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International Journal of Current Research
Vol. 6, Issue, 01, pp.4733-4738, January, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

EFFECT OF CHEMICAL MUTATION ON IMPROVEMENT OF RHIZOBIAL ISOLATE'S TOLERANCE TO ACIDIC AND ALKALINE SOIL CONDITION IN ETHIOPIA

Andarge Zelalem, Ameha Kebede and *Manikandan Muthuswamy

Department of Biology, Faculty of Natural and Computational Sciences, Haramaya University, Haramaya, Dire Dawa, Ethiopia

ARTICLE INFO

Article History:
Received 20th October, 2013
Received in revised form
08th November, 2013
Accepted 17th December, 2013
Published online 31st January, 2014

Key words:
Symbiotic effectiveness,
Mutagenesis,
Rhizobia,
Mutants,
Acidic,
Alkaline.

ABSTRACT

Background: The main aim of this study was to examine the effect of chemical mutagens on enhancement of extreme acid and alkaline condition of *Vicia* faba nodulating rhizobial isolates from Hararghe highlands in Ethiopia.

Methods: A total of 50 wild rhizobial isolates were isolated from different regions of Hararghe highlands soils by using pot experiment. All selected isolates showed significantly ($P \leq 0.05$) higher nodule dry weight (NDW) than the positive control. Above the 50 isolates only 10 highly performed isolates were subjected to chemical mutation. After the mutagenic treatment we selected only eight survived isolates, three isolates from sodium azide and five isolates form hydroxyl amine hydrochloride. The selected mutagen was subjected to study their symbiotic effectiveness.

Results: The sodium azide treated muatgens showed higher performance in terms of symbiotic effectiveness compared with hydroxyl amine hydrochloride. Chemical mutagen hydroxyl amine hydrochloride showed the acidic tolerance (pH 4) only three mutant isolates such as HUFBR12M4, HUFBR39M6 and HUFBR18M8. In case of sodium azide, it did not show any mutant isolates under acidic condition. In the meantime alkaline tolerance mutant isolates (HUFBR50M1, HUFBR31M2, HUFBR12M3, HUFBR12M4, HUFBR23M5, HUFBR39M6, HUFBR50M7, and HUFBR18M8) were observed in both the mutagenic chemicals (sodium azide and hydroxyl amine hydrochloride). All the isolates showed the pH range 10.5 to 12.

Conclusion: Finally, it was concluded that mutagenic chemical sodium azide is suitable for inducing chemical mutation to enhance acidic tolerance of mutant rhizobium in Faba bean and both the mutagenic chemicals (sodium azide and hydroxyl amine hydrochloride) are suitable for alkaline tolerance of mutant rhizobium in Faba bean.

INTRODUCTION

Soil acidification is, among the important environmental factors, emerging as an important land degradation issue. It is significant ecological process and one of the world's major soil management problems. In natural process, soil acidity is advanced weathering stage of soils and / or depletion of primary minerals in soils on geo-morphologically stable landforms in humid environments. It is a process by which soil pH decreases over time. Soils can be acidified under natural conditions over thousands of years especially in high rainfall areas. Moreover, they can also acidify rapidly over a few years under intensive agricultural practices (Richer and Markewitz 2001). The western and southern parts of Ethiopia, are dominantly covered by soils with pH<5.5(Schede 1989). In this area, the annual rainfall exceeds to potential evaporation. Similarly, the soil in areas such as Nedjo, Diga, Gimibi and Bedi in Oromiya, Chencha and Sodo in SNNP, and Gozamin and Senan region in Eastern Gojjam and Awi Zone in West Amhara region have acidic problems in the soil. Symbiotic nitrogen fixation is commonly limited by soil infertility, salinity, and acidity. Optimization of the benefits of legume inoculation with *Rhizobium* depends on the survival of rhizobia in soil. The introduction and persistence ability of a strains are affected by a number of abiotic factors like high salt, high water potential, high pH, and high temperature (Johri *et al*., 1999). The failure of legumes to nodulate under acid-soil conditions is common, especially in soils of pH less than 5.0. The inability of some rhizobia to persist under such conditions is one cause of nodulation failure (Bayoumi *et al.* 1995); in spite this elevated inoculation levels have enhanced the nodulation response under acidic conditions in some studies (Pijnenborg *et al*. 1991). The growth, nodulation, and yield of *V. faba* were improved after inoculation with strains of *R. leguminosarum bv. Viciae* in acid soils (Carter *et al.*, 1994). The amount of N_2 fixed by forage legumes on low-fertility acidic and alkaline soil is dependent on legume growth and persistence (Thomas *et al*. 1997). However, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic

***Corresponding author:** Manikandan Muthuswamy,*
Department of Biology, Faculty of Natural and Computational Sciences, Haramaya University, Haramaya, Dire Dawa, Ethiopia.

conditions will ensure the establishment of the symbiosis and also successful performance (Correa and Barneix 1997). Inoculation of *Vicia fab*a with efficient strains of the rhizobia has significant economic and ecological benefits in Ethiopia. In the present investigation, different rhizobia were isolated from nodules of *Vicia faba* (broad bean) growing on the soil of Harargh high lands in Haramaya University green house. The isolated rhizobia were assessed in their ability to grow in different acidic conditions; further more chemical mutagenesis was carried out to enhance tolerant rhizobia in acidic conditions. Thus the principal aim of this study was to examine the effect of chemical enhancement of tolerance to high acidic conditions in some Ethiopian broad bean nodulating *Rhizobium* bacteria.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from five major Faba bean growing districts of west and east Hararghe highlands. From each district 10 farm lands were selected, and from each farm land bulk samples from 10-15 cm depth were pooled, merged and collected in alcohol sterilized plastic bags. The collected samples were then transported to Haramaya University greenhouse for pot experiment. Upon arrival the soil samples were used for isolation of indigenous rhizobia by the host trap method (Somasegaran and Hoben 1994).

Collection of root nodules

Five seeds of *Vicia faba* (Faba bean), *Gachena* variety, which were obtained from Haramaya University research centre, were sown for each bulk sample in a sterile plastic pot at Haramaya University greenhouse. The pots were watered with distilled water every three days for 45 days. Then after 45 days the plants were uprooted from the pots and intact, pink, multi-lobed, and large nodules of plant were separated from the taproot with a portion of the root attached to the nodule and transported to the lab using a vial containing silica gel. The nodules were crushed with sterile glass rods in a large drop of water. The crushed nodule suspension was streaked on YEMA plates that contained Congo red. Then the plates were inverted and incubated at 28^{O}C for 3- 5 days (Lupwayi and Haque 1994). Presumptive tests were done by re-culturing the primary isolates into YEMA containing CR, peptone glucose agar (PGA), and acid alkaline production on BTB. Plates were examined for growth and single colonies were picked up and periodically purified by re-streaking on new YEMA plates. Pure isolates were then preserved on YEMA slants containing 0.3% (w/v) $CaCO_3$ and stored at 4^{O}C (Vincent 1970).

Chemical mutagenesis

Hydroxylamine hydrochloride and sodium azide were used as chemical mutagenic agents to induce mutation in ten selected *Rhizobial* isolates as described by O'Connel *et al*. (1990). Late-exponential phase cultures ($2x10^{8}$CFUml^{-1}) were used in all mutagenesis experiments. The cells of rhizobial isolates were first pelleted in an eppendorf microcentrifuge, washed once with phosphate buffered saline (0.876gm NaCl, 0.522gm K_2HPO_4, 0.136gm KH_2PO_4 per 100ml), and re-suspended to the original volume in phosphate- buffered saline solution. From the stock solution of each mutagenic substance (1.17g/ml), 0.0, 100, 200 and 300µl was added into each ml of rhizobial suspension. After mixing with a vortex, the cells were incubated at room temperature for 60 minutes. The mixture was then diluted and spread on TYEA medium without salt. The surviving colonies were transferred to other fresh TY slant media for preservation and further physiological tests. The mutants were selected based on their survival conditions.

pH Tolerance

The pH tolerance test was carried out on YEMA-medium according to Bernal and Graham (2001) and inoculated using inoculation loop containing about 10^{5}cells/ml and incubated at 28 ± 2^{O}C. Growth determined qualitatively as (+) for growth and (-) for no growth. The isolates were tested with the pH range of 4 - 10.5.

Symbiotic Effectiveness of Mutants on Sterilized Sand

In the determination of symbiotic effectiveness of mutants, the parameters such as number of nodules, nodule color, nodule dry weight and shoot dry weight were determined. A well washed, sulfuric acid immersed and autoclaved sand soil was filled into surface sterilized plastic pots (Subba Rao 1988). Surface sterilized (dipped into 95% ethanol and 35% H_2O_2 for 3 minutes) seeds of faba bean were planted (5 per pot). After a week, they were thinned to three and inoculated with 3 day's old 1ml of YEM broth culture (about 10^{8} to 10^{9} cells). The experiment was carried out in Haramaya Univeristy Horticulture laboratory using growth pouch and was laid out in complete randomized design (CRD) and replicated three times with two controls; the negative control (without N and *Rhizobium*) and the positive control (with 70 mg N liter^{-1} of distilled water). N was given as 0.05% KNO3 (w/v) by applying 120 ml during inoculation and 21 days later (Singleton and Tavares, 1986). All the pots were irrigated every day with distilled water and fertilized with quarter strength of Broughton and Dilworth N-free nutrient solution week^{-1} as described by Somasegaran and Hoben (1994). Forty five days after planting, screening was made using the parameters such as nodule number plant^{-1}, nodule color, nodule dry weight (mg plant^{-1}), shoots dry weight (g plant^{-1}) and symbiotic effectiveness (%) base. Nodule dry weight and shoot dry weights were determined by drying nodules and shoots in air and oven at 70^{O}C to constant weight and reported as mg and g plant^{-1}, respectively. The percent symbiotic effectiveness (SE) of the isolates was computed using the formula:

$$SE\ (\%) = \frac{\text{Shoot dry weight of innoculated plants}}{\text{Shoot dry weight of N supplied plant}} \times 100$$

(Beck *et al*., 1993)

Finally, the symbiotic effectiveness (SE) values of the isolates was rated as highly effective (>80%), effective (50-80%), less effective (35-50%), and ineffective for SE < 35% (Beck *et al*. 1993).

RESULTS

Isolation and presumptive identification test of the isolates

A total of fifty isolates were obtained from the nodules of faba bean plants grown at Haramaya University greenhouse. All isolates were found to be gram negative and did not absorb Congo red from YEMA-CR media. None of the isolates in this

Table 1. Determination of pH tolerance of the 50 wild Rhizobia isolates

Isolates	pH											
	4	4.5	5	5.5	6	7	8	8.5	9	9.5	10	10.5
HUFBR1	-	-	-	+	+	+	+	+	+	+	-	-
HUFBR2	-	-	-	+	+	+	+	+	+	+	-	-
HUFBR3	-	-	-	+	+	+	+	+	+	+	-	-
HUFBR4	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR5	-	-	-	+	+	+	+	+	+	+	-	-
HUFBR6	-	+	-	+	+	+	+	+	+	-	-	-
HUFBR7	-	-	-	+	+	+	+	+	+	-	+	-
HUFBR8	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR9	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR10	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR11	-	-	-	+	+	+	+	+	+	+	+	-
HUFBR12	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR13	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR14	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR15	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR16	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR17	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR18	-	+	+	+	+	+	+	+	+	-	-	-
HUFBR19	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR20	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR21	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR22	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR23	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR24	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR25	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR26	-	+	+	+	+	+	+	+	+	-	-	-
HUFBR27	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR28	-	-	-	+	+	+	+	+	+	+	+	-
HUFBR29	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR30	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR31	-	-	+	+	+	+	+	+	+	+	+	
HUFBR32	-	-	-	+	+	+	+	+	+	-	-	-
HUFBR33	-	-	+	+	+	+	+	+	+	+	+	-
HUFBR34	-	-	+	+	+	+	+	+	+	+	-	-
HUFBR35	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR36	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR37	-	+	+	+	+	+	+	+	+	+	+	-
HUFBR38	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR39	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR40	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR41	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR42	-	-	+	+	+	+	+	+	+	+	-	-
HUFBR43	-	-	+	+	+	+	+	+	+	+	-	-
HUFBR44	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR45	-	+	+	+	+	+	+	+	+	-	-	-
HUFBR46	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR47	-	+	+	+	+	+	+	+	+	-	-	-
HUFBR48	-	-	+	+	+	+	+	+	+	-	-	-
HUFBR49	-	-	+	+	+	+	+	+	+	+	-	-
HUFBR50	-	-	+	+	+	+	+	+	+	-	-	-

Table 2. Determination of pH tolerance of the survived mutant rhizobia isolates

Mutagen	Mutants Code	Acid Tolerance		Alkaline Tolerance				
		3.5	4	10.5	11	11.5	12	12.5
Sodium Azide	HUFBR50M1	-	-	+	+	+	+	-
	HUFBR31M2	-	-	+	+	+	+	-
	HUFBR12M3	-	-	+	+	+	+	-
Hydroxylamine	HUFBR12M4	-	+	+	+	+	+	-
Hydrochloride	HUFBR23M5	-	-	+	+	+	+	-
	HUFBR39M6	-	+	+	+	+	+	-
	HUFBR50M7	-		+	+	+	+	-
	HUFBR18M8	-	+	+	+	+	+	-

HUFBRM (HU, for Haramaya University, FBR, for Faba bean rhizobium, isolates number, M for mutation)

study showed growth on peptone-glucose agar (PGA). Failure of these isolates to absorb Congo red from YEMA-CR media and to grow on peptone glucose agar media presumptively indicated that they were root nodule bacteria (Lupway and Haque 1994). All isolates turned Yeast Extract Mannitol Agar containing bromothymol blue (YEMA-BTB) into deep and moderate yellow color after the incubation of 3-5 days at 28^{O}C. This showed that all were fast growers and acid producers (Table 1). The fact that almost all isolates displayed acid production on YEMA – BTB medium confirms the characteristics of fast growing rhizobia (Jacobsen, 1984; Somasagaran and Hoben 1994). Similarly, a study made by Aynabeba Adamu *et al*. (2001) on faba bean rhizobia isolated from Northern Shoa confirmed that all faba bean nodulating rhizobia were acid producing. (Table 1). The ability of the isolates to grow in alkaline media is in agreement with the finding of Surange *et al*. (1997) who reported that fast growing rhizobial isolates could grow with the pH range of 8.9 -12. In addition 34 (68%) of the isolates could grow on pH 5 and 6 (12%) of isolates grew at pH 4.5. However, all isolates that tolerate pH 4.5 such as HUFBR6, HUFBR18, HUFBR26, HUFBR37, and HUFBR45, were found to be sensitive to alkaline pH greater than 9 (Table 1). In this regard, Brockwell *et al*. (1991) showed that some strains of fast growing rhizobium isolates are very sensitive to pH less than 5.0. Isolate HUFBR47 was the most tolerant isolate which grows with wide range of pH (4.5- 10.0). Isolates which grew on pH 4.5 are the most desirable isolates which can be used in acidic soil. Finally, highly performed 10 faba bean nodulating rhizobia isolates were selected for mutagenesis.

pH Tolerance of mutant rhizobial isolates

As per the Table 2, the chemical mutagen hydroxyl amine hydrochloride showed the acidic tolerance (pH 4) only three mutatant isolates such as HUFBR12M4, HUFBR39M6 and HUFBR18M8. In case of sodium azide, it did not show any mutant isolates under acidic condition. Therefore, hydroxyl amine hydrochloride is an effective chemical mutagen to stimulate or enhance the acid tolerance in nodulating rhizobium in Faba bean. In the meantime alkaline tolerance mutant isolates (HUFBR50M1, HUFBR31M2, HUFBR12M3, HUFBR12M4, HUFBR23M5, HUFBR39M6, HUFBR50M7, and HUFBR18M8) were observed in both the mutagenic chemicals (Sodium azide and hydroxyl amine hydrochloride). All the isolates showed the pH range from 10.5 to 12.

Symbiotic effectiveness of Rhizobium mutated with sodium azide

The data indicated (Table-3) that all mutants were able to form nodules upon inoculation. Faba bean plants inoculated with mutant HUFBR31M2 produced a maximum nodule number of 180 per plant. But the same isolate wild type produced only 113 root nodules. This isolate showed a statistically significant difference from other mutants at ($P < 0.05$). The minimum nodule number (45/plant) was produced by plant inoculated with mutant HUFBR23M5. The mutagenic chemical sodium azide treated isolate showed minimum nodule number of 113%, even though this number was higher than the wild type isolates. Similar to this result William (1981) reported that some mutants of cow pea *Rhizobium* produced more nodule numbers than plants inoculated with the wild type isolates. Mutants showed variation in nodule dry weight. Plants inoculated with HUFBR12M3 showed significant difference than other isolates at ($P < 0.05$). The maximum nodule dry weight was produced by plants inoculated with mutant HUFBR12M3 (133mg/plant). The maximum nodule dry weight of the plants inoculated with mutant was more than the maximum nodule dry weight of plants inoculated with parental type *Rhizobium* isolate HUFBR12 (94.4 mg/plant). Therefore, sodium azide may be a potential mutagenic agent to induce the nodulation activity in Faba bean nodulating *Rhizobium*. In this study only one mutant treated with sodium azide (HUFBR50M7) showed higher shoot dry weight as compared to its parental type isolate of HUFBR50, but all other mutants recorded lower shoot dry weight than plants inoculated with its parental type. In this regard, Sharma and Yadav (2012) reported that shoot dry weight of pigeon pea plants infected with $PRODH^{-}$ mutants of *Rhizobium* sp. (*Cajanus*) was significantly lower for all mutants than the plants inoculated with parental strain.

Symbiotic effectiveness of Rhizobium mutated with hydroxyl amine hydrochloride

Only four isolates showed the nodulation among the selected isolates treated with hydroxyl amine hydrochloride. The mutant isolate (HUFBR50 M7) showed maximum nodule number, but the value was lesser than the mutant treated with sodium azide as well as the respective wild type (Table 3). The overall correlation in the sand experiment revealed that nodule number was associated positively and significantly ($r = 0.63$, $P < 0.0001$) with nodule dry weight. Shoot dry weight showed a

Table 3. Effect of chemical mutagen sodium azide and hydroxyl amine hydrochloride on nodulation and symbiotic effectiveness of Faba bean rhizobium isolates

	Mutant Code	Nodule number mean ± Std Dev	NDW (mg/pl) mean ± Std Dev	SDW (g/pl) mean ± Std Dev	Nodule Color	SE (%)
Sodium Azide	HUFBR50M1	139 ± 10^{b}	85 ± 7.9^{b}	1.79 ± 0.78^{b}	2	79.5
	HUFBR31M2	180 ± 13^{a}	63.3 ± 1.5^{cd}	$1.61\pm0.1b^{cd}$	2	71.6
	HUFBR12M3	113 ± 11^{cd}	133 ± 2.6^{a}	0.58 ± 0.03^{g}	1	25.8
	HUFBR12M4	102 ± 36^{d}	58.3 ± 3.5^{cd}	0.44 ± 0.05^{g}	1	19.6
	HUFBR23M5	45 ± 22^{e}	49.3 ± 21.5^{ed}	1.19 ± 0.08^{def}	2	2.9
	HUFBR39M6	65 ± 7^{e}	48 ± 6.6^{ed}	1.1 ± 0.2^{ef}	1	48.9
Hydroxyl Amine Hydro chloride	HUFBR50M7	123 ± 7^{bcd}	72.3 ± 4.9^{bc}	2 ± 0.15^{ab}	4	88.9
	HUFBR18M8	52 ± 3^{e}	55.7 ± 24^{cd}	1.2 ± 0.2^{efd}	2	53.3

positive correlation with nodule number at ($r = 0.1$, $p > 0.05$). Similar to this finding, Tejera *et al*. (2005) showed a positive and significant correlation of SDW with nodule numbers upon mutant inoculation on *Phaseolus vulgaris.* The mutants were also evaluated by nodule color examination scaled visually from 1 to 4 (Table 2). According to the scale 2- 4 i.e. pink to dark red colored nodules of plants displayed positive symbiosis (N fixation) while white nodules showed that poor nitrogen fixation. Seven (70%) of mutants displayed pink to dark red colored nodules hence, these mutants showed a positive symbiosis. Consequently, shoot dry weight accumulation of the treated plants in reference to N supplied positive control is used to evaluate the symbiotic effectiveness (SE) of the mutants. Accordingly, 10% of mutant HUFBR50M7 was highly effective, 60% of mutants (HUFBR50M1, HUFBR 31M2, HUFBR23M5 and HUFBR18M8) were effective.

DISCUSSION

As shown in Table 2, three mutants (30%) such as HUFBR12M4, HUFBR39M6 and HUFBR18M8 showed growth on TY agar medium which was adjusted to pH 4. These mutants were more tolerant to acid and alkaline condition than their perspective parental isolates. In this regard mutagen Hydroxylamine Hydrochloride induced extreme pH tolerant mutants. All mutants (100%) were able to grow on TY agar media adjusted to pH 10.5- 12. In agreement with this result, some mutants of *R. leguminosarum* have been reported to be able to grow at a pH as low as 4.5 (Chen *et al*. 1993). Furthermore, Priefer *et al*. (2001) reported that most mutant derivatives of Rhizobial strains nodulating *P*. v*ulgaris* grow reasonably well on TY agar media at pH 4. Hung *et al*. (2005) observed majority of rhizobia strain tolerated extreme pH in their medium from 3.5 to 12. Kulkarni and Nautiyal (1999) observed considerable growth on pH 9 for majority of rhizobial strains except 3 rhizobial strains that were well adapted to grow on pH 12.0. Raza *et al*. (2001) tested *Bradyrhizobium* sp (lupini) strains for pH range (4-10). Their overall results indicated that the isolates were tolerant to extremes of low and high pH since they grew over a range of pH from 4 to 10. Symbiotic effectiveness (%) measurement showed that 3 (20%), 1 (10%), 6 (60%) and 1 (10%) characterized to be ineffective, less effective, effective and highly effective, respectively (Table 3). Here, 60% of the mutants were identified to be symbiotically effective; likewise, 78% of the wild isolates were symbiotically effective. Similar to this result, AZR mutants with improved symbiotic effectiveness have also been reported in *R. leguminosarum bv.viciae* and in *R. phaseoli* (Membrillo-Hernandez *et al.* 1990). Two mutants (HUFBR12M3 and HUFBR12M4) have lost their symbiotic effectiveness due to the mutagens which could result in the loss of symbiotic effectiveness. These mutants induced nodulation upon inoculation on faba bean plants, but they were unable to fix nitrogen (Table 3). This finding is in line with Qing-Sheng *et al.* (1982) who reported that eight mutant strains of *R. leguminosarum* were identified which formed nodules on pea plants, but were unable to fix nitrogen.

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