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Relative efficiency, ureide transport and harvest index in soybeans inoculated with isogenic HUP mutants of *Bradyrhizobium japonicum*

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Summary. Differences between isogenic uptake hydrogenase (HUP) mutants of *Bradyrhizobium japonicum* in terms of nodule efficiency, N_2 fixation and N incorporation into various plant parts were studied in a monoxenic greenhouse experiment in order to confirm previous results with soybeans and beans inoculated with various HUP^+ and HUP^- strains. The HUP^+ revertant PJ17-1 of a HUP^- mutant (PJ17) of strain USDA DES 122 showed a completely restored relative efficiency (100% versus 78± 2% for the HUP^- mutant), higher nodule efficiency (N_2 fixed per g nodules), higher ureide-N transport rates, higher N contents in pods and higher N harvest indices. All these observations confirm previous experiments with HUP^+ and HUP^- strains.

Key words: N_2 fixation – Soybeans – *Bradyrhizobium japonicum* – HUP mutants – N transport – Ureide – Acetylene reduction assay (ARA)

There is conflicting evidence in the literature about the advantage of inoculating legumes with HUP^+ *Rhizobium* sp. strains and even the use of mutants has not completely clarified the problem. In controlled experiments with greenhouse-grown plants some authors found no differences between HUP^+ and HUP^- strains in plant growth (Gibson et al. 1981; Rainbird et al. 1983; La Favre and Focht 1985), while others found differences (Schubert et al. 1978; Albrecht et al. 1979; Zablotowicz et al. 1980; Hanus et al. 1981; La Favre and Focht 1983; Nelson 1983). Various field and greenhouse experiments carried out in Brazil have shown large differences between soybean plants inoculated with three HUP^+ or three HUP^- strains in terms of grain yields and especially seed N contents and N harvest indices. These differences were correlated ($r = 0.98$) with the percentage of ureide-N transported in the xylem sap (Neves et al. 1985), and similar differences in beans (*Phaseolus vulgaris*) showed good correlations ($r = 0.90$) of relative efficiencies $[1-(H_2/C_2H_4)\times 100]$ with the percentage of ureide-N in the xylem sap (Hungria and Neves 1986; Neves and Hungria 1987). The effects on N_2 fixation and total N incorporation were smaller than those on grain N content, indicating that HUP^+ strains not only fixed more N_2 but favoured N incorporation into the grain rather than into vegetative structures, resulting in higher N harvest indices (Neves et al. 1985).

Little information is available on differences between HUP^+ and HUP^- mutants of the same strain. Greenhouse experiments with hydroponically grown plants inoculated with an HUP^+ revertant (PJ17-1) of an HUP^- mutant (PJ17) showed recovery of the relative efficiency to 95–99% but no significant increase in plant dry weights of 35-day-old soybean plants (Drevon et al. 1987). Older plants (75 days) even showed decreases in dry weights due to inoculation with the HUP^+ revertant, which were attributed to O_2 depletion caused by the hydrogen oxidation (Drevon et al. 1987). Other authors have also failed to observe effects due to inoculation with HUP mutants, in peas, soybeans, and vetch (Eisbrenner and Evans 1983; Cunningham et al. 1985; Sorensen and Wyndaele 1986).

A number of reports, however, have shown increases in plant dry weights and total plant N incorporation in soybeans, peas, and mungbeans inoculated with HUP^+ strains compared with HUP-mutants or genetically engineered strains (Albrecht et al. 1979; Zablotowicz et al. 1980; Lepo et al. 1981; Pahwa and Dogra 1981; De Jong et al. 1982; Eisbrenner and Evans 1983). The effects were often greater for total N

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contents than for plant dry weights but in most of these reports the plants were compared in their vegetative growth stage. Hanus et al. (1981) reported results from plants grown to maturity in the field but the soil at this site was rich in available N. In this case beneficial effects of the HUP^+ strain over its non-revertible HUP^- mutants were observed only for the N% of seeds, and yields were not significantly increased. With the same set of mutant Zablotowicz et al. (1980) observed, in a greenhouse experiment, increases in seed dry weight and N contents of soybeans of 29 and 22%, respectively, which, however, were not significant.

In the present work we used larger soybean plants, grown until mid-pod-fill stage, to compare the effects of the HUP^- mutant and its HUP^+ revertant used by Drevon et al. (1987) on N_2 fixation and N distribution in shoots, in order to confirm their interrelationships with ureide-N transport and grain harvest indices.

Materials and methods

Soybean plants were grown in 900-ml Leonard jars (Vincent 1970) filled with a mixture of washed, sterilized sand and vermiculite (1:2, v:v). Seeds of soybean (*Glycine max* Merr.) cv. Santa Rosa were surface sterilized with 0.2% $HgCl_2$, inoculated with 1 ml log phase broth culture (10^8 cells ml^{-1}) for each set of 15 seeds and planted after 30 min. The inoculants were from a *Bradyrhizobium japonicum* HUP^- mutant (PJ17) of strain USDA DES 122 and its isogenic HUP^+ revertant (PJ17-1) (Lepo et al. 1981). The mutants were kindly supplied by Dr H. J. Evans, Laboratory for Nitrogen Fixation Research, Oregon State University. Each pot initially contained five seeds, which were thinned to two plants per pot 5 days after emergence. The experiment was laid out in a completely randomized block design with five replicates for each harvest and treatment. It was conducted in a greenhouse with air temperature varying between 22° and 32 °C under 13- to 14-h day natural illumination. Every 5 days N-free nutrient solution was applied, containing: KCl (2.00 mM), K_2HPO_4 (2.20 mM), $CaSO_4 \cdot 2H_2O$ (2.00 mM), KH_2PO_4 (0.29 mM), $MgSO_4 \cdot 7H_2O$ (2.00 mM), $CuSO_4 \cdot 5H_2O$ (0.30 µM), $ZnSO_4 \cdot 7H_2O$ (0.76 µM), $MnSO_4 \cdot 2H_2O$ (0.9 µM), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.008 µM), H_3BO_3 (11.56 µM), and $FeSO_4$ (18 µM), pH 6.0–6.2. Three harvests were taken, 28 (beginning of N_2 fixation), 48 (flowering) and 70 (mid-pod fill stage) days after emergence.

Xylem sap was collected during the first two harvests. At the third harvest not enough sap was exuded for collection. The plants were cut below the cotyledonary node, and the cut ends were rinsed with distilled water and dried with tissue paper. Thereafter, bleeding (xylem) sap was collected for 10 min in calibrated microcapillaries (20 and 50 µl) so that the exudation rate could be calculated. The sap was stored at −20 °C until analysis, and then ureide-N and total N were analysed colorimetrically according to Boddey et al. (1987). Plant tissues were extracted as described by Thomas and Schrader (1981) and then analysed for ureide-N as indicated above.

After sap collection, the plants were assayed by gas chromatography for acetylene reduction activity and for H_2 evolution. The roots of one plant per pot were cut off and incubated for 4 min in 300-ml glass bottles containing 12% (v:v) of acetylene. A 1-ml gas sample was removed at the beginning and at the end of the incubation period. Errors in the use of the acetylene reduction method in closed systems have been reported (Minchin et al. 1983); therefore an additional experiment was carried out, using intact plants in a continuous flow system run at a rate of 100 ml min^{-1} with 12% acetylene sampled after 1, 3, 4, 5, 7, 10, 15, and 30 min, and there was no significant decrease in the rate of acetylene reduction due to the presence of C_2H_2 (N. G. Rumjanek, unpublished data). However, effects due to plant disturbance (Minchin et al. 1986) were not checked, and therefore the results should not be used as a measure of N_2 fixation.

Simultaneously, the remaining plants were assayed for H_2 evolution on nodules detached from the root system with small root segments (±0.5 cm) (Schubert and Evans 1976) and incubated for 15 min under air. H_2 was analysed in a Varian 1420 with a thermal conductivity detector using a 100-cm stainless-steel column (0.32 cm external diameter) operated at 40 °C using Ar as a carrier gas.

Results

As expected there were no differences between the two HUP mutants in respect to nodule weights and acetylene reduction activity. The relative efficiency of nodules formed by the HUP^+ revertant was restored to 100% during the full growth period (Table 1). Acetylene reduction activity reached its maximum at the flowering stage, when the total N_2 fixed was 23% greater in plants inoculated with the HUP^+ revertant. Consequently, nodule-forming efficiency at the second and third harvests was also significantly greater in these plants (Table 1).

At the flowering stage not only the total N transport rate but also the percentage of ureide-N in the xylem sap were increased with the HUP^+ revertant resulting in a 37% greater total ureide-N transport (Table 2).

The difference in N_2 fixation between the two HUP mutants was reflected in the total N incorporation at flowering, 24 and 20% in leaves and stems, respectively (Table 3). Again, the increases in total ureide-N contents in the plant parts were larger than the increases in total N, indicating that there were other differences than the higher N_2 fixation in the HUP^+ revertant. The effect of the HUP^+ phenotype at the mid-pod-fill stage was observed only in the pods, and these differences would probably have been even more pronounced in field-grown plants at maturity; in Leonard jars full maturity could not be reached as soybean plants become seriously pot-bound once they get bigger. The efficiency of N translocation to the seeds was confirmed by the lower N loss in senesced leaves and by the higher N harvest indices of plants inoculated with the HUP^+ revertant (Table 3).

Discussion

Pronounced differences between *Bradyrhizobium japonicum* strains in relation to nodule-forming efficiency (mg N_2 fixed per g nodules) have been ob-

Table 1. Effects of HUP mutants[a] on nodulation and N_2 fixation of soybeans grown in monoxenic Leonard jars[b]

Days after emergence	Nodule dry weight (mg plant^{-1})		ARA[c] (μmol C_2H_4 plant^{-1} h^{-1})		Relative efficiency[d]		Total N_2 fixed (mg N plant^{-1})[e]		Nodule efficiency (mg N_2 fixed per g nodules)	
	HUP^{-}[3]	HUP^{+}[3]	HUP^{-}	HUP^{+}	HUP^{-}	HUP^{+}	HUP^{-}	HUP^{+}	HUP^{-}	HUP^{+}
28	560a	523a	29.4a	28.4a	81b	100a	124a	125a	221a	239a
48	795a	841a	103.7a	117.5a	78b	100a	163b	201a	205b	238a
70	657a	658a	16.5a	16.8a	77b	100a	268b	304a	408b	462a

[a] *Bradyrhizobium japonicum* PJ17 hydrogenase uptake negative mutant (HUP^{-}) of strain USDA DES 122 and its HUP positive (HUP^{+}) revertant PJ17-1
[b] Data are means of five replicate jars and values followed by the same letter are not significantly different ($P = 0.05$ by Tuckey's test)
[c] ARA, acetylene reduction activity
[d] Relative efficiency $= 1 - [(H_2)/(C_2H_4)] \times 100$
[e] Total N_2 fixed = leaf N + stem N + root N + pod N − N in seeds

Table 2. Effects of HUP mutants[a] on N transport in xylem sap at flowering stage of soybeans[b]

	HUP^{-}	HUP^{+}	Difference (%)
Xylem sap exudation rate (μl plant^{-1} min^{-1})	11.8a	12.6a	
Total N transport rate[c] (μg N plant^{-1} min^{-1})	1.91b	2.34a	22
% Ureide-N [(Ureide-N/Total N) × 100]	79.36b	88.80a	12
Ureide-N transport rate[c] (μg ureide-N plant^{-1} min^{-1})	1.52b	2.08a	37

[a] *Bradyrhizobium japonicum* PJ17 hydrogenase uptake negative mutant (HUP^{-}) of strain USDA DES 122 and its HUP positive (HUP^{+}) revertant PJ17-1
[b] Data are means of five replicate jars and values followed by the same letter are not significantly different ($P = 0.05$ by Tuckey's test)
[c] Estimated from the exudation rate data

served previously (Dobereiner et al. 1970), when some of the strains were considered "exceptionally efficient". These early observations seem particularly relevant today in view of the findings reported by Neves et al. (1985) and those in the present work. The HUP^{+} strains used in this work, just like those in the earlier experiments fixed significantly more N_2 than the HUP^{-} strains from the same amount of nodule tissue, indicating differences in nodule metabolism.

Differences between HUP^{+} and HUP^{-} strains have been attributed to the energy saving due to recycling of liberated H_2 coupled to ATP generation (Albrecht et al. 1979). Estimates covering a series of hypothetical situations, combining variable electron allocation with increasing hydrogenase uptake activities, demonstrated that the energy saving due to an active hydrogenase system could be as high as 30% (Pate et al. 1981) and therefore could not explain the much

Table 3. Effect of HUP mutants[a] on N distribution in soybean plants grown in monoxenic Leonard jars[b]

Days after emergence	Total N (mg N plant^{-1})			Ureide-N (μmol N plant^{-1})		
	HUP^{-}	HUP^{+}	Difference (%)	HUP^{-}	HUP^{+}	Difference (%)
48 (flowering stage)						
Roots	20.6a	21.1a		n.d.[c]	n.d.	
Stems	48.6b	58.6a	20	120.1b	197.1a	64
Leaves	112.0b	138.8a	24	30.7b	48.6a	58
70 (mid-pod-fill)						
Roots	15.2a	19.8a		n.d.	n.d.	
Stem	39.1a	34.2a		60.6a	69.8a	
Leaves	106.8a	114.3a		30.0a	37.2a	
Senesced leaves	11.6b	9.5a	18	n.d.	n.d.	
Pods	113.5b	144.4a	27	69.0b	87.5a	27
N harvest index[d]	0.40b	0.45a	12			

[a] *Bradyrhizobium japonicum* PJ17 hydrogenase uptake negative mutant (HUP^{-}) of strain USDA DES 122 and its HUP positive (HUP^{+}) revertant PJ17-1
[b] Data are means of five replicate jars and values followed by the same letter are not statistically different ($P = 0.05$ by Tuckey's test)
[c] n.d., not determined
[d] N harvest index = N in pods/total N in plants at mid-pod-fill stage

larger differences observed by some authors (Albrecht et al. 1979; Lepo et al. 1981; De Jong et al. 1982). The interpretation of these experiments is not only complicated by differences other than those in hydrogenase activity between strains, but also by the effects of HUP mutants on nodule N metabolism.

The pronounced differences between HUP^+ and partially or completely HUP^- strains observed by Neves et al. (1985) and Hungria and Neves (1987) in soybeans and beans were not only confirmed in the present work but also helped to explain the conflicting effects of the HUP^+ strains. The highly significant correlations between relative efficiency and ureide-N concentrations (percentage of ureide-N to total N) in the xylem sap observed by Neves et al. (1985) in field-grown plants, where no differences in total N_2 fixed were observed in spite of 56% yield increases, indicate that effects other than the HUP strain itself, but closely related to it, are responsible for the grain yield increases. In all those experiments, as also in the present work, the HUP^+ strains increased the proportion of ureide-N transported to the shoot. In the present experiment, since the plants were grown in an N-free sand/vermiculite substrate, this finding indicates that besides increasing N_2 fixation, the HUP^+ revertant induced a proportionate increase in ureide-N synthesis compared to the HUP^- mutant, confirming the main differences between HUP^+ and HUP^- strains reported by Neves et al. (1985). In addition, in the present work, the HUP^+ strains increased the N harvest indices, i.e. they reduced the N loss in senesced leaves and increased the efficiency of N translocation to the seeds. Although the biochemical basis for the relationship between HUP activity and nodule ureide-N metabolism remains unknown, the effect of the HUP strains on plant N distribution is likely to be related to a more efficient transport of ureide-N to the grain (Yoneyhama 1984a, b).

These effects can be of considerable economical importance. For example, in Brazil, a mean increase of only 10% in soybean yields due to the introduction of efficient HUP^+ strains could lead to an annual profit of more than one billion $ US.

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