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Influence of the *Bradyrhizobium japonicum* Hydrogenase on the Growth of *Glycine* and *Vigna* Species

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The effect of the *Bradyrhizobium japonicum* hydrogenase on nitrogen fixation was evaluated by comparing the growth of *Vigna* and *Glycine* species inoculated with a Hup mutant and its Hup$^+$ revertant. In all experiments, the growth of plants inoculated with the strain without hydrogenase was at least equal to the growth of the strain with hydrogenase. For *Glycine usuriensis* and *Glycine max* cv. Hodgson in liquid culture, the growth was higher with the Hup$^-$ strain. It is possible that reduced rates of nitrogen fixation in the presence of hydrogenase are due to O_2 depletion caused by the hydrogen oxidizing, since the oxygen pressure in the air appears to be a limiting factor of symbiotic nitrogen fixation in the soybean.

Nitrogenase simultaneously catalyzes the reduction of molecular nitrogen to ammonium and of protons to hydrogen (4). Most legume root nodules evolve this hydrogen (11, 24), except for the so-called Hup$^+$ (hydrogen uptake-positive) *Rhizobium* strains which possess a hydrogenase. This enzyme catalyzes hydrogen oxidation inside the bacteroids, partially or totally preventing the evolution of this gas outside the nodules. Hydrogenase activity has been postulated to be beneficial for symbiotic nitrogen fixation (8). The evaluation of this benefit has been based mostly on comparisons of nitrogen fixation and growth of the macrosymbiont inoculated with wild-type Hup$^+$ or Hup$^-$ strains. In some experiments the Hup$^+$ strains grew better than the Hup$^-$ strains (1, 3, 7, 14, 21). However, in other experiments, there were no significant differences (6, 13, 18, 20), or the results were contradictory (17). Moreover, the interpretation of such experiments is complicated by the probable interference of characteristics other than the hydrogenase activity which affect nitrogen fixation and which may differ significantly among the wild-type strains. A more accurate assay of the hydrogenase effect in the symbiosis requires the use of point mutant Hup strains differing from the Hup$^+$ parental strain only in the absence of hydrogenase activity. In this paper, we present the results of greenhouse comparisons of growth and yield of *Glycine* and *Vigna* species inoculated with a *Bradyrhizobium japonicum* Hup mutant (PJ17) and its Hup$^+$ revertant (PJ17-1). Data on the effect of external oxygen tension on the nodule acetylene-reducing activity (ARA) are also given.

B. japonicum PJ17 and PJ17-1 were obtained by Lepo et al. (19) from the native strain USDA 122 DES of the Beltsville collection. Their two-dimensional protein patterns were described previously along with some of their symbiotic properties in soybeans (9). The inoculants were prepared in yeast extract-mannitol medium (26). The plant cultivars were used: *Vigna unguiculata* 'TN 88-63', supplied by Van de Ven (CIRAD, Montpellier, France); *Vigna radiata* 'Avroc', supplied by H. Bannerot (Institut National de la Recherche Agronomique, Versailles, France); *Glycine max* 'Hodgson', supplied with *Glycine usuriensis* by A. Vidal (Institut National de la Recherche Agronomique, Montpellier, France). For the greenhouse experiment, the seeds were surface sterilized with 1.3% calcium hypochlorite for 30 min, rinsed five times in sterile distilled water, and transferred into sterile Bacto-Agar for germination at 28°C. Two days later they were inoculated (about 10^9 bacteria per seed) by a 30-min soak in the liquid inoculant and transferred into an aerated liquid medium (aeration rate, 400 ml of air liter of liquid medium^{-1} min^{-1}) as described previously (16). The temperature of the greenhouse was ca. 28°C during the 16-h diurnal period under daylight, which was complemented with mercury vapor lamps, and ca. 20°C during the nocturnal period. These experiments were repeated three times in a completely randomized experimental design.

ARA and hydrogen evolution were measured either on excised nodulated roots (10) or in situ (16). The apparent relative efficiency was computed by the method of Schubert and Evans (24). The response of ARA to the external O_2 pressure was assayed in the in situ design (16), with the root atmosphere being continually flushed with a gas flow mixture (rate, 70 ml min^{-1}) containing 10% C_2H_2 and various amounts of oxygen and nitrogen and the composition being monitored with Tylan (Z. I. de Chesnes Luzais, La Verpillère, France) flow meters.

The data were analyzed statistically with the test of Newman and Keuls.

In short-term greenhouse experiments, two *Vigna* species, *V. radiata* and *V. unguiculata,* and two *Glycine* species, *G. max* and *G. usuriensis* (an ancestor of the soybean), were inoculated with PJ17 and PJ17-1. Both strains were able to nodulate all of the hosts. The nodular mass was identical between the two strains whatever the macrosymbiont, although the fresh weight of nodules at harvest was significantly greater with *V. unguiculata* than with the other legumes (Table 1). The ARA values show that all symbioses were effective, and once again the activities of both strains were not significantly different. However, no effect of hydrogenase was expected in the presence of 10% C_2H_2 because H_2 synthesis by nitrogenase is inhibited by acetylene. The computation of the apparent relative efficiency shows that, with PJ17-1, almost all of the hydrogen is consumed inside the nodules, except with *V. radiata*. This last observation is in agreement with the previous record of a nondetectable hydrogenase activity of the Hup$^+$ strains CB756 with this host (10, 13). With PJ17, all nodules were evolving amounts of hydrogen which were consistent with

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TABLE 1. Growth, nodulation, nitrogenase activity, and apparent relative efficiency of *Vigna* and *Glycine* spp. inoculated with *B. japonicum* PJ17 (Hup^-) and PJ17-1 (Hup^+)

Plant species/ inoculant	Mean[a] ± SD			
	Shoot dry wt (g) plant^{-1}	Nodule fresh wt (g) plant^{-1}	C_2H_4 (μmol) h^{-1} plant^{-1}	Apparent relative efficiency
V. unguiculata				
PJ17	0.48 ± 0.03	0.27 ± 0.08	9.29 ± 2.99	0.42 ± 0.14
PJ17-1	0.51 ± 0.04	0.28 ± 0.08	7.37 ± 2.91	0.99 ± 0.01
V. radiata				
PJ17	0.16 ± 0.02	0.07 ± 0.02	6.36 ± 0.90	0.51 ± 0.07
PJ17-1	0.18 ± 0.05	0.05 ± 0.01	6.01 ± 2.05	0.70 ± 0.11
G. max				
PJ17	0.21 ± 0.02	0.09 ± 0.01	7.30 ± 0.99	0.64 ± 0.03
PJ17-1	0.22 ± 0.07	0.09 ± 0.01	7.24 ± 2.41	0.98 ± 0.02
G. usuriensis				
PJ17	0.23 ± 0.03	0.08 ± 0.03	5.90 ± 2.29	0.67 ± 0.06
PJ17-1	0.18 ± 0.03	0.07 ± 0.02	6.98 ± 1.70	0.95 ± 0.05

[a] The plants were harvested 35 days after sowing. Values given are the means and standard deviations of seven replicates.

an absence of hydrogenase. The dry weight of the shoots was not significantly different between the two strains, except for *G. usuriensis*, which showed greater growth in the absence of hydrogenase ($P < 0.05$).

In long-term experiments with only *G. max* cv. Hodgson, the growth of the plants was significantly greater ($P < 0.05$) with the Hup^- strain PJ17 compared with its Hup^+ revertant (Table 2). The ARA of the PJ17-1-nodulated roots at various physiological ages during the greenhouse experiments was described previously (16). This ARA was not significantly different from the PJ17 ARA during the experiments (data not shown).

The PJ17-nodulated plants of these experiments were subjected to increasing partial pressures of oxygen in their root environment. The ARA, lowest under 10% O_2, rose abruptly between 10 and 20% O_2 and was the highest at 30% O_2 (Fig. 1); above 40%, we know from more recent experiments (data not shown) that ARA starts declining. Thus, the nitrogenase activity of the soybean grown in aerated liquid medium was presumably limited by the oxygen content of the air.

In the greenhouse experiments, the poor yield of the noninoculated soybean (Table 2) established that nitrogen nutrition was the factor limiting plant growth. Therefore, the greater yield of the soybean inoculated with the Hup^- strain compared with the yield of the soybean inoculated with the Hup^+ revertant can be attributed to greater nitrogen fixation by the symbiotic pair that included the strain having no hydrogenase activity.

The two *B. japonicum* strains can be estimated to be very similar in their symbiotic properties, except for their hydrogenase capacity. Indeed, PJ17 is a point mutant since it can revert to the parental phenotype. The two-dimensional protein pattern of its bacteroids is similar to that of PJ17-1 bacteroids, except for one spot which is estimated to correspond to a component of the hydrogenase (9). The nodulation capacity of both strains is comparable irrespective of the host (Table 1). Consequently, the lower nitrogen fixation rate of the Hup^+ strain may be attributed to the presence of the hydrogenase.

These results with PJ17 conflict with the ones obtained

TABLE 2. Yield of *Glycine max* cv. Hodgson in greenhouse trial

Inoculant	Mean shoot dry wt[a] (g) ± SD
Control	5.23 ± 2.08
PJ17	24.80 ± 2.31
PJ17-1	17.33 ± 5.30

[a] Values given are of 11 replicates; plants were harvested 75 days after sowing.

with the Hup mutant PJ18 (12). However, in this latter case, the mutation, although it is a point mutation, has been characterized as a dominant mutation, since it cannot be complemented with a plasmid carrying the parental *hup* gene (15). This mutation may have affected a regulatory gene controlling functions other than just the hydrogenase activity, which is suggested by the major differences observed between the protein patterns of PJ18 and SR, the parental strain (9). Therefore, the results with PJ18 cannot be definitively attributed to hydrogenase. On the contrary, the results with PJ17 are in agreement with recent observations on the pea in which a Tn*5*-induced mutation in the *hup* gene carried by the plasmid PIJ 1008 did not decrease the nitrogen fixation activity (6).

The negative effect of hydrogenase in some of our experimental conditions could be explained by an oxygen limitation for bacteroid respiration. On excised nodules the external oxygen partial pressure corresponding to an optimal nitrogenase activity is about 0.5 atm (ca. 50 kPa) (2). On intact plants, our results, which agree with previous reports (5, 22), show that the optimal oxygen pressure is close to 0.3 atm (ca. 30 kPa), which is still above the partial pressure of oxygen in air (0.21 atm [ca. 21 kPa]). Therefore, the economy of carbon substrate caused by hydrogenase and dem-

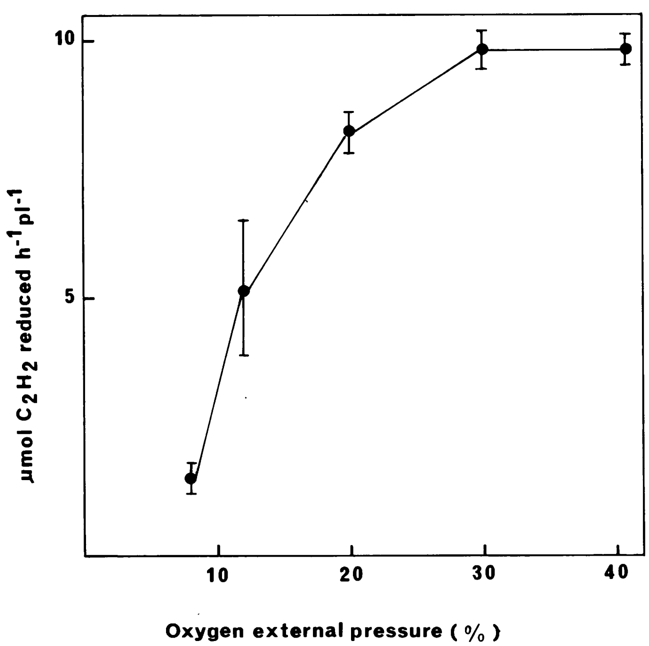

FIG. 1. Influence of external oxygen partial pressure in the root environment on soybean nodule nitrogenase activity. The symbiont is *B. japonicum* cv. Hodgson PJ17. Results are the means and standard deviations of eight replicates. Differences are highly significant ($P < 0.03$), except for the ARA at 30% O_2 and at 45% O_2.

onstrated by the lower CO_2 evolution rate of PJ17-1 nodules compared with PJ17 nodules in air (9) would not have a beneficial effect because the oxygen consumed in H_2 oxidation is probably lacking for the respiration of this extra amount of carbon substrate. Under such conditions of O_2 limitation, the preferential use of H_2 as a donor of electrons to the respiratory chain, known as the H_2 effect in hydrogenase bacteria (23), could limit nitrogen fixation by decreasing the ATP generation rate. In *R. sesbania* the P/O ratio of H_2 oxidation has been shown to be less than that of the carbon substrates (25).

These data may explain why the frequency of Hup^+ strains in the *Rhizobium* genera is lower than that in other genera of N_2-fixing procaryotes. In the latter procaryotes, the hydrogenase-catalyzed H_2 oxidation may help to protect the nitrogenase against O_2 (27). But in the *Rhizobium*-legume symbiosis, the free-O_2 internal tension seems to be at such a low level (2) that supplementary protection by the hydrogenase might have become a costly and unnecessary mechanism during the evolution of this symbiosis, at least in some hosts, such as the soybean and the temperate legumes.

To fully assess the hypothesis mentioned above on the effect of the hydrogenase in the *Rhizobium*-legume symbiosis, more work is needed with Hup^+ and Hup^- isogenic strains other than PJ17 and PJ17-1. It is also necessary to perform similar experiments on legumes other than the soybean and pea, for which the effect of the hydrogenase might differ significantly.

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