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Evaluating the Symbiotic Effectiveness of Common Bean (*Phaseolus vulgaris* L.) Nodulating *Rhizobial* Mutants on Extreme pH and High Salt Soil Conditions

Mulugeta Mekonnen*

Ethiopia Institute of Agricultural Research Center (EIAR), National Agricultural Biotechnology Research Center, Holetta, Ethiopia

Abstract

The intent of this study was to evaluate the symbiotic effectiveness of common bean (*Phaseolus vulgaris L.*)-nodulating mutant rhizobia isolates to extreme pH and high salt soil condition around Babile, eastern Ethiopia. After mutagenesis, from 50 identified wild rhizobia isolates, a total of 8 mutants were selected based on their ability to survive at extreme salt and pH conditions. The nodule number of mutants were positively and significantly correlated with nodule dry weight (r=0.85, p<0.0001) on sand culture. Six of the highly effective mutants were tested on unsterilized soil in controlled growth chamber. The correlation data on soil experiment displayed that nodule number was positively associated and significant (r=0.73, p<0.0001) with nodule dry weight (NDW) while shoot dry weight (SDW) was positively correlated with present N (r=0.8, p<0.0001) and total nitrogen content (r=0.9, p<0.0001). Physiological test of mutants also showed that- 5 (63%) and 3 (36%) of mutants were able to grow at salt concentrations of 11% and 12%, respectively. Notably, 3 (38%), 4 (50%), 2 (25%), and 2 (13%) of the mutants were able to grow at pH 4, 11, 11.5 and 12, respectively. Only the mutant isolates HUCRM2D (which tolerated 12% NaCl, pH4, and pH12), HUCRM5C (which tolerated 12% NaCl and pH 4), HUCRM3B (which tolerated 12% NaCl) and HUCRM9C (which tolerated 11% NaCl) were growing successfully at the indicated extreme conditions. Thus, on the basis of their symbiotic effectiveness and tolerance to extreme environmental conditions, these mutant isolates were recommended to be used as candidates for future development of rhizobial inoculants of common bean grown under saline and extreme pH conditions.

Keywords: Extreme soil conditions; Mutant *Rhizobia*; Symbiotic effectiveness; Tolerance

Introduction

Biological nitrogen fixation is one of the most vital processes used in the ecosystem to make available nitrogen for all living organisms. The major N_2-fixing systems are the symbiotic systems, which can play important role in improving the fertility and productivity of arid and semi-arid soils. The legume–rhizobium symbiosis is a classic example of mutualism where the rhizobia supply ammonia or amino acids to the plant and in return it receives organic acids (principally as the dicarboxylic acids malate and succinate) as a carbon and energy source. However, the performance of this association due to severe environmental conditions such as salt stress, high temperature, drought and acidity is highly hampered. These major stress factors suppress the growth and symbiotic characteristics of most rhizobia; however, several strains, distributed among various wild [1] and few mutant [2], species of rhizobia are showing tolerance to these effects. In addition to searching rhizobia that are tolerant and effective in extreme soil conditions, mutagenesis can generate better strains fastest capable functioning well at extreme abiotic conditions. The successful isolation, characterization and identification of rhizobium that have the desired characteristics through mutagenesis are not an easy task. To make the job done, first it is very important to identify the specific type of mutagen that suit for the particular type of bacteria. This is because one mutagen that is effective for one species of bacteria may not be effective for other species. Although the efficacies of a variety of mutagens for Rhizobium spp. have been reported [3], a comparison of results obtained with different mutagens is often difficult for a number of reasons. Primarily, the work has usually been carried out with different species or strains of *Rhizobium* under different experimental conditions. Moreover, the effectiveness of the mutagens has often been determined by detection of auxotroph using a non-selective procedure which is essentially non-quantitative. Furthermore, spontaneous mutation frequencies, with which the induced frequencies must be compared, are rarely given. Understanding this it is reasonably simple to mutate the identified wild rhizobium with a specific mutagen accurately in standardized laboratory. Then collecting the survivors (now mutants) and testing them for tolerance to extreme soil conditions is followed. If they are tolerant the symbiotic effectiveness test will be conducted according to the standard procedures. Almost no work has been done on evaluating the symbiotic effectiveness of rhizobium mutants on extreme soil conditions around to Babile, eastern Ethiopian. So, this study was undertaken with the aim of examining the symbiotic effectiveness of common bean nodulating mutant rhizobia around this region of Ethiopian.

Materials and Methods

Designation of the isolates

Each isolate was designated as HUCRM (Haramaya University Common bean *Rhizobium* Mutant) for the mutant isolates followed by a number (represent Kebele) and a letter (represent Genda or Village).

Mutagenesis

Chemical mutagenesis: Hydroxylamine hydrochloride and sodium azide were used as chemical mutagenic agents to induce mutation in *Rhizobium* isolates as described by O'Connel et al. [4]. Late exponential phase cultures (approximately 2×10^8 cfu ml^{-1}) were used in all mutagenesis experiments. The cells of rhizobial isolates were

***Corresponding author:** Mulugeta Mekonnen, Ethiopia Institute of Agricultural Research Center (EIAR), National Agricultural Biotechnology Research Center, Holetta, Ethiopia, Tel: + 251 0983506301; E-mail: mulebiotec@gmail.com

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first pelleted in an eppendorf micro centrifuge, washed once with phosphate-buffered saline (0.876 gm NaCl, 0.522 gm K_2HPO_4, 0.136 gm KH_2PO_4 per 100 ml), and re-suspended to the original volume in phosphate-buffered saline solution. From the stock solution of each mutagenic substance (1.17 g/ml), 0.0, 100, 200 and 300 µl was added into each ml of rhizobial culture suspension. After mixing with a vortex, the cells were incubated at room temperature for 60 minutes. The mixture was then diluted and spread plated on TY agar medium without salt. After three days of incubation at 28 ± 2°C, colonies of *Rhizobium* were observed and only those colonies that were grown in all the three different mutagenic chemical concentrations were selected as true mutants.

Physical mutagenesis: Physical mutagenesis was carried out according to the method described by Miller [5]. Late exponential phase cultures (approximately 2×10^8 CFU ml^{-1}). Pure culture of each *Rhizobium* isolate was first pelleted in an eppendorf micro centrifuge, washed twice; and re-suspended in phosphate buffer. The cell suspension (5 ml) was spread thinly on an open glass Petridis and exposed for 15 seconds to UV radiation at 2000 µw/cm^2 intensity. Each irradiated suspension was streaked on a Petridis containing TY agar media and incubated at 28°C for three days. The surviving colonies were transferred to other fresh media for preservation and further physiological tests. Thus, further screening was made to select desired mutants based on their ability to withstand extreme pH conditions, high salt concentrations, and temperatures higher than the usual.

Characterization of mutants' colony morphology

The mutants were also characterized by colony type, color and size by streaking a loopfull of 72 hours old culture (10^6 cells/ml) on TY media incubated at 28°C for 3-5 days [6].

Determination of symbiotic effectiveness of mutant *Rhizobial* isolates

The symbiotic effectiveness of the mutant rhizobial isolates on sterilized sand and unsterilized soils was determined using pot experiments in a growth chamber at Haramaya University Agronomy laboratory according to Subba Rao [7]. Percentage symbiotic effectiveness of isolates was calculated according the equation proposed by Lupwayi and Haque, [8] and described in section 3.8.1. Symbiotic effectiveness was then classified as in the classification used for the non-mutant isolates, i.e., ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80% [9].

Selection of salt and pH tolerant mutant *Rhizobium*

Serial dilution of pure culture of selected mutants was prepared. Following this, aliquots of the appropriate dilutions were spread on sterile TY agar media containing various concentrations of sodium chloride (9%, 10%, 11%, 12%, 14%, 16% and 18%) and measuring different levels of pH (pH 1-10) to test the levels of salt and pH tolerance of the test strains. Colonies that appeared on TY agar media containing concentrations of NaCl higher than the maximum required for the wild type *Rhizobium* (i.e., 10%) and colonies on media adjusted to pH values lower than the minimum (pH 4) or higher than the maximum (pH 10.5) required for the wild type *Rhizobium* were selected as salt and pH tolerant mutants, respectively [10,11].

Data analysis

Comparison between treatments was analysed 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.

Treatment	Salt tolerance (%)			pH range			
	11	12	14	3.5	4	11	11.5
HUCRM2D	+	+	-	-	+	+	+
HUCRM5C	+	+	-	-	+	+	-
HUCRM7D	-	-	-	-	-	+	-
HUCRM9A	+	-	-	-	-	-	-
HUCRM9C	+	-	-	-	-	-	-
HUCRM12E	+	-	-	-	-	-	-
HUCRM3B	+	+	-	-	-	-	-
HUCRM14A	+	-	-	-	+	+	+
% of tolerated mutant isolates	87.5	36	0	0	38	50	25
HUCRM=Haramaya University Common Bean Rhizobium Mutant							

Table 1: Salt, pH and tolerance of *Rhizobium* mutant isolates.

Treatment	Nodule number	Nodule dry weight	Shoot dry weight	SE (%)	Effectiveness
HUCRM2D	131 ± 11^a	0.21 ± 0.04^a	1.4 ± 0.4^a	233	HE
HUCRM5C	111 ± 11^b	0.2 ± 0.00^a	1.2 ± 0.1^b	200	HE
HUCRM7D	86 ± 31^c	0.2 ± 0.01^a	1.2 ± 0.1^b	200	HE
HUCRM9A	85 ± 1^c	0.2 ± 0.01^a	0.8 ± 0.1^c	133	HE
HUCRM9C	74 ± 1^c	0.08 ± 0.00^{b,c}	0.7 ± 0.0^c	117	HE
HUCRM12E	68 ± 1^c	0.08 ± 0.00^b	0.8 ± 0.0^c	133	HE
HUCRM3B	77 ± 1^c	0.1 ± 0.00^b	0.7 ± 0.0^c	117	HE
HUCRM14A	68 ± 1^c	0.1 ± 0.00^c	0.8 ± 0.0^c	133	HE
Control$^-$	0^d	0^d	0.3 ± 0.0^d	--	--
Control$^+$	0^d	0^d	0.6 ± 0.0^c	--	--
LSD (p<0.05)	18.328	0.0275	0.2469	--	--
Numbers in the same column followed by the same letters are not significantly different at p < 0.05 (Fisher's LSD). SE=Symbiotic Effectiveness; E=Effective and HE=Highly Effective; LSD=List Significant Difference; %SE=>80% is highly effective; 50-80% is effective.					

Table 2: Nodulation data of *Rhizobium* mutants on sterilized sand.

Treatment	Nodule number	Nodule dry weight	Shoot dry weight	Plant total nitrogen (%)	N Content (g/pl)
HUCRM2D	136 ± 39^{a}	0.2 ± 0.01^{a}	3 ± 0.5^{a}	2.7 ± 0.6^{a}	0.08 ± 0.01^{a}
HUCRM5C	106 ± 20^{b,c}	0.17 ± 0.04^{a-c}	2.9 ± 0.6^{a-c}	2.4 ± 0.6^{a-c}	0.07 ± 0.02^{a}
HUCRM7D	99 ± 21^{b,c}	0.15 ± 0.1^{c}	3.4 ± 0.6^{c}	2.6 ± 0.6^{a,b}	0.09 ± 0.02^{a}
HUCRM9A	100 ± 1^{b,c}	0.16 ± 0.00^{b,c}	1.9 ± 0.01^{b,c}	2 ± 0.01^{c,d}	0.04 ± 0.00^{b}
HUCRM12E	121 ± 1^{a,b}	0.19 ± 0.02^{a,b}	1.6 ± 0.01^{a,b}	2.3 ± 0.1^{a-c}	0.04 ± 0.00^{b,c}
HUCRM14A	95 ± 1^{b,c}	0.15 ± 0.00^{b,c}	1.9 ± 0.01^{b,c}	2.1 ± 0.1^{b-d}	0.04 ± 0.00^{b}
Control^{-}	91 ± 1^{c}	0.15 ± 0.00^{c}	1.6 ± 0.01^{c}	1.6 ± d0.01^{d}	0.02 ± 0.00^{c}
Control^{+}	77 ± 1^{c}	0.17 ± 0.00^{a-c}	1.9 ± 0.01^{a-c}	2.4 ± 0a-c.01^{a-c}	0.03 ± 0.01^{b,c}
LSD (p<0.05)	29.41	0.04	0.59	0.61	0.02
Numbers in the same column followed by the same letters are not significantly different at p < 0.05 (Fisher's LSD).					

Table 3: The effect of *Rhizobium* infection on the performance of common bean plants.

Description	Nodule number/nodule dry weight*		Shoot dry weight/N content*		Shoot dry weight/%N	
Mean	103.1	0.17 †	2.27	2.27 †	2.27	0.05 †
Std Dev	22.5	0.03 †	0.74	0.5 †	0.74	0.02 †
Minimum	76	0.09 †	1.5	1.6 †	1.5	0.02 †
Maximum	175	0.22 †	4	3.3 †	4	0.1 †
r value	0.72*		0.95*		0.78*	
*=Significance at p<0.001; 0.215†=Represents values for the 2nd parameter in a column						

Table 4: Correlation coefficient of selected parameters in soil experiment for mutant *Rhizobium*.

Results and Discussion

Isolation of mutant *Rhizobium* cells

Among the 50 presumptively tested and authenticated wild *rhizobium* isolates following the physical and chemical mutagenesis, a total of 65 survivors (22 from sodium azid, 24 from Hydroxylamine hydrochloride and 19 from uv) were isolated at Haramaya University Microbiology laboratory and Plant Science Pathology Laboratory (Appendix 2). Out of these, only 8 of the survivors have been selected as mutants for further studies based on their ability to survive at extreme conditions such as high salt concentrations and pH. Isolates HUCRM (2D, 5C, 7D, 9A, 9C, 12E) were mutants that developed from chemical mutagenesis. Among these, chemically induced mutant isolates HUCRM (2D, 9C, 5C) and HUCRM (7D, 9A, 12E) were induced through the effects of mutagenic chemicals sodium azide and hydroxylamine hydrochloride, respectively. HUCRM 3B and HUCRM 14A were the other mutant isolates that were obtained through physical mutagenesis at pathology laboratory. All chemically induced rhizobium mutants were isolated from Babille soils while the UV induced mutants were from both Fedis and Gursum, respectively.

Characterization of *Rhizobium* mutant isolates

Morphology and cultural characteristics of *rhizobium* mutant isolates: The colony morphology of the *rhizobium* mutant strains on TY agar plates were comparatively less mucoid and less elastic than that of the wild type on YEMA. In terms of size, the *rhizobium* mutant on solid TY agar media forms large spherical colonies than it was on YEMA medium after incubation for about 24 h. Similarly, Elizabeth et al. [12] and Kathryn et al. [13] reported that the rhizobium mutants showed less mucoid and less elastic morphology than that of the wild types. From this morphological difference between wild and mutant *rhizobium* it is possible to understand that the loss of elasticity and mucoidnes in mutant isolates may be due to the impact of the mutagens used during the course of mutagenesis.

Physiological characterization of *Rhizobium* mutant isolates

Salt tolerant *Rhizobium* mutants: From Table 1 below it is possible to observe how many of the mutant *Rhizobum* were tolerant to different sodium chloride concentrations on TY agar medium. 87.5% of the mutant isolates were successful to grow on a TY agar medium containing 11% NaCl, similarly, 36% and 0% of the isolates were tolerant to the same medium containing 12% and 14% of NaCl concentration. Mutant isolates like HUCRM (2D, 5C, 3B) were the most tolerant *Rhizobium* that grew on the entire medium containing different NaCl concentration except at 14% of NaCl. The most sensitive mutant isolate was HUCRM 7 D, which did not, grew in any of the given percentages of NaCl concentration. Isolates HUCRM (9A, 9C, 12E, 14A) were the next other sensitive mutant rhizobia that grew only at 11% of NaCl concentration on TY agar medium.

pH tolerance *Rhizobium* mutant isolates: In this study, 0%, 38%, 50%, 25%, 13% of the isolates were tolerated at pH of 3.5, 4, 11, 11.5, and 12 on sterile TY agar media. Isolate HUCRM2D were tolerated all the provided pH except at pH 3.5 and HUCRM14A was the second most tolerant isolate that tolerated all the given pH except at pH 3.5 and 12 (Table 1). The most sensitive mutant isolate that tolerated narrow ranges of pH condition were HUCRM9C and HUCRM5C all of them were isolated from wild rhizobium isolates of Babille soil. Mutant isolates HUCRM (7D, 9A, 12E and 3B) were not tolerated any of the given pH conditions.

Evaluation of symbiotic effectiveness of common bean *Rhizobium* mutant isolates: The symbiotic effectiveness of rhizobum mutants were evaluated at Haramaya University Agronomy laboratory under pouch experiment in growth chamber on both serialized sand and unsterilized soil.

Evaluation of symbiotic effectiveness of common bean *Rhizobium* mutant

Isolates under sterilized sand in pouch experiment: All of the mutant isolates were tested for their nodulation and relative symbiotic effectiveness on common bean Gofta (G-2816) variety in sterile pouch using sterilized sand culture, and they were efficient in nodulation and symbiotic effectiveness. Inoculated common bean showed significant (p<0.05) increase in all parameters investigated in this study as compared with the uninoculated negative control (Table 2). Concerning the outward appearances inoculated plants were

visibly different from the negative control. The control plant appeared relatively shorter, less dark green than the inoculated individuals. These indicate that the inoculated plants fixed atmospheric nitrogen well. In this study, the smallest nodule number record was 68 per plant for a plant inoculated with mutant isolates HUCRM12E and HUCRM14A. The highest nodules number record were 131 and 111 per plant for plants inoculated with isolates HUCRM2D and HUCRM5C correspondingly. Similarly in nodule dry mass 0.08g/plant was the smallest and 0.21g/plant was the highest record for plants inoculated with isolates HUCRM (9C,12E) and HUCRM2D respectively. The highest shoot dry matter accumulations 1.4 g/plant were recorded from the plant inoculated with isolates HUCRM2D from Babille soil and the least 0.7g/plant were recorded from the plant inoculated with isolates HUCRM3B and HUCRM9C from Fedis and Babille soil respectively. Furthermore, in this study, all isolates resulted in accumulation of shoot dry matter higher than the positive control (N^+). Plants inoculated with isolate HUCRM2D showed significantly ($p<0.05$) higher nodule number, nodule dry weight and shoot dry weight than any of the other plants. The superiority of isolate HUCRM2D could more likely be due to the contribution of *Rhizobium* species in increasing the biomass through plant growth promoting hormone production such as auxins and indole acetic acid beyond N fixation [14]. Correlation response among variables in the sand experiment for mutant rhizobia confirmed that nodule number were related positively and significantly ($r=0.85$, $p<0.0001$ with nodule dry weight. Similar correlation response was documented by Khondaker et al. [15] and Kassa Baye [16] who reported a correlation index of ($r=0.68$; $r=0.32$, $p<0.01$) for the association of nodule number with nodule dry weight with regard to inoculation of pea varieties. Concerning the symbiotic effectiveness, 100% of the mutant isolates were found to be highly effective (Table 2). This may show us the existence and abundance of indigenous wild *rhizobum* around Babile area which can be induced chemically and become effective in nodulation and biological nitrogen fixation of legumes.

Evaluation of symbiotic effectiveness of common bean rhizobium mutant isolates

Under unsterilized soil in pouch experiment: Six highly effective isolates HUCRM2D, HUCRM5C, HUCRM7D, HUCRM9A, HUCRM12E, and HUCRM14A from the sand culture were selected as inoculants for common bean and tested on Babille soil under growth chamber. The data showed that the different rhizobial inoculants displayed variation in nodule number, nodule dry weight, shoot dry weight, plant total nitrogen and N content of the inoculated common bean plants (Table 3). Isolates HUCRM (2D, 5C, 7D) showed significantly ($p<0.05$) higher N content than other plants. Isolate HUCRM2D induced the highest nodule number of 136 per plant followed by isolate HUCRM12E, HUCRM5C and HUCRM9A with nodule number of 121 per plant, 106 per and 100 per plant respectively. The lower nodule number was induced by plants inoculated with HUCRM14A with 95 nodules per plant. The highest nodule dry weight of 0.2 g per plant was induced by isolate HUCRM2D. On the other hand, the lowest nodule dry weight of 0.15 g per plant was recorded by plants inoculated with isolates HUCRM7D and HUCRM14A. This study (Table 4) showed that positive correlations were observed with respect to the number of nodules and dry weight of nodules ($r=0.73$, $p<0.0001$), dry weight of shoot and %N ($r=0.8$, $p<0.0001$), dry weight of shoot and N content ($r=0.9$, $p<0.0001$). In addition to nodule number and nodule dry weight, it was also observed that dry weight of shoot was influenced by inoculation of isolates (Table 3). The highest dry weight of shoot 3.4 g per plant was recorded with isolate HUCRM7D, which was 46% and 54% higher than the records in shoot dry weight of positive and negative controls, respectively. On the other hand, the lowest dry weight of shoot 1.6 g per plant was recorded with a plant inoculate isolate HUCRM12E.

Summary and Conclusion

Among the 50 identified rhizobia isolates exposed both for chemical and physical mutagenesis totally eight mutants were induced, HUCRM (2D, 5C, 9C), HUCRM (7D, 9A, 12E) and HUCRM (3B, 14A) were mutant isolates that induced through Sodium Azid, Hydroxylamine Hydrochlorid and UV respectively. Among these isolates common bean plants inoculated with mutant isolate HUCRM2D produced the maximum nodule number 131 per plant whereas the minimum number of nodules recorded was 68 per plant for the isolates HUCRM (12E, 14A). The highest and lowest dry weight of shoots was recorded for plants inoculated with HUCRM2D (1.4 g per plant) and HUCRM (3B, 9C) (0.7 g per plant), respectively. Plants inoculated with isolate HUCRM2D showed significantly ($p<0.05$) higher nodule number, nodule dry weight and shoot dry weight than any of the other plants. Depending on their shoot dry weight in reference to the N-fertilized control plant, the isolates displayed variation in effectiveness ranging from 117% to 267%. In this study, isolates were also found to have diversity in their response to various physiological responses. Isolates were relatively very sensitive to concentrations of NaCl beyond <11%. The above-mentioned results evidently made known the chance of having effective common bean rhizobial mutant isolates through chemical and physical mutagenesis. Correlation response among variables in the sand experiment for mutant rhizobia confirmed that nodule number were related positively and significantly ($r=0.85$, $p<0.0001$) with nodule dry weight. Symbiotic effectiveness (SE) test of six best mutant isolates was also carried out in Babile soil. Isolate HUCRM7D showed significantly ($p<0.05$) higher N content per plant. The highest dry weight of shoot 3.4g per plant was recorded with isolate HUCRM7D, which was 46% and 54% higher than the records in shoot dry weight of positive and negative controls, respectively. Positive correlations were observed with respect to the number of nodules and dry weight of nodules ($r=0.73$, $p<0.0001$), dry weight of shoot and %N ($r=0.8$, $p<0.0001$), dry weight of shoot and N content ($r=0.9$, $p<0.0001$). Among the highly tolerant mutants isolates HUCRM2D (which tolerated 12% NaCl, pH4, pH12), HUCRM5C (which tolerated 12% NaCl and pH 4), HUCRM3B (which tolerated 12% NaCl) and HUCRM9C (which tolerated 11%) are highly recommended for use as candidates of common bean inoculants upon further testing on field conditions.

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