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Ecology of Hup$^+$ *Rhizobium* strains of cow pea miscellany: native frequency and competence

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Abstract. *Rhizobium* strains nodulating summer legumes cow pea [*Vigna unguiculata* (L.)], green gram [*V. radiata* (L.) (Wilczek)], black gram [*V. mungo* (L.) (Hepper)] and cluster bean [*Cyamopsis tetragonoloba* (L.) (Taub)] and a winter legume chick pea [*Cicer arietinum* (L.)] were surveyed in the Northern Plains of India and screened for hydrogenase activity to determine distribution of *Hup* character in the native ecosystem. It was observed that 56% of the *Rhizobium* strains of summer legumes were Hup$^+$ whereas that of the winter legume, chick pea, were all Hup$^-$. *Ex planta* acetylene reduction activity was observed in most of the Hup$^+$ but not in the Hup$^-$ strains of any of the host species. In summer legume, mixed inoculation of Hup$^+$ and Hup$^-$ strains, under sterilized as well as unsterilized soil conditions, showed that the host species were predominantly nodulated with Hup$^+$ strain.

Key words: *Rhizobium* isolates – Uptake hydrogenase – Native ecosystem – Cow pea miscellany hosts – Hup$^+$ and Hup$^-$ *Rhizobium* strains – Acetylene reduction activity (ARA) – Competence

Uptake hydrogenase in *Rhizobium* is a desirable attribute of the bacterium to prevent plant energy losses during symbiosis (Albrecht et al. 1979; Emerich et al. 1979). Comparative inoculation studies with Hup$^+$ and Hup$^-$ strains in soybean, peas and 'cow pea group' hosts have shown anomalies from significant gains in plant weight and plant nitrogen to non-significant differences in the effectivity (Schubert et al. 1977; Zablotowicz et al. 1980; Hanus et al. 1981; Lafavre and Focht 1983). However, in cow pea where non-significant difference in effectivity has been observed, the Hup$^+$ *Rhizobium* strain has shown better economy of the plant photosynthate in nodules as compared to the Hup$^-$ strain (Rainbird et al. 1983). Ability to grow chemoautotrophically has been demonstrated in some of the Hup$^+$ *R. japonicum* strains (Hanus et al. 1979; Lepo et al. 1980), whether it has any advantage to the bacteria in soil ecosystem for survival and competence, is not known. In a random survey of commercial *Rhizobium* strains from different cross inoculation species, Schubert and Evans (1976) observed that *Rhizobium* strains of 'cow pea miscellany' are often Hup$^+$. In India a variety of grain legumes of cow pea miscellany host group, commonly known as pulse crops, have been cultivated in the Northern Plains for centuries. However, no study has been done with regard to either the frequency of Hup$^+$ rhizobia of these crops in native soils or to the competitive ability of Hup$^+$ with Hup$^-$ strains for nodulation. In this article we report the frequency of Hup$^+$ and Hup$^-$ *Rhizobium* strains, in native soil, nodulating five host species of cow pea miscellany and also, the competitive ability of a Hup$^+$ strain with a Hup$^-$ strain in mixed inoculation in three of the host species.

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Materials and methods

Isolation and screening of Rhizobium strains from native soil for Hup character

The pulse legumes, namely cow pea [*Vigna unguiculata* (L.)], green gram [*V. radiata* (L.) (Wilczek)], black gram [*V. mungo* (L.) (Hepper)] and Cluster bean [*Cyamopsis tetragonoloba* (L.) (Taub.)] are grown during summer season and chick pea [*Cicer arietinum* (L.)] during winter season in the Northern Plains of India. The soils of this belt are mostly saline or non-saline-alkali with a pH above 7.5–9.0 and are sandy to sandy loam in texture. Plant nodules for isolation of *Rhizobium* were collected at random from 30 different locations in Haryana and Rajasthan states. Nodule samples of summer legumes were collected between 30–45 days and of winter legume between 60–90 days after sowing the crops. *Rhizobium* strains were isolated from the nodules on yeast extract mannitol agar (YEMA) medium plates, containing Congo red dye by standard procedures (Vincent 1970). The plates were incubated at 28°C for 10–12 days to allow colonies to develop.

Isolated colonies from the plates were picked and transferred on YEMA slopes in duplicate. As a rule one colony was picked from the isolation plate used for each nodule. After 7 days of incubation at 28°C, one replication of YEMA slopes was stored for further studies while the second was used for inoculation in nitrogenase induction medium slopes of following composition: (gl^{-1}) yeast extract, 0.2; Mannitol, 5.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCO_3$, 0.5; sodium glutamate, 0.32; sodium malate, 1.6 and agar 15 (pH, 7.2). The slopes were prepared in 15 ml (1 cm dia) tubes containing 5 ml medium. Six tubes were inoculated for each isolate and incubated at 25°C for 8 days. Nitrogenase induction has been observed in the above medium between 7 and 10 days (Kundu et al. 1981). Nitrogenase induction was then determined by measuring acetylene reduction activity (ARA) in presence and absence of hydrogen in the atmosphere. The cotton plugs of the tubes were replaced with subaseals and in one set of tubes (3

Table 1. *Ex planta* acetylene reduction activity (ARA) in *Rhizobium* isolates from different hosts of cow pea miscellany

Host species	Total isolates	ARA positive		ARA enhanced in 5% H_2 atm (Hup^+)		ARA not enhanced in H_2 atm ($Hup^\pm$)		ARA negative	
	No.	No.	%	No.	%	No.	%	No.	%
Summer legumes									
Green gram	299	206	68.9	170	56.9	36	12.0	93	31.1
Black gram	168	113	67.3	91	54.2	22	13.1	55	32.7
Cow pea	283	181	64.0	159	56.2	22	7.8	102	36.0
Cluster bean	164	115	70.1	98	59.7	17	10.4	49	29.9
Winter legumes									
Chick pea	611	0	0.0	0	0.0	0	0.0	611	100.0

Rhizobium isolates which expressed ARA under the tested cultural conditions in nitrogenase induction medium were catagorized as ARA positive and those which fail to express ARA as ARA negative. Within the ARA positive group, isolates which showed enhanced ARA in 5% H_2 in air atm were catagorized as presumptive Hup^+ and those which did not show enhanced ARA in the H_2 atm, as $Hup^\pm$ (doubtful). Per cent values are based on the initial total numbers

replication per isolate) 10% air atm was replaced with C_2H_2, while in the second set the air atm contained 10% C_2H_2 and 5% H_2. The tubes were further incubated for 24 h and C_2H_4 formed was determined by Perkin-Elmer gas chromatograph using Porapak-N columns (2 M length) and dual FID, at 105° C oven and 110° C injector and detector temperature. The values were calculated as C_2H_4 formed per tube h^{-1}.

Randomly selected *Rhizobium* isolates (about 35–40% of the total) were further tested for nodulation under Leonard jar conditions to determine hydrogenase activity. Green gram var. K851 was used as a test host for *Rhizobium* isolates of green gram, black gram, cow pea and cluster bean. Seeds were surface sterilized with acidic alcohol (concentrated sulphuric acid : ethanol, 7:3) for 3 min and washed thoroughly with several changes of sterilized water. Surface sterilized seeds were inoculated with 7 days old cultures of different isolates grown in YEMA slopes. The inoculated seeds were sown in autoclaved Leonard jar assemblies containing washed river sand and Sloger's nitrogen free mineral salt solution (Sloger 1969). The jars were kept in a net house under day light conditions during July-August and quarter strenght sloger's N-free mineral salt solution was used for watering. The plants were uprooted after 35 days of growth, nodules were detached and 200 mg weighed samples were transferred into 15 ml assay tubes covered with subaseals. Nodule samples form each of the inoculated strain were incubated in air as well as air-H_2 (98:2) atm for 2 h at 28° C. H_2 production or consumption was then determined gas chromatographically by TCD using Molecular Seive 5A° columns (2 M length) at 70° C oven and 80° C injector and detector temperatures. Similarly, for chick pea *Rhizobium* isolates, chick pea var. C235 was used as a test host. The experiment was conducted during October and December. Nodule samples for hydrogenase activity assay were taken after 65 days of plant growth.

Determination of competitive ability of Hup^+ and Hup^- strains

Rhizobium strains S24 (Hup^+) and M11 (Hup^-) were used for competition studies under sterilized as well as unsterilized soil conditions. The strains were inoculated in 50 ml flasks containing 20 ml YEM broth and incubated for 6 days on a rotary shaker. Viable cell counts in the broth were determined by dilution plating. Based on viable counts, the broths of the two strains were mixed in the ratios of 2:1, 1:5 and 5:1 for seed inoculation. Surface sterilized seeds of green gram, black gram and cluster bean were inoculated by soaking the seeds in the broth for 30 min. The seeds were then sown in autoclaved Leonard jar assemblies as well as in earthen pots having 10 kg unsterilized farm soil. The jars and the pots were kept in a net house under normal sunlight conditions. The plants were uprooted after 30 and 45 days of sowing in the case of the jars and after 45 days in the case of the earthen pots to determine the competitive ability of the two strains. The nodules of the strain S24 were light pink coloured whereas that of the M11 were dark red coloured and therefore, in sterilized Leonard jars the two types of nodules could be counted in different treatment on the basis of nodule colour. In the experiment conducted in unsterilized farm soil, controls without inoculation, were also performed to determine the native frequency of the two types of nodules.

Results and discussion

Screening of Rhizobium isolates for ex planta ARA activity

A total of 914 *Rhizobium* isolates from the nodules of green gram, black gram, cow pea and cluster bean (summer legumes) and 611 isolates from the nodules of chick pea (winter legume) were obtained (Table 1). About 64–70% of the isolates of summer legumes expressed ARA under cultural conditions. All these isolates were of the slow growing type. The relative ARA values varied from 2 n mol h^{-1} $tube^{-1}$ to 150 n mol h^{-1} $tube^{-1}$. Only about 8–13% of these slow growing isolates which were having low ARA values (< 10 n mol h^{-1} $tube^{-1}$) did not show increased ARA in 5% H_2 atm. About 30–36% of the isolates did not show ARA activity and these were both of the fast as well as the slow growing type. The detection limit for C_2H_4 was upto 0.01 nmol in 0.5 ml of the gas sample used for assay. Also, none of the chick pea (winter legume) *Rhizobium* isolates expressed ARA under the tested cultural conditions. These isolates were all of the slow growing type.

Reduction of triphenyl tetrazolium chloride (TTC) and uptake of H_2 in low carbon substrate medium containing

arabinose and gluconate used for detection of *Hup* in *Rhizobium japonicum* (Maier et al. 1978), could not be used for screening cow pea miscellany rhizobia for *Hup* character. These strains reduced TTC nonspecifically and known Hup^- strains reduced the dye more strongly than Hup^+ strains in media supplemented with various carbon substrates. Also, 40–60% of the *Rhizobium* isolates from the host species used for the present investigation did not grow on pentoses. These tests for detection of *Hup* character under cultural conditions in *Rhizobium* strains of cow pea miscellany group were, therefore, not reliable. In our earlier study we found that some slow growing *Rhizobium* strains of green gram and black gram expressed ARA under cultural conditions in a simple medium having one of the citric acid cycle intermediates after 7–8 days of growth on agar slopes and this activity was enhanced in 5–10% H_2 atm. These strains were subsequently found to be Hup^+ (Dadarwal, 1980; Dadarwal et al. 1981). We, therefore, used the *ex planta* ARA induction and enhanced effect of H_2 atmosphere on ARA as criteria for initial screening of the different isolates for *Hup* character.

From the screening of *Rhizobium* isolates under cultural conditions it was presumed that isolates showing enhanced C_2H_2 reduction in H_2 atm could be Hup^+ whereas those which failed to express ARA under the tested conditions, as Hup^-. Isolates which showed low ARA, not enhanced in H_2 atm, were presumed to be doubtful for *Hup* character.

Ex planta activity of Rhizobium isolates VS Hydrogen uptake in nodules

In order to confirm the presence of hydrogenase activity, about 40% of the *Rhizobium* isolates of different host species from each of the presumptive categories, based on *ex planta* ARA, were tested for H_2 uptake in nodules (Table 2). It was observed that about 96% of the *ex planta* ARA positive isolates from green gram, black gram, cluster bean and cow pea (summer legumes), which showed enhanced ARA in H_2 atm, were Hup^+ showing H_2 consumption in nodules. Also, about 17.5% of the isolates which were presumed to be doubtful for *Hup*, were Hup^+. All of the isolates which were ARA negative under tested cultural conditions were Hup^- and produced H_2 in nodules. About 4% of the isolates which showed increased ARA in H_2 atm under cultural conditions and 25% of the isolates which showed low *ex planta* ARA (not enhanced in H_2 atm) neither produced hydrogen nor showed hydrogen uptake in air atm. In such strains H_2 uptake could be confirmed only by tritium exhange, facilities for which were not available. Randomly selected 230 *Rhizobium* isolates of chick pea (winter legume) which were all *ex planta* ARA negative were tested for hydrogenase activity in nodules of chick pea and all were found to produce H_2 in nodules indicating that these were Hup^-.

Host dependent *Hup* expression has been observed in cow pea miscellany species where hydrogenase activity has been reported to be not expressed in *V. radiata* nodules. (Gibson et al. 1981). Contrary to these observations, in our studies we observed that *V. radiata* cultivar K851 was the most promiscuous host (Dadarwal et al. 1977) and invariably showed H_2 uptake in nodules inoculated with Hup^+ strains and therefore, it was used as a test host.

Studies done with isogenic mutants of *Rhizobium* strains have shown that H_2 uptake is independent of nitrogenase induction under cultural conditions (Hanus et al. 1979).

Table 2. Frequency of Hup^+ *Rhizobium* strains in summer legumes of cow pea miscellany hosts characterized on the basis of H_2 uptake in nodules

Rhizobium group based on *ex planta* ARA	Original host species	Test host: green gram			
		Isolates tested	Hup^+	Hup^-	$Hup^\pm$
ARA postive (Hup^+)	Green gram	68	65	0	3
	Black gram	36	34	0	2
	Cow pea	64	62	0	2
	Cluster bean	40	38	0	2
	Total	208	199	0	9
	%		95.7	0.0	4.3
ARA positive ($Hup^\pm$)	Green gram	15	2	10	3
	Black gram	9	0	7	2
	Cow pea	9	2	4	3
	Cluster bean	7	3	2	2
	Total	40	7	23	10
	%		17.5	57.5	25.0
ARA negative	Green gram	37	0	37	0
	Black gram	22	0	22	0
	Cow pea	40	0	40	0
	Cluster bean	20	0	20	0
	Total	119	0	119	0
	%		0.0	100	0.0
	Total of all catagories	367	206	142	19
	%		56.1	38.7	5.2

Nodules of *Rhizobium* isolates showing H_2 uptake in 2 per cent H_2 in air atm were identified as Hup^+ and those showing increase in H_2 value in 2% H_2 in air atm as Hup^-. Nodule samples which showed nonsignificant differences in the initial values of 2% H_2 in air atm were grouped as not identifiable ($Hup^\pm$)

However, our present studies with cow pea miscellany rhizobia indicates that a majority of the *ex planta* ARA positive strains from native soil environment are Hup^+. Also, the known *ex planta* ARA positive strains of *R.* sp. 32H1, *R. japonicum* 61A106 and 61A76 were later on found to be Hup^+. Whether the Hup^+ *Rhizobium* strains acquire the ability to synthesize some specific nitrogenase inducing factors when they become autotrophs, or the oxygen protective role of hydrogenase allows nitrogenase expression under cultural conditions is not well understood. An important fact which has emerged from the present investigations is that the *Hup* character has been acquired only by rhizobia nodulating summer legumes and not by those of the winter legume chick pea, grown under similar soil and ecological conditions, except differences in seasonal temperature. The temperature during the summer when these crops are grown, rises as high as 45° C and does not fall below 30° C whereas during the winter season it does not rise above 25° C. From the *ex planta* ARA expression studies as well as from the hydrogen uptake studies in nodules, it could be concluded that in the native soil environment about 57–60% rhizobia nodulating the four summer legumes are Hup^+ whereas those of the winter legume chick pea are mostly Hup^-.

Table 3. Competitiveness of *Vigna Rhizobium* strains S24 (Hup$^+$) and M11 (Hup$^-$) in leonard jars

Host plant	Inoculum ratio	Nodulation percentage			
		30 days		45 days	
		S24	M11	S24	M11
Green gram K851	2:1	64.7	35.3	77.5	22.5
	1:5	39.9	60.1	42.4	57.6
	5:1	91.4	8.6	81.0	19.0
Black gram T9	2:1	73.5	26.5	74.6	25.4
	1:5	44.9	55.1	51.5	48.5
	5:1	82.8	17.2	87.2	12.8
Cluster been HG 75	2:1	81.5	18.5	79.3	20.7
	1:5	25.3	74.7	61.5	38.5
	5:1	77.4	22.6	89.4	10.6

Inoculum ratio indicates the proportion of viable cells of *Rhizobium* strain S24 and strain M11 (S24:M11) in the mixed broth used for seed inoculation. Clear distinction could be made in the colour of the nodules formed with strain S24 (light pink) and strain M11 (dark red). Therefore, based on the colour of nodule, the two types of nodules from each plant were counted. At each stage of observation a mean value of nine plants were taken and from the actual numbers, the percentage of two types of nodules were calculated

Table 4. Competitiveness of *Vigna Rhizobium* strains S24 (Hup$^+$) and M11 (Hup$^-$) in unsterilized soil

Host plant	Inoculum ratio	Nodulation percentage	
		S24	M11
Green gram K851	Native	90.2	9.8
	2:1	83.8	16.2
	1:5	72.1	27.9
	5:1	98.2	1.8
Black gram T9	Native	84.9	15.1
	2:1	87.1	12.9
	1:5	76.1	23.9
	5:1	86.4	13.6
Cluster bean HG 75	Native	71.2	28.8
	2:1	68.3	31.7
	1:5	39.4	60.6
	5:1	75.2	24.8

Observations were taken after 45 days of plant growth. Percentage of nodules of S24 and M11 were calculated as indicated in Table 3. Native frequency indicates the percentage of two types of nodules formed by native rhizobia in uninoculated treatments

Competitiveness of Hup$^+$ and Hup$^-$ Rhizobium strains under sterilized and unsterilized soil conditions

Competition studies were done only with summer legumes since Hup$^+$ strains were not observed among rhizobia of winter legume. Both of the *Rhizobium* strains S24 (Hup$^+$) and M11 (Hup$^-$) were earlier found to be symbiotically effective with green gram, black gram and cluster bean (Dadarwal et al. 1982) and therefore, these host species were taken for determining competitiveness for nodulation. Sterilized river sand was taken in Leonard jars to eliminate the competition with native rhizobia. It was observed that in Leonard jars the nodules formed by the two strains were not related to the initial inoculum ratios (Table 3). For example, in green gram when inoculum ratio for seed inoculation was 2:1 (S24:M11), at 30 days of plant growth the nodule ratio was also approximately 2:1 which increased to 3.5:1 after 45 days. At 1:5 ratio of inoculum the nodule ratio was 1:1.5 at 30 days which further narrowed to 1:1.3 after 45 days. A similar trend could also be seen in the two other host species. At 45 days of plant growth most of the nodules formed on lateral roots were of S24 which indicated that this strain survived better in the soil as compared to M11. Differences due to host species were not significant at 45 days of plant growth.

In farm soil, 10–30% of the nodules were dark red coloured (M11 type) and 70–90% were relatively white or light pink coloured (Table 4). In green gram and black gram the M11 strain did not compete for nodulation with native bacteria, even at an inoculum ratio of 1:5 (S24:M11). In cluster bean at a 1:5 inoculum ratio, 60% of the nodules were of the red type. Under unsterile soil conditions, however, the quantification of data based on nodule colour could not be justified, especially for Hup$^+$ strain S24 because the native population of similar nodule type were quite large. The competitive ability of Hup$^-$ strain M11 further decreased in unsterilized soil as compared to sterilized soil conditions. Trinick et al. (1983) studied competitiveness of a slow and a fast growing strain in mixed inoculation in cow pea and observed that at higher temperature the slow growing strain predominates for nodulation over the fast growing. Although, we did not study the effect of temperature on nodulation patterns, we noticed that the air temperature in the net house varied from 30–40°C during plant growth. In broth culture both the strains were found to grow upto 40°C, with M11 growing faster then S24. The high temperature was therefore, not a limitation in survival of the red nodule forming strain.

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