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EVALUATING THE SYMBIOTIC EFFECTIVENESS OF COMMON BEAN (*Phaseolus Vulgaris L.*) NODULATING RHIZOBIAL ISOLATES ON EXTREME PH AND HIGH SALT SOIL CONDITION IN *HARARGHE* LOWLANDS AND MID ALTITUDES, EASTERN ETHIOPIA

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Abstract

The intent of this study was to evaluate the symbiotic effectiveness of Common Bean (Phaseolus Vulgaris L.)-Nodulating Rhizobial Isolates to extreme pH and high salt soil condition in Hararghe Lowlands and Mid Altitudes of Eastern Ethiopia. A total of 50 isolates were obtained from soil samples of three wered as in Hararghe lowlands and mid altitudes using the host trap method and were presumptively identified as rhizobia. The physiological tests revealed that among the 50 wild isolates 43(86%), 37(74%), 29(58%), 6(12%), 4(8%), and 2(4%), grew at 2%, 4%, 6%, 8%, 9%, and 10% NaCl concentration, respectively. All of the isolates grew in the range of pH 5.5 - 8.5. 2 (4%), and 38(76%) of the isolates managed to grow at pH4.5 and pH 5 while 48(96%), 43(86%), 40(80%) and 37 (74%) of the isolates were able to grow at pH 9, pH9.5, pH10 and pH 10.5 respectively. The correlation data on soil experiment displayed that nodule number was associated positively and significantly ($r = 0.73$, $p<0.0001$) with NDW while SDW was positively correlated with percent N ($r = 0.8$, $p<0.0001$) and total nitrogen content (($r = 0.9$, $p<0.0001$)). Among the observed rhizobium isolates, HUCR (3D, 3A), HUCR 2D and HUCRM 2D showed the highest symbiotic effectiveness. Thus, on the basis of their symbiotic effectiveness and tolerance to extreme environmental conditions, these wild isolates were recommended to be used as candidates for future development of rhizobial inoculants of common bean grown under saline, extreme pH conditions.

Keywords: Biological nitrogen fixation (BNF), Common Bean, Rhizobia, Tolerance, symbiotic effectiveness.

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INTRODUCTION

As many sub-Saharan countries' soil, now a day the Ethiopian soil also faces low fertilities problem due to the deficiency or absence of important nutrients like nitrogen that has a great impact on the fertilities and productivities of the soil. The declining in the amount of this important nutrient in the soil is largely related with many environmental factors and other human Interventions that can limit their presence to the maximum. Regarding to this soil fertilities problem due to the deficiency or absence of nitrogen, many researchers are recommending that the use of microorganisms(biological nitrogen fixation) is the wisest, economical and environmentally friendly way to solve it.

Biological nitrogen fixation (BNF) is the process whereby atmospheric nitrogen (N=N) is reduced to ammonia in the presence of nitrogenase. Nitrogenase is a biological catalyst found naturally only microorganisms such as the symbiotic Rhizobium and Frankia, or the free living Azospirillum and Azotobacter (Bloom, 2011).

Biological nitrogen fixation is brought about by free-living soil microorganisms and by symbiotic associations of microorganisms with leguminous and some higher plants. The curiosity of this study is on the legume-*Rhizobium* symbiosis. Leguminous plants don't pull nitrogen from the air by themselves. They actually need help from a common bacteria called Rhizobium which live in the root nodules. Rhizobia infect root hairs of the leguminous plants such as beans to help it draw nitrogen from the air and produce the nodules. The nodules become the home for bacteria where they obtain carbohydrate which is the source of energy and hydrogen from the host plant and take free nitrogen from the air and process it into combined nitrogen. In return, the plant receives the fixed N from nodules and produces food and forage protein. There are so many environmental stresses that affect biological nitrogen fixation process; the main ones are air or soil temperature, soil pH, soil fertility, soil salinity, soil moisture, and drought (Hirsch *et al.*, 2001).

Even if the host legume appears to be the limiting factor for establishing *Rhizobium*-legume symbiosis under acidic conditions, soil pH affects both the host plant and the bacteria involved in the symbiotic relationship. In general, a soil pH between 6.0 and 7.0 is optimum pH for their symbiotic relationship to be established. Therefore, maintaining the soil pH in this range tends to promote optimal plant growth and optimal BNF in many legumes. Salt stress is also one of the major environmental stresses adversely affecting legume production in arid and semi-arid regions.

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High soil salinity can deleteriously affect symbiotic association between legume and *Rhizobium* by osmotic stress and ionic toxicity and imbalance. Increased salt concentration affect the soil micro-biota by causing osmotic stress on them. Additionally saline soils are generally deficient in nutrients and microbial activities and population is low (Thomas, 1995). The legume-*Rhizobium* symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than are rhizobia (Hirsch *et al.,* 2001). In contrast to their host legumes, some rhizobia can survive in the presence of extremely high levels of salt both in culture and in soil. Thus, organisms such as *S. melilotit*olerate 200-300mM (milimole) NaCl, while nodulation and nitrogen fixation in their host can be inhibited at 50-100mM salt concentration Serraj and Sinclair, (1998); Hirsch *et al*. (2001) also indicated that, nodulation and nodule dry weight of *Trifolium*Spp. *alexandrium*inoculated by *R. trifolii*was depressed significantly with consistent increase in salinity.

Few trials have been done on evaluating the symbiotic effectiveness of common bean (*Phaseolus Vulgaris L.*)- nodulating rhizobia lisolates on extreme pH and high salt soil condition in Hararghe lowlands and mid altitudes, eastern Ethiopia. So this study was undertaken with the aim of examining the symbiotic effectiveness of common bean nodulating rhizobial in this region of Ethiopian.

MATERIALS AND METHODS

Description of the Study Area

Rhizobia were isolated from soils of pulse growing areas in the lowlands and mid altitudes of East Hararghe Zone around Babile, a small town located 529 kilometers away from Addis Ababa, Ethiopia, and approximately 25 km east of the ancient city of Harar. The lowlands and mid altitudes of East Hararghe zone generally included altitude ranging from 1300 m.a.s.l to 1600 m.a.s.l.

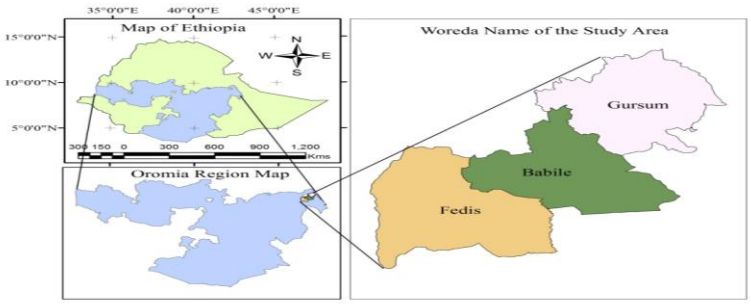

Fig.1: The geographical location of the sampling sites within the map of Ethiopia.

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Study Design

The experiments included isolation and characterization of nitrogen fixing bacteria (rhizobia) from soil samples and determination of their symbiotic effectiveness in pot experiments. The rhizobial isolates were also tested for their tolerance to salinity and extreme pH conditions in the laboratory. The pot experiments used for screening of the rhizobia from soil samples were laid down in randomized complete block design (RCBD) with three blocks or replications in a green-house. As controls, each block contained two pots, negative control with no chemical fertilizer (no KNO_3) and no inoculum (Con^-) and positive control with chemical fertilizer (Con^+) (0.05% KNO_3) and absence of inoculum.

Soil Sample Collection

The soil samples were collected from ten major potential pulse-growing Eastern Hararghe lowland and mid altitude areas in April, 2012. In each area, five bulk samples (about 3 kg of soil) were randomly collected using auger and sterile polyethylene bags from a depth of 30 cm of the surface for the isolation of indigenous rhizobia by trap method as indicated in Somasegaran and Hoben (1994). Soil collection spots were geo-referenced using the Universal Transverse Mercator (UTM) geographic coordinating system. Aseptic precautions were exercised to avoid contamination of the soil samples until used for green house pot experiment and for soil pH analysis.

Isolation of Rhizobia

Five seeds of common bean (*Phaseolus vulgaris L.*) Gofta (G-2816) variety was sown for each bulk sample in a sterile plastic pot at Haramaya university green house. The pots were watered every three days for 45 days. After 45 days, the plants were uprooted from the pots and intact, pink, red or brown multi-lobed and large nodules of the plants separated from the taproots with portions of the roots attached to the nodules and transported through vials containing silica gel. In this manner, more than 50 isolations were made at Haramaya University Microbiology Laboratory. The nodules were thoroughly washed with distilled water so as to remove gross surface contamination. After overnight soaking in distilled water, the nodules were again immediately immersed in 70% ethanol and 0.15 acidified $HgCl_2$ for 3 minutes and 1 minute, respectively (SubbaRao, 1999). Surface sterilized nodules were washed at least 10 times by successively washing with sterile water to remove the contaminants completely. The nodules were placed in 0.1N NaCl solution and a few drops of distilled sterile water and crushed with a

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sterile glass rod. The suspension was then placed on YEMA (Yeast Extract Mannitol Agar) and incubated at 28^{o}C for 3 to 5 days.

Purification and Preservation of the Isolates

All isolates were tested for presumptive purity using gram staining and by growing them on peptone glucose agar (PGA) and YEMA medium containing 25ppm (0.0025%) of Congo red (YEMA-CR). The isolates were also inoculated on YEMA containing 25 g/ml bromothymol blue which serve as useful criteria for distinguishing the two aligned genera *rhizobium* and *Bradyrhizobium*. The isolates were preserved on YEMA slants containing 0.3 % (w/v) $CaCO_3$ in a slant culture and stored at 4^{0}C for future use (Somasegaran and Hoben, 1994).

Designation of the isolates

Each isolate was designated as HUCR (Haramaya University Common bean *Rhizobium*) followed by a number (represent Kebele) and a letter (represent Genda or Village).

Physiological Characterization of the *Rhizobium* Isolates

Determination of Salt and pH Tolerance

The *in vitro* pH tolerance among *Rhizobium* isolates was determined using the method described by Lupwayi and Haque (1994). Media plates of YEMA containing various concentrations (2%, 4%, 6%, 8%, 9%, 10%, 11%, and 12%) of sodium chloride or TY agar media adjusted to pH levels of 4-10.5 (for pH tolerance) were prepared and inoculated with appropriately diluted pure cultures of the isolates. The plates were then incubated at 30^{o}C for four days. Finally, the growth of the rhizobial strains were qualitatively evaluated as negative (-) for no growth and positive (+) for growth and in the same manner the susceptibility to NaCl were recorded as a positive (+) for growth or negative (-) for absence of growth.

Authentications and Evaluation of Symbiotic Effectiveness of Common bean rhizobia

The effectiveness of wild rhizobial strains were tested using sterile sand and unsterilized field soil culture systems at Haramaya University green house.

Authentication and evaluation of symbiotic effectiveness of wild common beanrhizobia in sterilized sand culture

Fifty pure isolates that qualified the presumptive test were further tested for authentication in the green house. Fine graded river sand was washed in tap water and immersed in concentrated sulfuric acid ($H_2 SO_4$) for two days. It was washed in several changes of tap and distilled water to get rid of the last traces of the acid and autoclaved for 1.5 hours before filling into plastic pots of 3 kg capacity which were surface sterilized with 95% ethanol (Lupwayi and Haque,

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1994). Two hundred fifty common bean seeds, Haramaya University Gofta (G-2816) variety, of uniform size and color were surface sterilized by suspending for 10 minutes in 3-5% H_2O_2 with washings of several changes of distilled water (Lupwayi and Haque, 1994). The sterilized seeds were then transferred to distilled water and incubated at 25^oC for 3 days of germination. Five surface sterilized and pre-germinated seedlings were transferred into each pot, which were later thinned to three after a week of planting. Each seedling was inoculated with 1ml culture of each isolate adjusted to an inoculum size of 10^9cell/ml.

The pots with seedlings were subsequently supplied with distilled water every two days and fertilized once a week with quarter strength of Broughton and Dilworth N-free medium described in Somasegaran and Hoben (1994).

Forty five days after sowing, the plants were harvested and their roots were scored for nodulation. The shoots of plants as well as the nodules were then oven dried to determine their dry weight at 70^0C for 48 hrs at HU Microbiology laboratory. Symbiotic effectiveness (SE) of the isolates was calculated according to the equation:

$$SE = \frac{Inoculated plant SDM}{N-Fertilized plant SDM} \times 100$$

Then, symbiotic effectiveness (SE) of isolates were rated as ineffective, lowly-effective, effective and highly effective, when the calculated values were <35%, 35-50%, 50-80%, and >80 %, respectively (Beck *et al*., 1993).

Evaluation of Symbiotic Effectiveness of Wild Common bean Rhizobia in Unsterilized soil

The effectiveness of five selected isolates was determined in the pot experiment using unsterilized soil at Haramaya University greenhouse. The soil was well mixed, sieved and air–dried. Three Kg of this soil was distributed to plastic pots. Twenty five common bean of "Gofta (G-2816) Variety" was surface sterilized as before and rinsed in five changes of sterile distilled water. Five un-germinated seeds were sown in each pot and later thinned down to three after germination for one week. After a week, each seedling was inoculated with 1 ml of each isolate grown for 72 hrs in YEM broth. The experiment was set up in replicates in the green house and the pots were arranged in complete random design with each block consisting of negative control (without nitrogen and without inoculum) and positive control (without inoculum but with nitrogen). The nitrogen fertilizer (KNO_3) was given at a concentration of 0.5 g / l per week until the plants were harvested. All the pots were watered every two days.

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Plant total nitrogen content analysis

The modified Kjeldhal method was used to evaluate the nitrogen content of the plant samples (*SahlemedhinSertsu and TayeBekele, 2000*). In this method, plant samples were first ground and initially 0.3 g of the resulting material measured in 100 ml digestion tube for analysis. Following this, 0.5 g of a mixture of 10 g K_2SO_4, 2 g $CuSO_4.5H_2O$ and 0.2 g Selenium was added to the ground sample and used as a catalyst. A mixture of sulfuric and salicylic acid (7 ml) was also added to the ground sample and permitted to react for 30 minutes. In addition, 0.5 g of $Na_2S_2O_3.5H_2O$ was added and shaken to react for five more minutes. A blank consisting of 0.5 g salt mixture and 7 ml of sulfuric-salicylic mixture was also prepared. The digestion of the ground sample was undertaken at a temperature of 300^0C for 2hrs until the content turned to colorless. Then after, the sample was distilled by adding 75 ml of 40% NaOH and its nitrogen collected with a flask containing 20 ml boric acid until the volume reached 110 ml. Ultimately, the distillate was titrated using 0.1 N H_2SO_4 and the reading of the burette used to calculate the percentage nitrogen using the formula:

$$\text{Nitrogen (\%)} = \frac{(a1-b2)\ X\ N\ X\ 0.014}{S} \quad X\ 100$$

Where, a_1 = ml of titrant used for the sample

b_2 = ml of titrant used for the blank

N= Normality for the acid

S = weight of the plant material

Total N uptake was calculated as:

$$\text{N content} = \frac{\%N\ X\ \text{shoot dry wight}}{100}$$ (Ogutcu*et al*., 2008)

Data Analysis

Comparison between treatments was analyzed using one-way ANOVA (Fisher's LSD tests) (SAS.9.1).The data that used in the analysis were nodule number, nodule dry weight, shoot dry weight,% of nitrogen, content of nitrogen.

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RESULTS AND DISCUSSION

Isolation and Presumptive Test for Identification of Rhizobia

Overall 50 Common bean rhizobial bacteria were obtained from nodules that were induced from soils of Babile (40 isolates), Fedis (5 isolates) and Gursum (5 isolates). Hundred percent of the isolates were found to be gram negative, rod shaped bacteria which did not absorb Congo red on YEMA-CR medium and were not grown on Peptone Glucose Agar (PGA). According to SubbaRoa (1999) growth of bacteria on PGA media is a useful criterion for the identification of true Rhizobia from the root nodule. These 50 isolates turned Yeast Extract Mannitol Agar medium containing bromothymol blue (YEMA-BTB) into moderately yellow, yellow and deep yellow color after 48 hours of incubation showing that all the isolates were fast growing acid producing rhizobia. Somasegaran and Hoben, (1994) and Gupta (2000) also confirmed that colonies of fast growing *Rhizobium spp.* can show little or no Congored absorption when incubated in the dark and should turn the YEMA-BTB to yellow after 3-5 days of incubation.

Authentication of rhizobia

To determine the potential of the isolates that were induced from the soils of eastern Ethiopian lowland and mid-altitude to form nodules, they were re-inoculated on the host plant on sterile pouch and grown in a growth chamber. Hundred percent or all the 50 isolates formed nodules and were authenticated as root nodule bacteria.In line with this, Wortmann*et al*. (1998),AlemayuhuWorkalemahu (2006), and AntenehArgaw (2007) indicated that all collected rhizobia from common bean nodules were authenticated as root nodule bacteria. AlemayuhuWorkalemahu (2006) andAntenehArgaw (2007) also confirmed the existence of native bean-nodulating rhizobia in Ethiopian soils.

Physiological Characterization of the *Rhizobium* Isolates

Salt tolerance

The isolates displayed growth differences on YEMA medium adjusted to different NaCl concentrations. Among the 50 isolates 43(86%), 37(74%), 29(58%), 6(12%), 4(8%), and 2(4%), were grown at 2%, 4%, 6%, 8%, 9%, and 10% of NaCl on YEMA medium, respectively. The most salt tolerant isolates were HUCR9A and HUCR7B from Babille grown at 10% NaCl concentration. On the other hand, isolates HUCR (9C, 10A, 10B, 10C, 12B, 12C) from Babile were found to be within a narrow range of salt tolerance up to 2% of NaCl concentration and HUCR(3C,5D,12E,13A,13B,13C,13E) were the most sensitive isolates which did not grow at 2%

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of NaCl concentration. In the same way, Hungria and Vargas (2000) reported that usually fast growing rhizobia grew well between 3-5 % concentrations of NaCl. It is obvious that *Rhizobiumphaseoli*is amongst the salt tolerant rhizobia and a number of isolates have been reportedto tolerate high salt concentrations between 4%-5%. Likewise, twoisolates of common bean from southern Ethiopia tolerated higher concentration of saltas reported by AlemayehuWorkalemahu (2006).

pH Tolerance

The tolerance to pH changes varied amongst the tested isolates (Table 6). In this study growth was evidently detected from pH 4.5-10.5. 100% of the isolates were found to grow in the range of pH 5.5-8.5. Four percent of isolates were adapted to grow at pH 4.5, whereas 80% of the isolates managed to grow at pH 10. Only two isolates from Babile HUCR (2C,5C) managed to grow on all the tested pH values whereas, the isolates that were found to be sensitive at pH 10.5 were isolates HUCR(2A,5E,7E,10A,10B,10C,10E,12D,13A,13C,13D) from Babille and HUCR (3A,3E) from Fedis. All isolates from Gursum, Fedis and 96% of the isolates from Babile failed to grow at pH <5. Similarly, Bordeleau and Prevost (1994) showed that fast growing rhizobia are commonly susceptible to low pH but grow best nearly at neutral to basic pH. In line with this, AlemayehuWorkalemau (2006), Anteneh Argaw (2007) and Esubalew Sintie (2011) and other similar domestic researchers revealed that acid producing rhizobia are poor in tolerance to low soil pH conditions.

Evaluation of Symbiotic Effectiveness of Common bean rhizobia

Evaluation of the symbiotic effectiveness of common bean rhizobia under sterilized sand in pot experiment

The inoculated plants showed significant variations ($p < 0.05$) in nodule number, nodule dry weight and shoot dry weight. Each of the presumptive rhizobial isolates were tested for their ability to cause nodulation and their relative symbiotic effectiveness on common bean, Gofta (G-2816) variety, in sterile pots using sterilized sand culture in a greenhouse. All the 50 rhizobial isolates in this study were able to induce nodulation on common bean (*Phaseolus vulgaris L.*) and were subsequently authenticated as true rhizobial isolates (Vincent, 1970). In line with this, Rahmani*et al*. (2011), EsubalewSintie,(2011) and KassaBaye,(2011) on common bean, with lupine and field pea legumes reported that all their rhizobial isolates were successful in nodulating their host plants .

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Visual observation of the outward appearances of inoculated plants had also shown that they were visibly different from the negative control. The control plants (specially the negative control) appeared relatively dwarf and pale green while the inoculated plants appeared deep green with long shoots, many branches and pink to red nodules, indicating that the later performed better than the control in terms of atmospheric nitrogen fixation.

The least number of nodules was recorded for the plant inoculated with isolate HUCR13C (28/plant) and the highest was for the plant inoculated with isolate HUCR2A (140/plant) both from Babille*woreda*. Similarly, 0.04g/plant and 0.3g/plant were the smallest and highest nodule dry mass recorded for plants inoculated with isolates HUCR (5C, 6A,9B, 9E 10B) and HUCR3A ,respectively. In line with this, AntenehArgaw (2007) reported that the smallest and the highest nodule dry mass were 0.041gm/pl and 0.215 gm/plant in eastern Ethiopian soils.

The accumulated shoot dry matter in the infected host plant was used to evaluate the relative symbiotic effectiveness of the isolate. According to Somasegaran and Hoben (1994), Lupwayi and Haque (1994), and Peoples *et al.* (1992) shoot dry matter is a good indicator of the relative symbiotic effectiveness of the isolates and a positive correlation between the nitrogen fixing capacity of legumes and their shoot dry matter accumulation. However, nodule dry weight measurements were fairly better related to shoot dry weight than nodule number. Such correlation of nodule number and nodule dry weight with shoot dry weight is a good indicator of the nitrogen fixing efficiency of isolates and, hence, these parameters can be used to determine the symbiotic effectiveness of legume nodulating-rhizobia (Peoples *et al.*, 1992).

The highest (1.4g/plant) and the least (0.3g/plant) shoot dry matter accumulations were recorded from plants inoculated with isolate HUCR3D and isolate HUCR5A, respectively. Plants inoculated with isolates HUCR2A, HUCR3C and HUCR3D showed significantly ($p<0.05$) higher nodule number, nodule dry weight and shoot dry weight than any of the other inoculated plants respectively.

Correlation among variables in the sand experiment for wild rhizobia confirmed that nodule number were correlated positively and significantly ($r = 0.4$, $P < 0.0001$) with nodule dry weight. A similar correlation result was documented by Khondaker*et al*. (2003) who reported a correlation index of $r = 0.32$, for the association of nodule number with nodule dry weight with regard to inoculation of pea varieties

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Regarding the relative symbiotic effectiveness 46%, 52% and 2% of the isolates were found to be highly effective, effective and ineffective respectively (Table 1). Considering the site of location, all isolates from Gursum, 61% of the isolates from Babille and 17% of the isolates from Fedisworeda were highly effective. Four percent of the isolates from Fedis and 96% of the isolates from Babilleworeda became effective.

The highest scores of effectiveness of symbiotic nitrogen fixation were displayed by HUCR (3D,3 A,2D,2C,3C,14D,14C,6B,7B,9C,13A,13B,14A,14E,2A,6C,13C,2B,2E,3E,7C,13D,14B) shoot dry mass >0.8g/plant. Isolate HUCR5A from Babille soil obtained to be ineffective which was observed to be smaller than the ineffective equivalent of 0.414g/plant showed by negative control. In this study, 98% of the isolates were categorized into effective and very effective groups. In a similar work,AlemayuhuWorkalemahu, (2006), Anteneh Argaw, (2007) reported that more effective isolates were obtained from wide range of geographical locations and pH ranges of south and east Ethiopia soils respectively. Hopefully this is an indication for the survival and abundance of more effective soil rhizobia in Ethiopia.

Table 1.The symbiotic effectiveness of common bean (Phaseolus vulgaris L.) rhizobial isolates and their ability to produce root nodules on sterilized sand culture.

Treatments	Nodule No/ plant	Nodule dry weight/plant(gm)	Shoot dry weight/plant(gm)	SE (%)	Effectiveness
HUCR2A	140 ± 17^{a}	0.18 ± 0.02^{c}	0.9 ± 0.04^{el}	94	HE
HUCR2B	62 ± 19^{gm}	0.09 ± 0.01^{eg}	0.8 ± 0.010^{ho}	83	HE
HUCR2C	61 ± 22^{hm}	0.19 ± 0.01^{c}	1.2 ± 0.580^{cd}	125	HE
HUCR2D	102 ± 20^{b}	0.24 ± 0.02^{b}	1.3 ± 0.230^{b}	136	HE
HUCR2E	69 ± 15^{dk}	0.18 ± 0.01^{c}	$0.8\pm0.001^{g-n}$	83	HE
HUCR3A	82 ± 18.6^{cf}	0.3 ± 0.010^{b}	1.3 ± 0.002^{c}	136	HE
HUCR3B	36 ± 1^{ps}	0.2 ± 0.001^{d}	0.7 ± 0.001^{lq}	73	E
HUCR3C	86 ± 13.1^{bd}	0.2 ± 0.010^{a}	1.2 ± 0.002^{b}	125	HE
HUCR3D	56 ± 12.9^{io}	0.09 ± 0.002^{eg}	1.4 ± 0.001^{a}	158	HE
HUCR3E	39 ± 11.9^{os}	0.05 ± 0.002^{nq}	0.8 ± 0.001^{hn}	83	HE
HUCR5A	64 ± 10.6^{fm}	0.05 ± 0.001^{nq}	0.3 ± 0.001^{t}	31	I
HUCR5B	69 ± 15^{dk}	0.14 ± 0.003^{d}	0.5 ± 0.001^{qt}	52	E
HUCR5C	85 ± 11.1^{be}	0.04 ± 0.003^{pq}	0.6 ± 0.001^{os}	63	E
HUCR5D	43 ± 13.1^{ns}	0.05 ± 0.001^{mq}	0.7 ± 0.001^{jq}	73	E
HUCR5E	68 ± 17.6^{dk}	0.14 ± 0.001^{d}	0.6 ± 0.001^{pt}	63	E
HUCR6A	70 ± 16.6^{dk}	0.04 ± 0.003^{pq}	0.7 ± 0.003^{mr}	73	E
HUCR6B	103 ± 12.5^{b}	0.05 ± 0.002^{mq}	1.0 ± 0.002^{di}	104	HE
HUCR6C	77 ± 13.5^{ch}	0.05 ± 0.001^{nq}	0.9 ± 0.001^{fm}	94	HE
HUCR6D	35 ± 7.2^{ps}	0.06 ± 0.001^{lq}	0.6 ± 0.002^{pt}	63	E
HUCR6E	69 ± 8.4^{dk}	0.05 ± 0.001^{oq}	0.7 ± 0.100^{kq}	73	E
HUCR7A	61 ± 12.3^{hm}	0.06 ± 0.001^{oq}	0.7 ± 0.001^{mr}	73	E
HUCR7B	90 ± 35.4^{bc}	0.05 ± 0.001^{pq}	1.0 ± 0.001^{dj}	104	HE
HUCR7C	93 ± 20.5^{bc}	0.06 ± 0.002^{lq}	0.8 ± 0.001^{fn}	83	HE

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HUCR7D	66±15.6^{fl}	0.06±0.002^{lq}	0.6±0.001^{os}	63	E
HUCR7E	67±13.5^{el}	0.05± 0.001^{oq}	0.5±0.001^{rf}	52	E
HUCR9A	65±2.7^{fm}	0.06±0.001^{kq}	0.6±0.001^{os}	63	E
HUCR9B	71±3.1^{dj}	0.04±0.001^{pq}	0.8±1.000^{ip}	83	E
HUCR9C	63±2.7^{gm}	0.05±0.001^{pq}	1.0±1.000^{dg}	104	HE
HUCR9D	61± 3.2^{gn}	0.07±0.001^{in}	0.6±0.003^{ns}	63	E
HUCR9E	78±4^{ch}	0.04±0.001^{pq}	0.7±0.001^{kq}	73	E
HUCR10A	61±3.2^{gn}	0.08±0.002^{fj}	0.6±0.001^{ns}	63	E
HUCR10B	53±3.1^{jp}	0.04±0.001^{q}	0.6±0.001^{os}	63	E
HUCR10C	52±2.1^{kp}	0.07±0.002^{gl}	0.6±0.001^{os}	63	E
HUCR10D	63±2.5^{gm}	0.06±0.002^{jp}	0.6±0.001^{ps}	63	E
HUCR10E	51±6.5^{kp}	0.05±0.001^{mq}	0.7±0.003^{mr}	73	E
HUCR12A	49±2^{lr}	0.09±0.002^{eh}	0.6±0.001^{pt}	63	E
HUCR12B	74±1^{ci}	0.07±0.002^{hm}	0.8±0.001^{iq}	83	E
HUCR12C	80±1^{cg}	0.05±0.001^{pq}	0.7±0.002^{kq}	73	E
HUCR12D	56±3.6^{io}	0.05±0.002^{mq}	0.8±0.002^{jo}	83	E
HUCR12E	51±3^{kq}	0.1±0.0100^{l}	0.7±0.002^{mr}	73	E
HUCR13A	52±17.2^{kp}	0.18±0.060^{c}	1±0.0010^{kq}	104	HE
HUCR13B	56±5.1^{io}	0.1±0.001^{eg}	1±0.0020^{jq}	104	HE
HUCR13C	28± 2^{s}	0.1±0.002^{li}	0.9±0.002^{go}	94	HE
HUCR13D	54±3.5^{jp}	0.1±0.001^{mq}	0.8±0.001^{jq}	83	HE
HUCR13E	55±3^{jo}	0.1±0.002^{fj}	0.7±0.001^{kq}	73	E
HUCR14A	70±6.7^{dk}	0.1±0.100^{fk}	1.0±0.001^{dj}	104	HE
HUCR14B	32±3.2^{qs}	0.1±0.002^{ei}	0.8±0.001^{ho}	83	HE
HUCR14C	36±2.1^{qs}	0.05±0.001^{mq}	1.1±0.002^{ce}	114	HE
HUCR14D	31± 2.1^{rs}	0.07±0.002^{jn}	1.2±0.001^{cd}	125	HE
HUCR14E	47± 2^{mr}	0.10±0.002ef	1.0±0.001^{dh}	104	HE
Control(-)	0^{t}	0r	0.414±0.002st	43	
Control(+)	0^{t}	0r	0.959±0.001^{ek}	100	
LSD(p<0.05)	18.95	0.03	0.23		

Key: Numbers in the same column followed by the same letters are not significantly different at p<0.05(Fisher's LSD). SE= Symbiotic effectiveness; I= ineffective; E= Effective and HE= Highly Effective; LSD= list significant difference. % SE= >80% is highly effective, 50-80 % is effective, and < 35% is ineffective.

Table 2: Rating of effectiveness of isolates by sampling site (woreda)

Samplin g*woredas*	**%H E**	**%E**	**Total**	**%I E**	**Total**	**HE isolates**	**E Isolates**
Babile	35%	62.5	97.5	2.5	100	2D,2C,6B,7B,9C,13A,13B2A,6C,13C,2B,2E,7C13D	9B,12B,12D,5D,6A,6E,7A,9E,10E,12C,12E,13E,5C,5E,6D,7D,9A,9D,10A,10B,10C,10D,12A,7E,5B
Fedis	80	20	100	0	100	3D,3A,3C,3E	3B
Gursum	100	0	100	0	100	14D,14C,14A,14E,14B	None

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Evaluation of Symbiotic Effectiveness of Common bean Rhizobia underunsterilized soil in pot experiment

From the sand culture HUCR(3D,3A) from Fedis, HUCR(2D,2C) from Babille and HUCR14D from Gursum were the five highly effective isolates selected as inoculants for Common bean plant and tested on Babille soil under greenhouse condition at Haramaya University main campus. In all parameters except in % N the inoculated plants showed significant difference ($P<0.05$). In this study the different rhizobial inoculants showed variation in nodule number, nodule dry weight, shoot dry weight, N percent and N contents on the inoculated common bean plants (Table 3). In line with this, Amarger*et al.,(*1996) reported that symbiotic effectiveness of rhizobial isolates showed variation in nodulation ,shoot dry weight, nodule dry weight and total nitrogen of legumes as it could be influenced by soil aeration, inoculums size and viability, soil nutrient level, soil pH, temperature and absence or presence of native rhizobia in the soil. Similarly, Giller (2001) also explained that the variation in nodulation, nodule dry weight, shoot dry weight and percent of nitrogen in the soil cultures may be attributed to the difference of nitrogen in the soil, and additional nodulation by the native rhizobia of soils and other rhizospher effects on plant growth.

HUCR3A was the isolate that induced the highest nodule number (120 per plant) followed by isolates HUCR2C and HUCR2D with nodule number of 117 per plant and 110 per plant respectively. The least number of nodules was induced by plants inoculated with isolates HUCR3D and HUCR14D with nodules number 52 per plant and 99 per plants correspondingly. All isolates except HUCR 3D showed significantly ($p <0.05$) higher nodule number than both positive and negative controls and isolate HUCR 14D showed significantly ($p<0.05$) higher shoot dry weight than other plants.

This study, showed that positive correlations were observed with respect to the number of nodules and dry weight of nodules ($r = 0.8$, $p<0.0001$), dry weight of shoot and N content ($r = 0.73$, $p<0.0001$) and %N and N content in shoot of common bean ($r = 0.43$, $p<0.04$). Lalande*et al*., (1990) and Mungai and Karubiw (2008) observed positive significant correlation ($r=0.96$, $p< 0.01$; $r=0.3$, $p<0.06$) between shoot dry weight and N content using *Rhizobium leguminosarum* by *phaseoli* isolates.

Comparing the nodule number of the controls with the inoculated plants, the least nodule number for the inoculated plant (52 per plant) was greater than the nodule number of positive control by 85% and the nodule number of negative control by 42%. Even comparing the nodule number of

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the positive control with the negative control, the negative control nodule number was greater than the nodule number of the positive control by 73 %. Possibly beside other several influencing factors, inhibitory effects of fertilizer and nitrogen treatment were responsible for the inadequate symbiosis between the positive control and the rhizobia in the soil Giller, (2001); Ümmühan and Uyanoz, (2012).

In this study, 0.23 g/plant and 0.16 g/plant were the highest and the lowest nodule dry weight record induced by plants inoculated with isolate HUCR3A and HUCR14D correspondingly. Contrary to this, on common bean Ümmühan and Uyanoz, (2012) reported that 1.56 and 0.41 g/plant were the highest and the lowest dry weight of nodule, which is by far greater than the present result. The average nodule dry weight value obtained in this study 0.16 g/plant is found to be higher than the mean nodule dry weight values of other legumes such as Faba bean 0.032g/plant by Zerihun Belay,(2006) and 0.058g/plant by GetanehTesfaye,(2008) on Ambagiorgis and Sebeta soils, respectively. At the same time it is also higher than the nodule dry weight value 0.085g/plant and less than the nodule dry weight value 0.227g/plant of White Lupin (*Lupiusalbus L*.) by EsubalewSintie, (2011) on Holeta and Sekela soil respectively.

HUCR14D was the isolate that induced the highest shoot dry weight 2.2 g/plant followed by isolates HUCR3A and HUCR2D with shoot dry weight of 1.3 and 1.1 g/plant in that order. The least shoot dry weight was 0.5 g/plant induced by isolate HUCR3D. The highest shoot dry weight 1.96 g/plant which is documented for Awash Melka Variety on Melkassa soil by AntenehArgaw, (2007) is smaller than the highest shoot dry weight 2.2 g/plant of this study by 12% but the highest shoot dry weight of this study is less than the maximum shoot dry weight 3.07 g/ plant of Ayenew variety on Melkassa soil by 28%. On Babille soil, the highest shoot dry weight 1.14g/ plant of Awash Melka variety and 1.87 g/plant of Ayenew variety which is announced by the same researcher is smaller than the maximum shoot dry weight of the present study by 48% and 15% respectively. The recorded maximums shoot dry weight 2.2 g/plant of this study on Babille soil is greater than the shoot dry weight of the positive and negative control by 23% and 77% correspondingly.

Concerning % N, 2.5% N/gm for a plant inoculated with isolate HUCR3D and 2.1% N/gm for a plant inoculated with isolate HUCR14D were the highest and the lowest record for this study. Here the % N of the negative control decreased from lowest value % N of this study by 24%.

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Table 3: Nodulation data of selected effective wild isolates of common bean rhizobia with Gofta (G-2816) variety on Babile soil

Isolates	Nodule No/plant	Nodule dry weight/plant(gm)	Shoot dry weight/plant(gm)	Nitrogen (%)	N Content (g/pl)
HUCR 3D	52 ± 2^{b}	0.18 ± 0.01^{bc}	0.5 ± 0.001^{d}	2.5 ± 1^{a}	0.02 ± 0.003^{cd}
HUCR 3A	120 ± 10^{a}	0.23 ± 0.05^{a}	1.3 ± 0.6^{bc}	2.3 ± 0.05^{a}	$0.03\pm0.01^{a,b}$
HUCR 2D	110 ± 35^{a}	0.21 ± 0.02^{a}	1.1 ± 0.001^{c}	2.4 ± 0.1^{a}	0.03 ± 0.00^{b}
HUCR 2C	117 ± 18^{a}	0.2 ± 0.00^{ab}	0.9 ± 0.001^{c}	$2.2\pm0.14^{a,b}$	0.02 ± 0.00^{c}
HUCR14D	99 ± 1^{a}	0.16 ± 0.04^{c}	2.2 ± 0.001^{a}	$2.1\pm0.01^{a,b}$	0.03 ± 0.01^{b}
Control(-)	$30\pm1^{b,c}$	0.08 ± 0.001^{d}	0.5 ± 0.01^{d}	1.6 ± 0.01^{b}	0.01 ± 0.00^{d}
Control(+)	8 ± 1^{c}	0.05 ± 0.01^{d}	1.7 ± 0.3^{b}	2.5 ± 0.01^{a}	0.04 ± 0.00^{a}
LSD($p<0.05$)	26.7	0.04	0.42	0.67	0.01

Key: Levels connected by same letter are not significantly different ($p<0.05$).

SUMMARY AND CONCLUTION

Overall fifty rhizobial isolates were induced from soils collected around Babille including some parts of Fedis and Gursumworedas of east Hararghae lowlands and mid altitudes using common bean as a trap host. The indigenous rhizobia were isolated following the standard procedures. All isolates pass through purification, cultural characterization and presumptive tests. The presumptive test, result showed that the whole isolates were gram negative, rod shaped, and acid producing bacteria. In the same way, no isolate growth was noticed on CR and PGA media.

Authentication was done in a greenhouse on sterilized sand through inoculation of the selected seed of common bean Gofta (G-2816) variety which was arranged in RCBD with three replication and two controls(positive and negative). All the isolates induced nodulation and proved to be true root noduleting bacteria. Among the inoculated plants, common bean plants inoculated with isolate HUCR2A produced the maximum nodule number 140 per plant whereas the minimum number of nodules recorded was 28 per plant for the isolate HUCR13C. In addition, there was a significant variation in dry weight of nodule per plant with different *Rhizobium* isolates. The highest and lowest dry weight of shoots were recorded for plants inoculated with HUCR3D (1.4 g per plant) and HUCR3A (0.33 g per plant), respectively.

Depending on their shoot dry weight in reference to the N-fertilized control plant, the isolates displayed variation in effectiveness ranging from 31% to 158%. In this study, isolates were also found to have diversity in their response to various physiological responses. It was found that similarity exist among isolates in terms of tolerance to 5.5-8.5 pH ranges whereas, isolates were very sensitive to concentrations of NaCl beyond 8%.

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Effectiveness rating result also showed that, 1(2%), 23(46%) and 26(52%) of the isolates were found to be ineffective, effective and highly effective, correspondingly. The above mentioned results evidently make known the abundance and existence of effective common bean rhizobial isolates in eastern Hararghae lowlands and mid altitude. Correlation response among variables in the sand experiment for wild rhizobia confirmed that nodule dry weight were related positively and significantly ($r = 0.4$, $P < 0.0001$) with shoot dry weight.

Symbiotic effectiveness (SE) test of five best isolates was also carried out in Babile soil. All isolates except HUCR 3D showed significantly ($p < 0.05$) higher nodule number than both positive and negative controls and isolate HUCR 14D showed significantly ($p<0.05$) higher shoot dry weight than other plants. The significant ($P < 0.05$) highest dry weight of shoot (2.2g/ plant) was recorded with isolate HUCR14D, which was higher than the records in shoot dry weight of uninoculated positive and negative controls by 23% and 77%, respectively. Positive correlations were observed with respect to the number of nodules and dry weight of nodules ($r = 0.8$, $p< 0.0001$), dry weight of shoot and N content ($r = 0.73$, $p<0.0001$) and %N and N content in shoot of common bean ($r = 0.43$, $p<0.04$).

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