebele) and the least mean nodule number (45.05 p^{-1}) was obtained by GCR9 (Berji Keble).

Inoculated plants shown significant ($P< 0.05$) difference on plant height on sand culture in greenhouse (Table 8). The minimum plant height (21.32 cm) was recorded from the negative control, which was significantly lower than rhizobial isolates treated plants. *Rhizobium* inoculated common bean plants and (+N) control under the greenhouse performed similarly on plant height. This might be due to inoculated isolates maximized as applied N-fertilizer. The mean plant height ranges from (29.47 cm/p) to the maximum (42.52 cm/p). The present result was almost in line to that of [18] An increase in plant height might be due to the increased vegetative growth with help of fixed nitrogen. [17] suggested that this increase in plant height might be due to the fact that some rhizobial isolates produced plant growth promoting hormone in addition to fixing nitrogen. There was

strong correlation among plant height (r= 0.563, *p<0.001)* with nodule dry weight, shoot dry weight (r=0.87, p<0.001) and symbiotic effectiveness (r=0.86, p<0.001) (Table 9).

Rhizobium with common bean, showed significant variation in nodule number per plant ($P < 0.05$) within treatment and negative and positive Controls (Table 8). All inoculated plants except (GCR9) treatments formed red and pinkish color nodules with the dark green leaves. [24] suggested that nodules with a pink color indicate effective nodules where as white and greenish nodules infer ineffective in fixation of nitrogen. In the present study N-fertilized (positive control) and Negative control) did not form nodule (Table 8). The nodule number of isolates treated plant ranges between (41 nodules plant^{-1}) GCR11 the smallest to (159.62 nodules plant^{-1}) by GCR19 the largest score followed by GCR16 (136.37 nodules plant^{-1}). The average nodule number produced by common bean plants in this study was (79.02 nodules plant^{-1}). The mean nodule number per plant record indicated except two GCR9 and GCR25 all the rest 83.4% isolates exhibited more than 50 nodules per plant each. The highest mean nodule number (159.62^{-1}) was recorded for isolate GCR19 and the least mean nodule number (41.0^{-1}) was obtained by GCR9. Studies indicated that variation in nodulation could be due to low rhizobial density, incompatibility of the rhizobia and edaphic factors that hinder the effectiveness of the rhizobia [23]. Present result was agreed with the finding of [21] treated plants with rhizobia were increased nodule number and averaged to (87.5 plant^{-1}). Nodulation showed positive correlation with all investigated parameters except root length (Table 9).

a b

Figure 6. *Plant inoculated with rhizobia and the control treatments.*

Table 8. *Effect of Rhizobium isolates on Nodule number, Nodule dry weight, Shoot dry weight, Shoot length and Root length Per root system of common bean (Phaseolus vulgaris) under glasshouse condition.*

Treatment	NN/p	NDWg/p	SDWg/p	SHcm/p	RLcm/p	SE%	Eff
GCR1	63.602^{f}	0.446^{dc}	0.947^{d}	30.917^{f}	11.433^{bc}	50.690^{d}	E
GCR2	85.480^{e}	0.629^{b}	1.869^{b}	40.489^{b}	11.761^{bac}	98.933^{cb}	HE
GCR4	63.410^{f}	0.385^{de}	1.857^{b}	38.430^{c}	11.786^{ba}	99.247^{cb}	HE
GCR7	91.795^{d}	0.273^{f}	0.967^{d}	33.285^{e}	11.836^{ba}	51.217^{d}	E
GCR8	82.556^{e}	0.381^{de}	1.630^{c}	36.813^{d}	11.918^{ba}	87.423^{c}	HE
GCR9	45.217^{h}	0.204^{f}	0.646^{e}	30.908^{f}	12.364^{ba}	34.527^{e}	IE
GCR11	109.777^{c}	0.645^{b}	1.045^{d}	36.757^{d}	11.669^{bac}	55.830^{d}	E
GCR14	52.166^{g}	0.293^{fe}	0.992^{d}	34.294^{e}	12.213^{ba}	53.033^{d}	E
GCR16	136.371^{b}	0.469^{dc}	2.265^{a}	42.523^{a}	11.788^{ba}	121.13^{a}	HE
GCR19	159.628^{a}	0.885^{a}	2.120^{a}	41.165^{b}	12.384^{ba}	113.44^{a}	HE
GCR25	41.926^{h}	0.243^{f}	0.662^{e}	29.476^{g}	13.547^{a}	35.500^{e}	LE
GCR28	94.333^{d}	0.484^{c}	1.765^{cb}	38.432^{c}	11.644^{bc}	94.380^{cb}	HE

Treatment	NN/p	NDWg/p	SDWg/p	SHcm/p	RLcm/p	SE%	Eff
(+) control	--------^{i}	-----^{g}	1.870^{b}	40.457^{b}	10.980^{bc}	100.00^{b}	-
(-) control	--------^{i}	-----^{g}	0.602^{e}	21.321^{h}	9.900^{c}	32.220^{e}	-
$LSD_{(0.05)}$	5.848	0.093	0.22	1.202	1.879	12.32	
Mean value	73.304	0.381	1.374	35.376	11.802	73.398	
CV	4.770	14.619	9.85	2.032	9.521	10.039	

N.B: Means in the same column followed by same letter are not statistically significant at $p<0.05$, HE= highly effective, E= effective, LE=lowly effective, IE=Ineffective. NN nodule number, NDW Nodule dry weight, SDW Shoot Dry weight SH shoot height RL, root length and SE, symbiotic effectiveness.

There was significant variation ($P < 0.05$) in shoot dry weight among treatments (Table 9). The shoot dry weight of inoculated plants varied from (0.646 g/p) to (2.265 g/p) by isolates GCR9 and GCR16 respectively. This implies that inoculation with rhizobia increased shoot dry weight up to (67.28%) over the negative control. The minimum shoot dry weight, (0.602 g/p) was recorded from the negative control plant. Maximum shoot dry weight (2.265 g/p) was recorded from plant inoculated with isolate GCR16 (Alenga keble) followed with (2.12 g/p) isolate GCR19 (Alenga). This might be due to the effectiveness of inoculated rhizobia in nitrogen fixation and other plant growth promoting hormone production such as auxin and indole-3-acetic acid [26].

The present finding was similar with the finding of [10, 17, 26] reported that inoculation of common bean with rhizobial isolate had increased the shoot dry weight under controlled environment. Shoot dry weight used indirectly to estimate nitrogen fixation for screening of effective rhizobia [5] Shoot dry weight was strongly correlated with shoot length (r = 0.87, $p < 0.001$) and symbiotic effectiveness (r= 0.98, $p< 0.01$) (Table 9). This finding was in concord to [2, 18].

There was significant difference ($P< 0.05$) in nodule dry weight among the treatments (Table 9). The recorded mean nodule dry weight varied between, 0.204 g/p and 0.885 g/p by isolates from (Berji and Alenga) GCR9 and GCR19 respectively. The minimum mean nodule dry weight 0.204 g/p recorded by isolate GCR9 (Berji). The maximum mean nodule dry weight (0.885 g/p) recorded by isolate GCR19 (Alenga) followed by nodule dry weight 0.645 g/p isolateGCR11 (Kuja). The result showed significant difference in nodule dry weight among treated plant (Table 8). [3, 8] reported that high nodule dry weight could be generally a prerequisite for increasing N_2-fixation in legumes, rather than number of nodules. [25] reported *Rhizobium* inoculation significantly increased nodule dry weight of common bean. The present finding was in agreement to [2, 6, 20, 21] suggested that inoculation of rhizobia isolates on common bean showed increase in nodule dry weight under controlled environment. In present study nodule dry weight was positively correlated with shoot dry weight (r=0.48, $p<0.01$), plant height (r=0.56, $p<0.001$) and symbiotic effectiveness (r= 0.48, $p<0.01$) (Table 9).

The relative effectiveness expressed as, percentage of shoot dry mass of the inoculated over the N- fertilized (positive control), shown (Table 8). Result revealed that 50 percentage or (6) rhizobial isolate recorded highly effective (87.42% - 121.13%).

It is interesting to note that (16.7%) of authenticated isolates scored their symbiotic effectiveness greater than (100%). [24] stated that shoot dry matter is a good indicator of relative effectiveness of isolate rather than nodule number which is less reliable indicator of strain effectiveness. This result, underlines the importance for local screening of *rhizobium* isolates in order to improve N_2–fixation and production of common bean at the study area.

In the present study Root length of, rhizobia treated plants over the negative and positive control was not significantly ($p<0.05$) affected by the treatment. The correlation analysis revealed that root length was not correlated with any of the investigated parameters. This result was in agreement to [27] reported that inoculation of rhizobia isolates on common bean has no significant effect and not correlated with any of investigated parameters. Similar result was obtained by [18] reported that inoculation of rhizbia isolates on faba bean has no effect on root length of the plant and was not positively correlated with other investigated parameter.

Table 9. *Correlation coefficients amongst investigated parameters of common bean (Phaseolus vulgarius).*

Variable	NN	NDW (g/p)	SDW (g/p)	SL (cm/p)	RL (cm/p)	SE%
NN	1.000					

Variable	NN	NDW (g/p)	SDW (g/p)	SL (cm/p)	RL (cm/p)	SE%
NDW (g/p)	0.87806***	1.000				
SDW (g/p)	0.53016***	0.48751**	1.000			
SL (cm/p)	0.61012***	0.56375***	0.87143***	1.000		
RL (cm/p)	0.21993 ns	0.17743 ns	-0.03971 ns	0.14872 ns	1.000	
SE%	0.53078***	0.48762**	0.99899***	0.8698***	-0.05156 ns	1.000

*, **, ***, significant at $P<0.05$, $P<0.01$, $P<0.001$, respectively, ns: not significance at the $P<0.05$ NN = Nodule number, NDW = nodule dry weight, SDW = shoot dry weight = SL =shoot length, RL = root length, SE =symbiotic effectiveness.

4. Conclusion

Isolation, Characterization and evaluation of effectiveness of nitrogen fixing rhizobia from Legume crops (*Phaseolus Vulgarius* L.) of the study area as, well as observing the factors affecting the rhizobia, legume and symbiosis providing effective rhizobia is essential as it may result into the identification of supper inoculants (Bio-fertilizers) for improving legume growth and yield and later providing economic benefit to legume producers. Consequently, more research and prominence is required to this inexpensive and eco-friendly technology for majority of smallholder farmers in Ethiopia. Further investigation should be carried out to evaluate their effectiveness under different environmental conditions both, in greenhouse and field trials. In addition for further emphasis molecular characterization of the rhizobial isolates should be seen.

Abbreviations

NDW	Nodule Dry Weight
NN	Nodule Number
SL	Shoot Length
SDW	Shoot Dry Weight
RL	Root Length
YEMA	Yeast Extract Manitol Agar

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Conflicts of Interest

The authors declare no conflicts of interest.

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