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Rhizobium strains expressing uptake hydrogenase in different host species of cowpea miscellany

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Abstract. Uptake hydrogenase activity in nodules of green gram (*Vigna radiata* (L.) (Wilczek)), black gram (*Vigna mungo* (L.) (Hepper)), cowpea (*Vigna unguiculata* (L.) and cluster bean (*Cyamopsis tetragonoloba* (L.) (*Taub*.)), formed with two Hup^+ (S24 and CT2014) and one Hup^- (M11) *Rhizobium* strains, was determined at different levels of external H_2 in air atmosphere. Nodules of all the 4 host species formed by inoculation with strains S24 and CT2014, showed H2 uptake but not those formed with strain M11. H_2 uptake rates were higher in 1 and 2% H_2 in air atmosphere (v/v) than at 5 or 10% levels in all the host species. Variations in the relative rates of H_2 uptake were observed both, due to host species as well as due to *Rhizobium* strains. However, no host dependent complete repression of the expression of H_2 uptake activity was observed in nodules of any of the host species formed with Hup^+ strains.

Keywords. *Rhizobium* strains; uptake hydrogenase; nitrogenase; cowpea miscellany hosts; hydrogenase repression.

Introduction

Nitrogenase evolves H_2 concurrent to N_2 reduction which consumes ATP and reductant (Bulen and Lecomte, 1966; Orme-Johnson *et al.,* 1977). In the absence of uptake hydrogenase, a considerable amount of plant energy is lost by way of H_2 evolution in root nodules of legumes (Schubert and Evans, 1976; Bethlenfalvay and Phillips, 1979; Evans *et al.,* 1981). *Rhizobium* strains possessing uptake hydrogenase (Hup^+) are therefore, desired to conserve the plant energy losses. Such strains have been identified in host range infective groups of *R. leguminosarum* (Dixon, 1968; Ruiz-Argueso *et al.,* 1978; Dejong *et al.,* 1982), *R. japonicum* syn. *Bradyrhizobium japonicum* (Schubert and Evans, 1976; Albrecht *et al.,* 1979; Ruiz-Argueso *et al.,* 1981) and *Rhizobium* sp. cowpea miscellany (Schubert *et al.,* 1977; Gibson *et al.,* 1981; Dadarwal *et al.,* 1985).

Extensive *in planta* as well as *ex planta* studies have shown that some of the Hup^+ strains from the 3 host infective groups have physiological, agronomical and/or ecological advantages over the Hup^- strains (Dixon, 1972; Evans *et al.,* 1981; Eisbrenner and Evans, 1983; Minamisawa *et al.,* 1983; Dadarwal *et al.,* 1985; Lambert *et al.,* 1985). Although under complete anaerobiosis and in the absence of alternate electron acceptors, H2 production (conventional hydrogenase activity) has been reported recently in barley and maize roots (Torres *et al.,* 1986), normally respiring plant systems have so far not been known to either produce or utilize H_2. However repression of bacterial uptake hydrogenase has been reported in nodules of

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Abbreviations used: YEMA, Yeast extract mannitol agar; ARA, acetylene reduction activity; RE, relative efficiency.

some of the host species belonging to these cross inoculation groups (Dixon, 1972; Gibson *et al.,* 1981; Keyser *et al.,* 1982; Lopez-et *al.,* 1983; Garg *et al.,* 1985). The ambiguity has been reported more in cowpea miscellany hosts where species like *V. unguiculata* and *V. radiata* have been described as expression positive (Schubert *et al.,* 1977; Drevon *et alb.,* 1983; Lopez *et al.,* 1983; Thimmaiah and Lodha, 1986) as well as negative (Gibson *et al.,* 1981; Garg *et al.,* 1985), when nodulated with genetically Hup^+ *Rhizobium* strains. Present studies have shown that in cowpea miscellany host species, H_2 concentration in the incubation atmosphere is a major factor affecting bacterial uptake hydrogenase activity expression, which varies from species to species.

Materials and methods

Rhizobium strains

Effective *Rhizobium* strains S24 (Hup^+) and M11 (Hup^-), originally isolated from green gram nodules (Dadarwal *et al.,* 1979) and CT2014 (Hup^+), originally isolated from cluster bean nodules were used in the present studies. The strains were maintained on yeast extract mannitol agar (YEMA) slopes (Vincent, 1970) by periodic subculturing.

Host species

The legume species used were green gram (*Vigna radiata* (L.) (Wilczek)) cv. K 851, black gram (*V. mungo* (L.) (Hepper)) cv. T9, cowpea (*V. unguiculata* (L.)) cv. N 1 and cluster bean (*Cyamopsis tetragonoloba* (L.) (*Taub*.)). Seeds of these legumes were surface sterilized with acidic alcohol (concentrated sulphuric acid: ethanol, 7:3, v/v) for 3 min and washed thoroughly with several changes of sterilized water. Surface sterilized seeds were inoculated with 7 day old growth of *Rhizobium* strains from agar slopes. The inoculated seeds were sown in autoclaved chillum jar assemblies (Dahiya and Khurana, 1981) containing washed river sand and Slonger's nitrogen free mineral salt solution (Sloger, 1969). Uninoculated seeds were sown as controls. Fifteen replicated jars were sown for each treatment. The jars were kept in a net house under day light conditions during July–August. Quarter strength Sloger's nitrogen free mineral salt solution was used periodically for watering. The plants were uprooted at appropriate stages of growth to determine nitrogenase and hydrogen uptake activities.

Nodule nitrogenase activity

Weighed samples (200 mg) of detached nodules were transferred in 15 ml assay tubes and stoppered with suba seals. The inner atmosphere in the tubes was made to contain 10% C_2H_2 in air atmosphere (v/v). The tubes were incubated for 1 h at 28°C and C_2H_4 formed was determined by Sigma-3B gas Chromatograph (P/E), using Porapak N columns (2 M length) and dual flame ionisation detector (FID) at 105°C oven and 110°C injector and detector temperatures.

Uptake hydrogenase activity

For hydrogenase assay, nodule samples (200 mg) were incubated in 15 ml assay tubes containing 1, 2, 5 or 10% H_2 in air atmosphere (v/v). Consumption of H_2 was then determined by gas chromatography by thermal conductivity detector (TCD) using Molecular Sieve 5 A° columns (2 M length) at 70°C oven and 80°C injector and detector temperatures. The gas atmosphere in tubes was always made on the basis of v/v.

In case of green gram, black gram and cowpea observations for nitrogenase activity, hydrogen uptake and plant dry weight ratios were taken after 30 and 45 days, whereas, for cluster bean these parameters were observed after 35 and 50 days of plant growth. Plants of each treatment at both the stages of observation were dried and plant dry weight ratios of different treatments were calculated by comparing with respective uninoculated controls.

Results and discussion

Time course of acetylene reduction activity and H_2 uptake

Figure 1 shows the relative acetylene reduction activity (ARA) and H_2 uptake by nodules of green gram and black gram formed by inoculation with the Hup^+ strain S24. Nodules of black gram showed higher ARA than green gram which decreased abruptly in both the species after $1\frac{1}{2}$ h of incubation. The optimum time of incubation for ARA was therefore taken as 1 h in all the 4 legumes at both the stages of plant growth. When 10% H_2 was added in air atmosphere (v/v), black gram nodules showed H_2 uptake within 1 h, while those of green gram showed only after

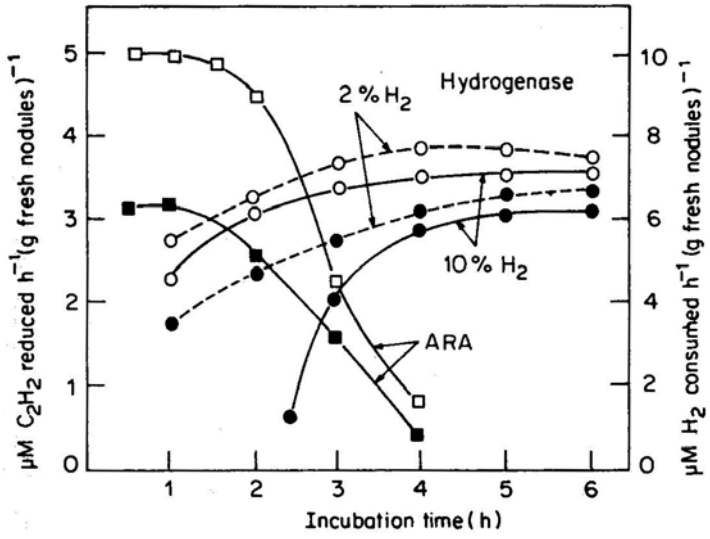

Figure 1. ARA in nodules of green gram (■) and black gram (□) in 10% C_2 H_2 in air atmosphere and hydrogen uptake in 10% H_2 (green gram ● black gram ○) and 2% H_2 in air atmosphere (green gram -- ● --; black gram -- ○ --) inoculated with Hup^+ *Rhizobium* strain S24.

$2\frac{1}{2}$ h of incubation. However, nodules of both the legumes showed H_2 uptake within 1 h of incubation when H_2 level in air atmosphere was kept to 2% (v/v). Although the relative H_2 uptake was higher in nodules of black gram than green gram, the uptake activity continued upto 6 h in 10% as well as in 2% H_2 in air atmosphere, in both the legumes. Since the H_2 uptake were measured in air atmosphere, the uptake should be in excess of the H_2 produced (theoretically) by nitrogenase.

In order to determine the effect of H_2 concentration on uptake activity, nodules of all the 4 host species formed with different *Rhizobium* strains were incubated in 1, 2, 5 and 10% of H_2 in air atmosphere (v/v) for 2 h and the relative uptake values were determined (table 1). Nodules of all the 4 legume species formed with strains S24 and CT2014 (Hup^+) showed H_2 uptake upto 5% and those of black gram upto 10% of H_2 in air atmosphere. H_2 uptake rates were higher in nodules containing 1 to 2% H_2 as compared to 5 or 10%. Nodules formed by inoculation with *Rhizobium* strain M11 which was Hup^- (Dadarwal and Kundu 1981), did not show H_2 uptake, irrespective of H_2 levels or host species.

Table 1. Effect of external H_2 levels in air atmosphere on H_2 uptake by nodules of different cowpea miscellany hosts.

Host species	*Rhizobium* strain	H_2 uptake at different H_2 levels (v/v)*			
		1%	2%	5%	10%
Green gram (30 days)	S24 (Hup^+)	2·36	3·99	1·14	—
	CT2014 (Hup^+)	3·61	4·20	1·54	—
	M11 (Hup^-)	—	—	—	—
Black gram (30 days)	S24 (Hup^+)	4·05	6·47	6·94	4·24
	CT2014 (Hup^+)	5·82	7·85	9·57	7·02
	M11 (Hup^-)	—	—	—	—
Cowpea (30 days)	S24 (Hup^+)	3·61	4·72	3·04	—
	CT2014 (Hup^+)	4·72	6·82	3·17	1·02
	M11 (Hup^-)	—	—	—	—
Cluster bean (35 days)	S24 (Hup^+)	2·81	2·32	1·10	—
	CT2014 (Hup^+)	3·74	6·74	2·44	—
	M11 (Hup^-)	—	—	—	—

*μmol H_2 consumed h^{-1} (g nodules)$^{-1}$; (–) indicates absence of detectable H_2 uptake. Figures are average values of 5 replications. H_2 uptake measurements were done after 2 h of incubation.

Garg *et al.* (1985) used 10% H_2 in air atmosphere for detection of H_2 uptake in nodules of 5 host species of the cowpea miscellany and reported H_2 uptake only in black gram. Above studies have shown that the H_2 concentration in the external air atmosphere is one of the critical factors affecting H_2 uptake by nodules in short period exposures and this level varied with host bacterium combination. Secondly, H_2 uptake in air atmosphere included the H_2 produced by nitrogenase, therefore, in first 1 h of incubation when nitrogenase activity is high, observed absolute H_2 uptake rates are either low or even absent when added H_2 concentration in the incubation atmosphere is high (figure 1). Results of 1 to 2 h observations, in the absence of tritium exchange studies, therefore, could be misleading in determining the *Hup* phenotype of *Rhizobium* in nodules.

Variations in the relative rates of H_2 uptake were observed, both due to host species as well as due to *Rhizobium* strains. Evidently, black gram nodules formed with strain S24 or CT2014 showed higher H_2 uptake rates than the other 3 host species. Likewise, nodules formed by inoculation with strain CT2014 consumed more H_2 than those formed with S24 in all the 4 legumes (table 1). Such variations in the relative efficiency (RE) have been observed among hosts of cowpea, peas, as well as soybean groups nodulated with different Hup^+ strains (Dixon, 1972; Gibson *et al.,* 1981; Lopez *et al.,* 1983).

Table 2 shows relative ARA, H_2 uptake in 2% H_2 in air atmosphere and effectivity of the 3 *Rhizobium* strains with the 4 host species. Nodules formed with the two Hup^+ strains S24 and CT2014 showed H_2 uptake activity at both the stages of plant growth but no uptake was detected in nodules formed by inoculation with strain M 11. All the 3 strains formed effective symbiosis with green gram, black gram and cowpea. In cluster bean only strain CT2014 formed effective symbiosis while strains S24 and M 11 were relatively less effective as evident from lower plant dry weight ratios. The symbiotic effectivity therefore, did not affect the *Hup* phenotype of the *Rhizobium* in nodules.

Gibson *et al.* (1981) used specific strain combinations in cowpea, green gram, black gram and two other host species of cowpea miscellany and found only green gram producing phenotypically Hup^- nodules. Lopez *et al.* (1983) observed 7 strains

Table 2. Nitrogenase activity, H_2 uptake and effectivity of different *Rhizobium* strains in the cowpea miscellany hosts.

Host	*Rhizobium*	Nitrogenase[a] activity		H_2 uptake[b]		P.D. ratio[c]	
		30d	45d	30d	45d	30d	45d
Green gram	S24	2·84	3·78	3·99	5·40	4·32	7·32
	CT2014	4·24	3·92	4·20	3·34	4·62	6·95
	M11	5·32	5·47	—	—	3·14	6·42
Black gram	S24	4·73	5·26	6·47	8·65	3·81	5·97
	CT2014	5·82	8·44	7·85	8·33	4·84	6·31
	M11	6·12	9·40	—	—	3·59	5·82
Cowpea	S24	4·42	8·02	4·72	2·12	2·84	5·72
	CT2014	7·85	6·47	6·82	4·41	4·63	5·41
	M11	2·06	3·13	—	—	2·43	3·52
Cluster bean[d]	S24	2·80	6·42	2·32	5·94	1·92	2·45
	CT2014	9·02	13·42	6·74	10·47	2·57	5·72
	M11	1·14	3·21	—	—	1·42	2·14

(–), No detectable activity.

[a] μmol C_2H_2 reduced h^{-1} (g-nodules)$^{-1}$ in 10% C_2H_2 in air atmosphere (v/v) incubated for 1 h.

[b] μmol H_2 consumed h^{-1} (g fresh nodules)$^{-1}$ in 2% H_2 (v/v) in air atmosphere incubated for 2 h.

[c] $\text{P.D. ratio} = \dfrac{\text{Dry wt. plant}^{-1} \text{ in inoculated jar}}{\text{Dry wt. plant}^{-1} \text{ in control jar}}$

[d] Observations in cluster bean were taken after 35 and 50 days of plant growth as the crop is relatively of longer duration than the other 3 species.

expressing similar levels of H_2 uptake in nodules of soybean and cowpea but only 3 expressed uptake activity in green gram bacteroids. Likewise, Garg *et al.* (1985) observed *Hup* expression in only one out of 5 host species of cowpea miscellany examined. In all these studies nodule samples were incubated in air atmosphere having 10% H_2 uniformly, without determining the critical level of H_2 tolerance in various legumes which could be a reason for apparent Hup^- phenotype. Enhanced ARA in 2 to 5% H_2 in air atmosphere has been considered as an indirect way to denote *Hup* expression in nodules formed with Hup^+ strains in cowpea miscellany hosts in the absence of tritium exchange assay or detectable H_2 uptake over the rates produced by nitrogenase (Dadarwal *et al.,* 1985; Thimmaiah and Lodha, 1986). Although host strain combinations having Hup^+ phenotype in one and Hup^- in the other can not be ruled out due to complexity of symbiotic process, yet in nature *Rhizobium* strains could be found which have ability to express H_2 uptake activity in a wide variety of host species as has been found in the present studies. Recently, Lambert *et al.* (1985) transferred cosmid born *Hup* genes of *R. japonicum* to *R. meliloti* and *R. trifolii* and found that the recombinants expressed *Hup* genes in alfalfa and clover hosts respectively, indicating that in some of the host dependent *Hup* expression experiments, gaseous atmosphere could be a factor preventing H_2 uptake determination.

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