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RELATIONSHIPS BETWEEN HYDROGEN UPTAKE ACTIVITY AND NITROGEN FIXATION BY *Rhizobium leguminosarum*-FABA BEAN SYMBIOSIS

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ABSTRACT

Nitrogen fixation in faba beans is associated with nodule bacteria *Rizobium leguminosarum.* In this study, hydrogen uptake activity was well distributed in *Rhizobium leguminosarum* isolated from nodules of faba bean (*Vicia faba*). Two *Rhizobia* effective strains namely, ICARDA 441 and ARC 207, exhibiting significantly higher hydrogen uptake activity were subjected to mutagensis with nitrosoguanidine. The respective mutation frequencies were 0.20 and 0.22%. Three negative Hup mutants each of ICARDA 441 and ARC 207 were compared parental with the wild type strains under pot experiments to evaluate the significance of the hydrogen uptake system in biological nitrogen fixation .The all parameters tested, nodulation statues, plant growth characteristics, nitrogenase activity and nitrogen content were significantly reduced in the plants treated with negative hydrogen uptake mutants strains.

Keywords: Effectiveness, faba bean, Hydrogenase activity, Nitrogenase Activity, *Rhizobium leguminosarum*, Rhizobium mutation.

INTRODUCTION

The formation of efficient nodules depends on a complex relationship between the two partners the host and the rhizobial strains. The survival of rhizobia in the soil and the high computability between the two partners leeds to good nodulation and high grain yield. Because of the large indigenous population of rhizobia which in many localities are not fully effective in nitrogen fixation, it is not surprising that inoculation with selected rhizobial strains commonly results in highly significant agronomic response, (Gibson, 1969; Roughly, 1970 and Nutman, 1971). Symbiotic properties, such as ability to induce nodule formation on the root of a legume host and ability to fix nitrogen within a nodule, have long been to be relatively unstable in certain laboratory cultures of *Rhizobium*. The plasmid genes in *Rhizobium* may be involved in the establishment of symbiosis and host range specificity (Denarie *et al*., 1981).

The reduction of atmospheric N_2 to NH_3 by rhizobia, an energy intensive reaction, is catalyzed by the nitrogenase enzyme. Moreover, 30-50 % of the nitrogenase electron flux is lost as H_2 from most nodulated legumes (Schubert and Evans, 1976). However, some *Rhizobium sp.* strains contain an oxidizing hydrogenase induced in bacteroids together with a nitrogenase (Dixon, 1976). The presence of hydrogenase renders a strain capable of hydrogen uptake which leads to ATP formation under oxygen consumption (Dixon, 1976). Thus the hydrogenase reaction supports nitrogen fixation by sparing energy (Emerich *et al*., 1979). Legumes inoculated with negative

hydrogen uptake rhizobial strains recorded lost a mean of 32% of the nitrogenase electron flux as hydrogen, whereas nodules formed by efficient positive hydrogen uptake rhizobial strains provided lost a mean of 3.8 % of electron flux as hydrogen (Evans *et al*., 1981).

Inoculations of legume seeds with positive hydrogen uptake rhizobial strains have shown increase 11% in total N content in the soybean (Maier *et al*. 1978) and 21-46% in the mungbean (Pahwa and Dogra 1981). Plant dry matter with positive hydrogen uptake rhizobial strains produced 50% more than those inoculated with negative hydrogen uptake rhizobial strains (Ahmad and McLaughlin, 1985). On the other hand (Cunningham *et al*. 1985) reported that hydrogen uptake characteristics have little or no significant effect on growth and nitrogen fixation.

In this work, pot experiments were conducted to investigate the effect of positive and negative hydrogen uptake *Rhizobium leguninosarum* strains in nodulation and nitrogen fixation on faba bean plants.

MATERIALS AND METHODS

***Rhizobiun liguminosarum* cultures:**

Several different specific rhizobial strains were used. These strains were isolated and identified by ICARDA and ARC culture collection on YEM medium according to Vincent (1970).

Screening for hydrogen uptake:

The strains were stabbed into an hydrogen uptake medium containing triphenyltetrazolium chloride (TTC) and incubated at 28° C. The strains that reduced TTC until the fifth day were denoted positive hydrogen uptake and the others negative hydrogen uptake strains (Maier *et al*. 1978).

Mutagensis:

N-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) was used as a mutagen to mutant the Hup loci of wild type positive Hup strains. On the basis of dose response curves obtained by using the Cannon (1980) method, 3.5×10^{8} cells per ml of positive Hup strains were tested with 0.2 ml NTG (10 mg per ml) for 30 min. The presence or absence of the hydrogen uptake system in the mutant colonies was recorded as described above.

Pots experiment:

Pots containing sterilized sandy loam soil (pH 7.4) were served to investigate the comparison of nitrogen fixing activity of wild type positive hydrogen uptake strains with negative hydrogen uptake mutants. Seeds surface sterilized were separately inoculated with wild type and mutant rhizobial cultures and sown in pots. Each treatment had three replicates in a random block design. The plants were harvested at full bloom of the crop, nodulation and growth characteristics including; chlorophyll content, nitrogenase activity, and N content, were recorded. The number and dry weight of nodules, and dry weight of shoots were determined according to Vincent (1970).

Nitrogenase activity was determined in terms of C_2H_2 reduction activity by Gas Liquid Chromatography (Hardy *et al*., 1968). The chlorophyll content was determined from flag leaves of the plants by acetone extraction (Witham *et al*., 1971). The total nitrogen content of the plants was estimated by micro-Keldahl method (Burris and Wilson 1957).

RESULTS

Mutagenesis:

Rhizobium leguminosarum strains namely ICARDA 441 and ARC 207, subjected to mutagenesis by nitrosoguanidine, recorded mutation frequencies of 0.20 and 0.22%, respectively. After being stored for two months in refrigerator at 4°C, the mutant colonies were restricted on hydrogen uptake medium to check for reversion. The mutant colonies which reverted back and restored the ability to recycle hydrogen was 54 and 48 %, respectively. From each parent strain ten mutant colonies were randomly selected for comparison with their parents.

Screening for hydrogen uptake system:

Results in Table 1 shows that the screening of rhizobial strains used, to ability reduce TTC. These strains were therefore denoted positive hydrogen uptake and the strains failed to reduce TTC surved as negative hydrogen uptake.

Table 1: Reduction of triphenyltetrazolium chloride (TTC) by *Rhizobium leguminosarium* strains.

Rhizobial strains	TTC reduction (days of inocubation)				
	1	2	3	4	5
ICARDA 400	red	red	deep red	deep red	deep red
ICARDA 414	red	deep red	deep red	deep red	deep red
ICARDA 441	red	deep red	deep red	deep red	deep red
ICARDA 481	red	deep red	deep red	deep red	deep red
ARC 201	slight red	slight red	slight red	red	red
ARC 202	slight red	red	red	red	deep red
ARC 207	red	deep red	deep red	deep red	deep red
ARC 211	creame	creame	creame	slight red	red
ARC 212	slight red	slight red	red	red	red
ARC 221	cream	cream	cream	slight red	slight red

The data presented in Table 2 show that the comparison of positive hydrogen uptake rhizobial strains with their mutants produced by nitrosoguanidine. The observations recorded after storing the colonies for 2 months at 4°C on yeast extract mannitol agar, TTC triphenyltetrazolium chloride.

Table 2: Comparison of Hup$^+$ rhizobial strains with their mutants produced by Nitrosoguanidine (NTG).

Rhizobial strains	Polysaccharide production	Nodulation capability	Nitrogenase activity	TTC reduction
Parent strain 441	+	+	+	+
Mutant strain 1	+	+	+	+
Mutant strain 2	+	+	+	-
Mutant strain 3	+	-	-	+
Mutant strain 4	+	+	-	-
Mutant strain 5	+	+	+	-
Mutant strain 6	+	+	+	-
Mutant strain 7	-	+	-	-
Mutant strain 8	-	-	-	-
Mutant strain 9	+	+	+	+
Mutant strain 10	+	+	+	-
Parent strain 207	+	+	+	+
Mutant strain 11	+	+	+	-
Mutant strain 12	-	+	-	-
Mutant strain 13	-	+	+	+
Mutant strain 14	-	+	+	-
Mutant strain 15	+	-	-	-
Mutant strain 16	+	+	+	-
Mutant strain 17	+	+	+	+
Mutant strain 18	+	+	+	-
Mutant strain 19	-	+	+	+
Mutant strain 20	+	-	-	-

Comparison of nitrogen fixing efficiency:

The data recorded in Table (3) showed clearly that inoculation with the three mutants of parent strain ICARDA 441 produced lower nodule number plant^{-1} than the nodule number obtained with the wild type Hup$^+$ strain.

Table (3): Comparison of nitrogen fixing efficiency between Hup$^-$ rhizobial strains and their mutants.

Rhizobium strains/ mutants	Nodules No. plant^{-1}	Nodule dry weight (mg plant^{-1})	Shoot dry weight (g plant^{-1})	Nitrogen content (%)	Nitrogenase activity (μ mol C2H4 h^{-1} g -1 dry weight of nodules)	Chlorophyll content of leaves (mg g^{-1} tissue)
ICARDA 441	70	65.0	3.26	2.51	72.5	2.17
Mutant 2	59	50.7	2.96	2.38	46.3	1.93
Mutant 5	55	50.0	2.81	2.35	45.4	1.87
Mutant 6	51	47.7	2.73	2.29	43.6	1.58
ARC 207	84	72.2	3.78	2.74	82.7	2.34
Mutant 11	64	57.6	3.11	2.50	71.2	2.14
Mutant 16	60	50.3	2.98	2.41	65.8	1.86
Mutant 18	58	48.6	2.76	2.26	41.17	1.51
L. S. D. 5%	3.9	5.6	0.5	0.3	3.8	0.15

Similary, the dry weight of nodules, nitrogen content and nitrogenase activity were also significantly higher with the Hup^+ strain. Results from plants inoculated with ARC 207 and its mutants were quit similar to those from plants inoculated with ICARDA 441.

DISCUSSION

The ability to recyle H_2released during the nitrogenase reaction is known to influence N_2 - fixation efficiency, as the cell economizes on energy. In the present investigation, TTC reduction was used to screen *Rhizobium leguminosarum* strains for H_2 uptake. This method has been successfully used by many workers (Pahwa and Dogra, 1981; Brewin, 1984 and Sekhon *et al*., 1992). *In vitro* the beneficial effect from H_2 recycling in legumes, Hup^+ and Hup^- inoculants that are isogenic except for H_2 oxidation characteristics should be used (Evans *et al*., 1981). This is possible only by inducing mutations in a Hup^+ strain so as to isolate Hup^- mutants. Nitrosoguanidine, which was used for mutagenesis of wild type Hup^+ strains ICARDA 441 and ARC 207, has been reported as a storing mutagen, resulting in a high frequency of mutations (Bhandal *et al*., 1989). This was confirmed during the present investigation since the mutation frequencies of both parent strains were very high.

The inoculation with Hup^+ strain increased the N_2 fixing efficiency than the inoculation with Hup^- strains. This demonstrated was attributed to the ability of this strains to conserve carbohydrates, which leads to additional dry matter accumulation. This results support those reported by other researchers working with Hup+ strains (Rainbird *et al*. 1983; Truelsen and Wyndale 1984). The chlorophyll content of the leaves in the present study also increased with Hup^+ strains, probably because of a direct correlation between photosynthesis and N_2 fixation (Fakir *et al*., 1986). The higher nitrogenase activity in nodules formed by Hup^+ strains was attributed to the ability of these strain to recycle H_2, conserve energy, and convert photosynthesis into the ATP needed for N_2 fixation (Dixon 1972; Sawhney *et al*., 1985). The N content of plants inoculated with Hpu+ strains was also higher than that of the plants inoculated with Hup- strains. This may reflect a rapid transport of fixed N from the roots of Hup+ inoculated plants to the other parts of the plant (Minamisawa *et al*., 1983).

CONCLUSION

The present work indicated that the presence of an H_2 uptake system in rhizobia can markedly increase the yield and total N content of nodulated legumes.

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العلاقة بين نشاط امتصاص الهيدروجين وتثبيت الأزوت الجوي بواسطة رايزوبيم ليجيومينوزاريم علي نبات الفول البلدى

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تم دراسة العلاقة بين نشاط امتصاص الهيدروجين وعملية تثبيت ازوت الهواء الجوى بواسطة سلالتين من رايزوبيا الفول البلدى ذات كفاءة ونشاط عالى فى عمليتى امتصاص الهيدروجين وتثبيت ازوت الهواء الجوى هما السلالة رقم ٤٤١ والسلالة رقم ٢٠٧. حيث تم تعريض كلتا السلالتين لمادة مطفره كيميائيا هى نيتروزوجوانيدين وذلك للحصول على طفرات وراثيه سالبه وموجبة لنشاط امتصاص الهيدروجبن. حيث سجل معدل الطفور لكلتا السلالتين ٢٠ و٢٢% على التوالى. وتم اختيار ثلاثة طفرات سالبه لنشاط امتصاص الهيدروجين من كل سلاله من السلالتين تحت الدراسة لمقارنتها مع الآباء لمعرفة مدى العلاقة بين معدل امتصاص الهيدروجين وتثبيت الازوت الجوى. وقد وجد أن الطفرات السالبة لامتصاص الهيدروجين سجلت معدل أقل فى الوزن الجاف للنبات ومحتوى النيتروجين الكلى فى النبات وكذلك عدد العقد الجذرية على النبات ونشاط أنزيم النيتروجينيز داخل العقد الجذرية وذلك مقارنة بالآباء الموجبة لنشاط امتصاص الهيدروجين.