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EFFECT OF CHEMICAL MUTAGENESIS ON SALT TOLERANCE OF COMMON BEANS (*PHASEOLUS VULGARIS* L.) NODULATING *RHIZOBIUM*

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Abstract

The chemical mutation is one of the important factors in the enrichment of the salt resistance capacity of the rhizobium isolates. Therefore, this study focused on 10 isolates of rhizobium collected from soil samples of Babile, Hararghe region, Ethiopia. All the collected isolates turned into a moderately yellow, yellow, and deep yellow color in yeast extract mannitol agar medium (YEMA) containing bromothymol blue after 48 h of incubation. It indicated that all the isolates were acid-producing *Rhizobia*. Moreover, based on colony morphology and diameter, 70% of the isolates displayed large mucoid colonies, and 30% of the isolates showed large watery colonies in YEMA media. Among the 10 isolates, 9(90%), 9(90%), 6(60%), 6(60%), 2(20%), and 1(10%), were grown at different salinity levels such as 2%, 4%, 6%, 8%, 9% and 10% of NaCl, correspondingly. The most salt-resistant wild isolate was HUCR 6 collected from Babile soil grown at 10% NaCl salinity level. A total of six mutants were considered after chemical mutagenesis based on their capacity to survive in the extreme salinity levels (11 to 14%). Mutant isolates such as HUCRM 4, HUCRM 5, and HUCRM 6 were the most tolerant *Rhizobium* that grew at the salinity level of 11 to 14% NaCl. The most sensitive mutant isolate was HUCRM 10 followed by the isolates HUCRM 8 and HUCRM 9 was the next sensitive mutant *Rhizobia* that grew only at 11% of NaCl concentration. Compared to the *Rhizobium* wild isolates, the mutant isolates were observed to be more tolerant to a medium containing higher concentration of NaCl, as high as 11% to 14%. Besides, 80% of the mutant isolates demonstrated effective nodulation with the common beans. The mutant isolate (HUCRM 4) showed better performance in relation to root nodule performance of *Rhizobium* species and increased the plant biomass production. In this study, mutant isolates HUCRM 6, which is tolerated to 14% NaCl, and HUCRM 4, HUCRM 5, and HUCRM 6 isolates tolerated at 12% salinity level. Finally, based on their symbiotic efficiency and tolerance to extreme salt levels, these mutant isolates (HUCRM 4, HUCRM 5 and HUCRM 6) were encouraged to be used for the development of *Rhizobium* inoculants of common beans grown under extreme saline conditions.

Key words: Chemical mutagenesis, common bean, nitrogen fixation, nodulation, rhizobia, salt tolerance.

Introduction

Common bean (*Phaseolus vulgaris* L.) is considered an important cash crop in Ethiopia, it is one of the important protein sources for farmers in many lowland and mid-altitude zones in Ethiopia. It also plays a vital role in export earnings are estimated nearly 85% of export (Negash 2007). In Ethiopia, 9.5% of the total export value from agricultural source contributed by common bean and stand the third rank as an export commodity (FAOSTAT 2010). The common bean is a significant factor contributing to agriculture since its capacity to produce root nodules in symbiosis with rhizobia, which change atmospheric nitrogen into the useable form of ammonia and also add a significant amount of organic material. Therefore, addition of common bean into the cropping systems has the prospective to avoid the usage of chemical source nitrogenous fertilizer (Houngnandan et al. 2000).

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In arid and semi-arid regions soil salinity is one of the most significant ecological pressures pessimistically disturbing the production of legumes. High salinity causes an imbalance of osmotic stress and ionic toxicity which leads to the effect of the symbiotic association between the legume and *Rhizobium* bacteria. Moreover, high salt concentration affects the soil micro-biota due to the variation of osmotic pressure. Furthermore, salty-soils is not favorable for microbial growth and population, which leads to a lack of availability of nutrient contents and microbial activities (Hirsch et al. 2001). However, some *Rhizobia* such as *Sinorhizobium meliloti* can live in high salinity levels (200-300 mM NaCl) in culture and in the soil of their host legumes. Particularly, salt stress reduces cytosolic proteins like leghemoglobin, which leads to the affect of *Rhizobium* respiration and the fixation nitrogen process. According to Bordeleau and Prevost (1994), the harmful consequence of salt stress on N_2 fixation by legumes is directly associated with decreases in shoot and root nodule growth and dry weight. Innumerable bacterial species have adjusted in the salty environments by intracellular accumulation of organic solutes like osmolytes. These osmolytes are permitted to offset the dehumidification consequence of low water exertion in the medium but not to intrude with macromolecular structure or function. *Rhizobia* make use of this medium of bibulous stress adaption. Smith et al. (1994), Zahran (1991) elucidate that the Rhizobial cells adapted to elevated salt stress by altering their cellular morphology. Soil salinity is one of the severe risks to agricultural activity in arid and semi-arid regions. Moreover, 40% of the world's land surface can be characterized as having the potential for salinity problems, these areas are restricted to the tropics and Mediterranean regions. Consequently, the positive effect of *Rhizobium*-legume symbiosis under salty conditions desires the isolation and development of salt-tolerant *Rhizobium* inoculants (Bordeleau and Prevost 1994). Thus, the major objective of this study was to examine the role of chemical mutagenesis in the enhancement of tolerance to high salinity in common bean nodulating rhizobia isolated from soils of Babile, Hararghe region, Ethiopia.

Material and Methods

Soil sample collection

In this research, soil samples were collected from ten selected sites in Eastern Hararghe Highland, particularly in Babile District. The locations for soil collection were geo-referenced using the Universal Transverse Mercator (UTM) geographic coordinating system. Accordingly, 3 kg of soil samples were arbitrarily collected in sterile polyethylene bags. The basic sterilized safeguards were implemented to rectify the infection of the soil samples until used for pot experiments.

Initiation of nodulation

The sieved (0.2 mm particle size) sterilized soil samples were filled in plastic pots to stimulate nodulation *Phaseolus vulgaris* L. Five seeds of Gofta (G-2816) were sown in each pot, and the pots were irrigated every once in three days up to 45 days. The root was detached after 45 days, the plants were uprooted and collected root nodules were immediately kept in vials containing silica gel.

Isolation of *Rhizobia*

The collected root nodules were allowed for sterilization by using 70% ethanol after this treated with acidified $HgCl_2$ (0.15%) for 3 minutes according to Rao (1999). Root nodules were washed at least 10 times with sterile distilled water to remove the contaminants completely. The nodules were then placed in 0.1N NaCl solution with a few drops of sterile distilled water and crushed them with help of a glass rod for collecting milky fluid or suspension. After this, suspensions were placed on a YEMA medium plate and incubated for 3 to 5 days at 28^{o}C.

Purification and preservation of the isolates

The selected isolates were frequently sub-cultured for purity on YEMA medium in 0.3 % (w/v) $CaCO_3$ on YEMA and kept at 4ºC for future use (Rao 1998).

Gram staining

The selected isolates were subjected to gram stain for detection of gram-positive bacteria as specified by Lupwayi and Haque (1994). An alkaline broth test was also carried out to differentiate *Rhizobia* from the related contaminant genera (Rao, 1998) by growing bacterial isolates on an alkaline medium (K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.1 g; $CaCO_3$, 0.05 g; yeast extract 1.0 g; mannitol, 10 g; and water, 1000 ml; pH adjusted to 11.0 by adding approximately 28 ml of NaOH and 1ml of 1.6% Thymol blue).

Congo red absorption test

The selected isolates were streaked on the YEMA-Congo red (CR) and incubated at 28 ± 2ºC in the dark covered with aluminum foil for 3-5 days to detect Congo red absorption by the isolates (Vincent 1970). Congo red stock solution was prepared by dissolving 0.25 g in 100 ml of distilled water. From this, 10 ml was added to a liter of YEMA and autoclaved (at 121ºC for 15 minutes with16 pounds pressure per square inch).

Peptone glucose test

The selected isolates were streaked onto the peptone glucose medium, which was growing on yeast extract mannitol broth culture. The medium was prepared by mixing 10.0 g of peptone, 5.0 g of glucose, 15.0 g of agar, and 10 ml of Bromcresol Purple in one liter of distilled water and the pH was adjusted to 6.7 (Lupwayi and Haque 1994). Finally, the culture was incubated at 28ºC for 3-5 days.

Designation of the isolates

The isolates were named HUCR and HUCRM (Haramaya University Common bean *Rhizobium*) for the wild and mutant isolates respectively, followed by different numbers.

Characterization of the *Rhizobium* isolates

48 hour old broth culture of *Rhizobium* isolates was streaked on YEMA medium and incubated at 28±2ºC for 3-5 days (Somasegaran and Hoben 1994). After 5 days, colony diameter, texture, and color were recorded as large mucoid (LM), large watery (LW), small dry (SD), elastic, and buttery, as indicated in Martinez-Romero et al. (1991).

Acid-base production

A loop full of each isolate from 48 hours old culture broth was streaked on the YEMA–BTB (medium and incubated for 3-7 days for determination of acid or alkaline production by *Rhizobium* isolates.

Chemical mutagenesis

Chemical mutagenic agents (hydroxylamine hydrochloride and sodium azide) were used to induce mutation described by O'Connell et al. (1990). The late exponential phase cultures (approximately 2 × 108 CFU/ml) were used for chemical mutagenesis. The selected *Rhizobium* isolates were first pelleted by using micro-centrifuge, washed with phosphate-buffered saline solution (0.876 g NaCl, 0.522 g K_2HPO_4, 0.136 g K_2HPO_4 per 100 ml), and again mixed and make into the original volume in phosphate-buffered with saline solution. Finally, different concentration of (0.0, 100, 200, and 300 µl) mutagenic substance was added to each ml of Rhizobial culture. The mixture was mixed with the help of a vortex after proper mixing the suspensions were subjected to incubation for 60 minutes under room temperature. After 3 days of incubation at 28±2ºC,

colonies of *Rhizobium* detected in different concentrations of chemical mutagenesis were carefully chosen as survivors.

Determination of salt tolerance of *Rhizobium*

Determination of salt resistance based on various salinity levels such as 2%, 4%, 6%, 8%, 9%, 10%, 11%, and 12% (Ahmed et al. 2010) were prepared and inoculated in YEMA medium plates with appropriately diluted pure cultures. The plates were then incubated at 30ºC for four days. Finally, the growth of the Rhizobial strains was assessed as negative (-) for no growth and positive (+) for growth (Lupwayi and Haque 1994).

Selection of salt tolerant *Rhizobium* mutants

The selected mutants were prepared for pure culture by using serial dilution methods. Afterward, suitable dilutions were spread on sterile tryptone yeast agar media containing 11, 12, and 14% salinity levels to test the levels of salt tolerance.

Evaluation of symbiotic effectiveness of common bean *Rhizobium* in sterilized sand culture

The selected mutant isolates that were capable of the presumptive test were further tested for confirmation. The sandy soil was collected from the riverside and washed in water and kept for two days with concentrated sulfuric acid. After this, several times soil was washed by using distilled water to remove traces of the acid and then subjected to autoclaving for 90 minutes to remove all the contaminants form the soil sample. Finally, plastic pots (3 kg capacity) were surface sterilized with 95% ethanol before filling the soil (Lupwayi and Haque 1994). After this, sterilized seeds were transferred to petri dishes containing distilled water and incubated at 25ºC for 3 days for germination. Five pre-germinated seedlings were transferred into each pot, each seedling was inoculated with 1ml culture of isolate adjusted to an inoculum size of 109 cells/ml. After the 3rd leaf emerged seedlings were thinned to three in each pot and the seedlings were maintained (Somasegaran and Hoben 1994). After 45 days of sowing, the plants were collected and their roots were counted for nodulation. The shoots of plants, as well as the nodules, were then oven-dried to determine their dry weight at 70ºC for 48 hours. The symbiotic effectiveness (SE) of the isolates was by the following equation:

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

According to Beck et al. (1993), the symbiotic effectiveness (SE) of isolates was ranked as ineffective (<35%), less-effective (35-50%), effective (51-80%), and highly effective (>80%).

Result and Discussion

Isolation and presumptive test for identification of *Rhizobium*

In this study, a total of 10 isolates were changed their color after 48 hours of incubation into a medium yellow, yellow, and dark yellow. This indicated that all the selected isolates were acid-producing *Rhizobia*. In line with this Argawe (2007) also reported that *Rhizobium* spp. growing on yeast extract mannitol agar medium containing bromothymol blue turns to yellow after 3-5 days of incubation. However, Congo red was not absorbed by all the isolates on the YEMA-CR medium, subsequently; these isolates were not grown on peptone glucose agar (PGA) which is indicated by rod-shaped, gram-negative bacteria.

Authentication of *Rhizobia*

All of the 10 isolates also produced nodules and were authenticated as root nodule bacteria (Vincent 1970).

Morphology, cultural characteristics and generation time of the isolates

Colony morphology, texture, color, margin, shape, and diameter were the foundations used to characterize all isolates (Table 1). Almost all isolates showed a smooth margin, round colonies shape varying from flat to domed and even conical, a shape with a white-opaque, milky, and watery translucent color on the agar surface. In this study, five isolates were established their colony diameters in the range of 1.5 - 2.5 mm, four isolates showed a range of 3.0 - 3.5 mm, and one isolate was found at 5.5 mm, respectively. On the basis of colony characteristics and diameter with YEMA medium 30% of the isolates exhibited large watery (LW) colonies and 70% of the isolates displayed large mucoid (LM) colonies. However, after frequent re-streaking, a few of the isolates formed a second form of colonies which were a bit less than the colonies produced by the parent cultures but with the same texture. In line with this, Somasegaran and Hoben (1994) explained that after 6-7 days, the colonies of the fast growers were between 1.0 and 5.0 mm in diameter. Moreover, isolates of HUCR 5 from the Babile district showed the largest diameter of 5.5 mm. whereas; isolates HUCR (7, 8, 9) showed the smallest diameter of 1.5 mm in the study area.

Table 1. Cultural characterization of common bean Rhizobial isolates from Babille, grown on YEMA-CR and YEMA-BTB media.

Isolates	Colony morphology	Colony texture	Growth on YEMA-BTB	Colony diameter (mm)	Altitude (m.s.l)	Soil pH
HUCR 1	LM	Elastic	Yellow	2.5	1546	7.6
HUCR 2	LM	Buttery	Yellow	3.0	1538	6.9
HUCR 3	LM	Buttery	Yellow	3.5	1452	8.4
HUCR 4	LM	Buttery	Yellow	3.0	1448	8.3
HUCR 5	LM	Buttery	Yellow	5.5	1438	7.9
HUCR 6	LM	Elastic	Yellow	2.5	1536	8.3
HUCR 7	LM	Elastic	Yellow	1.5	1534	7.7
HUCR 8	LW	Elastic	Yellow	1.5	1538	7.9
HUCR 9	LW	Elastic	Yellow	1.5	1540	7.9
HUCR 10	LW	Elastic	Yellow	3.0	1562	7.9

LM = Large mucoid, LW = Large watery.

Salt tolerance

The salt resistance isolates showed different growth patterns on the YEMA medium adjusted to different salinity levels (Table 2). In this research, 10 isolates, 9(90%), 9(90%), 6(60%), 6(60%), 2(20%), and 1(10%), were grown at 2%, 4%, 6%, 8%, and 9% of NaCl, correspondingly. In Babille soil especially, isolate HUCR 6 showed the highest salt tolerance at 10% salinity level. In addition to this, isolates HUCR 1, HUCR 2, HUCR 3, HUCR 4, HUCR 5, HUCR 6, HUCR 7, HUCR 8, and HUCR 10 were found to be within a narrow range of salt tolerance up to 2% of NaCl salinity level and HUCR 9 isolates were the most sensitive isolates which did not grow at 2% salinity level. Argaw (2007) elucidated that frequently fast-growing *Rhizobia* grew well between 3-5% concentrations of NaCl. Therefore, it is clear that *Rhizobium phaseoli* are among the salinity-resistance *Rhizobia* and a number of isolates have been reported to tolerate high salt concentrations between 4%-5%. Similarly, Workalemahu (2006) also reported salt isolates from southern Ethiopian soil.

Table 2. Tolerance of the wild isolates to different salt concentrations.

Isolates	Salt concentrations								
	2%	4%	6%	8%	9%	10%	11%	12%	Salt tolerance range
HUCR 1	+	+	-	-	-	-	-	-	2-4
HUCR 2	+	+	-	-	-	-	-	-	2-4
HUCR 3	+	+	+	-	-	-	-	-	2-6
HUCR 4	+	+	+	+	-	-	-	-	2-8
HUCR 5	+	+	-	-	-	-	-	-	2-4
HUCR 6	+	+	+	+	+	+	-	-	2-10
HUCR 7	+	+	+	-	-	-	-	-	2-6
HUCR 8	+	+	+	-	-	-	-	-	2-6
HUCR 9	-	-	-	-	-	-	-	-	-
HUCR 10	+	+	+	-	-	-	-	-	2-6

(+) = presence of growth, (-) = Absence of growth.

Isolation of mutant *Rhizobium* cells

In this study, 10 wild *Rhizobium* isolates were subjected to chemical mutagenesis, and after chemical mutation 6 isolated were identified as survivors (2 from sodium azide, 4 from Hydroxylamine hydrochloride). Additionally, based on their ability to grow in the highest salinity levels. The isolates such as HUCRM 4, HUCRM 5 (sodium azide) HUCRM 6, HUCRM 8, HUCRM 9, and HUCRM 10 (hydroxylamine hydrochloride) were developed from chemical mutagenesis by using sodium azide and hydroxylamine hydrochloride.

Characterization of *Rhizobium* mutant isolates

In this experimental analysis, TY agar plates revealed the *Rhizobium* mutant strains colony morphology was relatively less mucoid and less elastic than that of the wild type on YEMA medium. This may be due to the impact of the mutagens. However, *Rhizobium* mutants confirm less mucoid and less elastic morphology than

that of the wild types reported by Elizabeth et al. (2011). Correspondingly, the size of the mutant colony on solid TY agar media forms larger spherical shape colonies.

Salt tolerance of *Rhizobium* mutant isolates

The mutant isolates such as HUCRM 4, HUCRM 5, and HUCRM 6 showed the highest salt-resistance *Rhizobium* that grew in the medium containing different salinity levels like 11 to 14% NaCl. Among the isolates, HUCRM 10, is the most sensitive mutant isolate in NaCl salinity levels. Isolates HUCRM 8 and HUCRM 9 showed the next level of salt-susceptible mutant *Rhizobia* that grew only at 11% of the salinity level. Compared to the non-mutated *Rhizobium* isolates, the mutant isolates were identified to be more resistance to high concentrations of sodium chloride (11% to 14% of salinity levels). The wild isolates HUCR 6 showed the highest salt-tolerant at 10% NaCl in the YEMA medium. Hamid et al. (2011) suggested that Rhizobial mutants were tolerated 12% NaCl salinity level (Table 3). Furthermore, compared with mutant derivatives, wild isolates were sensitive to a higher salinity level.

Table 3. Salt tolerance of Rhizobial mutants isolates.

Treatment	Salt tolerance (%)		
	11	12	14
HUCRM 4	+	+	-
HUCRM 5	+	+	-
HUCRM 6	+	+	+
HUCRM 8	+	-	-
HUCRM 9	+	-	-
HUCRM 10	-	-	-

HUCRM = Haramaya University common bean *Rhizobium* mutant, + = presence of growth, - = Absence of growth.

Evaluation of relative symbiotic effectiveness of common bean *Rhizobium* mutant isolates under sterilized sand in pouch experiment

The results showed that significant ($p<0.05$) increase in mutant isolates of inoculated common beans in all the selected growth parameters studied. Related to the colony morphology, noticeably a change was observed from the control plate (Table 4). The *Rhizobium* inoculated plant showed comparatively dark green and increased height as compared with the un-inoculated plant. These may be due to the effect of *Rhizobium* and its contribution of nitrogen. However, mutant isolates HUCRM 8 showed less number of root nodules (68 per plant). While the mutant, isolates such as HUCRM 4 and HUCRM 5 showed the highest nodule numbers 131 and 111 per plant, respectively. Correspondingly, the nodule dry weight, 0.08 g/plant was the minimum in isolates such as HUCRM 8 and HUCRM 9. The highest nodule dry weight (0.22 g/plant) was reported with HUCRM 5 isolates. The lowest value of 0.7 g/plant were noticed from the plant inoculated with isolates HUCRM 9. Whereas the maximum shoot dry weight (1.6 g/plant) was noticed from the common bean plant inoculated with mutant isolates HUCRM 4.

The results showed that maximum nodule number, nodule dry weight, and shoot dry weight was reported in mutant isolate (HUCRM 4) inoculated plants (Table 4). According to Zafar-ul-Hye et al. (2007), this is may be

due to the effect of induction of mutation which increased the efficiency of *Rhizobium* and increasing the plant growth and development, especially promoted the plant growth hormones, such as auxins and indole acetic acid beyond N fixation process. Association response between variables in the soil culture for mutant *Rhizobia* established that nodule numbers were related positively and significantly ($r = 0.85$, $p<0.0001$) with nodule dry weight. Comparable result were explained by Khondaker et al. (2003) and Baye (2011) 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 relative symbiotic effectiveness, 100% of the isolates were found to be highly effective (Table 4). This might be due to the existence and abundance of indigenous wild *Rhizobium* around the Babile area, which can be induced chemically and become effective in nodulation and biological nitrogen fixation of legumes.

Table 4. Evaluation of symbiotic effectiveness of common bean *Rhizobium* mutant isolates under sterilized sand.

Treatment	Nodule number	Nodule dry weight	Shoot dry weight	SE (%)	Effectiveness
HUCRM 4	131±11^{a}	0.22±0.00^{a}	1.6±0.4^{a}	177	HE
HUCRM 5	111±11^{b}	0.14±0.04^{a}	1.2±0.1^{b}	133	HE
HUCRM 6	85±1^{c}	0.21±0.01^{a}	0.8±0.1^{c}	89	HE
HUCRM 8	68±1^{c}	0.08±0.00^{b}	0.8±0.0^{c}	89	HE
HUCRM 9	74±1^{c}	0.08±0.00^{b,c}	0.7±0.0^{c}	78	E
HUCRM 10	77±1^{c}	0.1±0.00^{b}	0.7±0.0^{c}	78	E
Control^{-}	0^{d}	0^{d}	0.4±0.0^{d}	-	-
Control^{+}	0^{d}	0^{d}	0.9±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 of significant difference, % SE = >80% is highly effective; 51-80 % is effective.

The mutant isolate HUCRM 6, which is tolerated to 14% NaCl, and HUCRM 4, HUCRM 5, and HUCRM 6 isolates tolerated at 12% salinity level. Moreover, based on their symbiotic efficiency and tolerance to extreme salt levels, these mutant isolates (HUCRM 4, HUCRM 5 and HUCRM 6) were encouraged to be used for the development of *Rhizobium* inoculants of common beans grown under extreme saline conditions.

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