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Hydrogen Reactions of Nodulated Leguminous Plants

II. EFFECTS ON DRY MATTER ACCUMULATION AND NITROGEN FIXATION[1]

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ABSTRACT

The interaction between the ATP-dependent evolution of H_2 catalyzed by nitrogenase and the oxidation of H_2 via a hydrogenase has been postulated to influence the efficiency of the N_2-fixing process in nodulated legumes. A comparative study using soybean (*Glycine max* L. Merr.) cv. Anoka inoculated with either *Rhizobium japonicum* strain USDA 31 or USDA 110 and cowpea (*Vigna unguiculata* L. Walp.) cv. Whippoorwill inoculated with *Rhizobium* strain 176A27 or 176A28 cultured on a N-free medium was conducted to address this question. Nodules from the Anoka cultivar inoculated with USDA 31 evolved H_2 in air and the H_2 produced accounted for about 30% of the energy transferred to the nitrogenase system during the period of active N_2 fixation. In contrast the same soybean cultivar inoculated with USDA 110 produced nodules with an active hydrogenase and consequently did not evolve H_2 in air. A comparison of Anoka soybeans inoculated with the two different strains of *R. japonicum* showed that mean rates of C_2H_2 reduction and O_2 consumption and mean mass of nodules taken at four times during vegetative growth were not significantly different.

When compared to Anoka inoculated with USDA 31, the same cultivar inoculated with USDA 110 showed increases in total dry matter, per cent nitrogen, and total N_2 fixed of 24, 7, and 31%, respectively. Cowpeas in symbiosis with the hydrogenase-producing strain 176A28 in comparison with the same cultivar inoculated with the H_2-evolving strain 176A27 produced increases in plant dry weight and total N_2 fixed of 11 and 15%, respectively. This apparent increase in the efficiency of N_2 fixation for nodulated legumes capable of reutilizing the H_2 evolved from nitrogenase is considered and it is concluded that provision of conclusive evidence of the role of the H_2-recycling process in N_2-fixing efficiency of legumes will require comparison of *Rhizobium* strains that are genetically identical with the exception of the presence of hydrogenase.

In regard to their capability to catalyze nitrogenase-dependent H_2 evolution and H_2 oxidation via a hydrogenase, leguminous symbionts that have been tested may be classified into two categories (13, 15). The first includes symbionts that evolve H_2 produced by the nitrogenase system. The second group is like the first, with the exception that H_2 produced via the nitrogenase system is recycled via a hydrogenase. So far it appears that all *in vivo* N_2-fixing systems reduce protons during the N_2-fixing process. Our investigations have concentrated on testing the premise that the capacity of nodules to recycle the H_2 from the nitrogenase system would be expected to improve the efficiency of energy utilization during N_2 fixation and therefore increase the quantity of N_2 fixed and plant yield.

The function of hydrogenase within legume nodules has been discussed by Dixon (6, 7) who has proposed the following possible roles: (*a*) serves as a means for reducing H_2 concentrations in nodules below inhibitory levels; (*b*) acts as a mechanism for protecting nitrogenase from O_2 inactivation by using H_2 as a substrate to support respiration; and (*c*) provides a system whereby the H_2 evolved from nitrogenase is metabolized and a portion of the otherwise wasted energy is conserved (4, 6). The proposal that H_2 metabolism leads to ATP synthesis or to available reducing power which increases the efficiency of energy utilization within the nodule has been favored in most discussions (1, 6–8, 15). Dixon (6) has shown that the oxidation of H_2 via hydrogenase in pea nodule bacteroids apparently is linked to ATP production. At this time there are no published reports supporting the conclusion that hydrogenase in nodules is capable of providing the reducing potential via the appropriate electron carriers, to support N_2 fixation directly. If energy is the major limitation of N_2 fixation in leguminous species as has been proposed (4, 9), then the coupling of the oxidation of H_2 to energy-yielding processes conceivably could increase the rate of N_2 fixation. Alternatively the conservation of energy through H_2 recycling processes might decrease the demand for photosynthate and consequently contribute to increased dry matter production. The possibility also must be considered that oxidation of H_2 via hydrogenase is not coupled to useful energy-producing processes and therefore is not beneficial to the plant.

This paper describes an investigation for the purpose of comparing the yield and efficiency of N_2 fixation by legumes with nodules that recycle H_2, with those that lack an active hydrogenase and evolve the H_2 produced during N_2 fixation.

MATERIALS AND METHODS

Seeds were surface-sterilized by immersion for 1 min in 95% ethyl alcohol and then 5 min in 0.2% acidified $HgCl_2$ (17). Residual $HgCl_2$ was removed by rinsing 10 times with sterile distilled H_2O. Seeds were placed on sterile water agar plates in the dark at 26 C where they germinated. After 2 days soybean seedlings (*Glycine max* L. Merr cv. Anoka) were selected and inoculated with *Rhizobium japonicum* strains USDA 110 or USDA 31 and cowpea seedlings (*Vigna unguiculata* L. Walp. cv. Whippoorwill) with *Rhizobium* strains 176A28 or 176A27 and transplanted into surface-sterilized 20-cm plastic pots filled with Perlite. The pot cultures with seedlings were kept moist for 3 days and then irrigated with N-free nutrient solution as described previously (13). Plants were grown in the greenhouse with supplemental fluorescent lighting of about 5380 lux at 0.73 meter on a 16-hr photoperiod. The day temperature was maintained near 29 C, the night temperature near 24 C. Ten replicate pot cultures each containing four soybean plants or five cowpea plants were used for each strain at each harvest date. Experiments were arranged in a completely randomized design in the greenhouse.

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Rates of C_2H_2 reduction, H_2 evolution, and O_2 utilization of excised nodules were performed as described previously (14) except rates of C_2H_2 reduction were measured on separate samples of nodules. Gases were obtained from the same sources as listed previously (14). At least 2 days prior to each experiment, plants in the pot cultures were transferred into a growth chamber (day temperature, 29 C; night temperature, 24 C; light intensity, 22,000 lux and photoperiod, 16 hr). Samples of nodules with attached root pieces (about 0.3 g) were excised and used for C_2H_2 reduction and for H_2 evolution and O_2 uptake measurements. The remaining nodules were removed and weighed. The entire plants including nodules were dried at 70 C, weighed, and ground. After Kjeldahl digestion of 0.2-g samples, nitrogen contents were determined on diluted aliquots of the digested material by use of an ammonia-sensitive electrode (Orion 95-10-00) as described by Bremner (3). Analyses of variance were calculated for the data presented with the exception of relative efficiencies.

RESULTS

Experiments with Soybeans. Based on previous findings (13, 15) two soybean cultivar-*R. japonicum* strain combinations were selected to determine the effect of the capability to recycle H_2 produced by the nitrogenase system in nodules, on dry matter accumulation and total N_2 fixation. The Anoka cultivar inoculated with USDA 31 is known to exhibit a rapid rate of net H_2 evolution from nodules while the same cultivar inoculated with USDA 110 shows little or no H_2 evolution in air and has a capability of utilizing an exogenous supply of H_2. Plants from Anoka soybean plants inoculated with USDA 31 and cultures of the same cultivar inoculated with USDA 110 were harvested at 18, 27, 34, and 41 days after planting. No significant differences in nodule weights were observed between the two symbiont combinations at any of the four sampling dates (Fig. 1A). Likewise mean rates of respiration (O_2 consumption) were not measurably different for samples of nodules from Anoka soybeans inoculated with the two

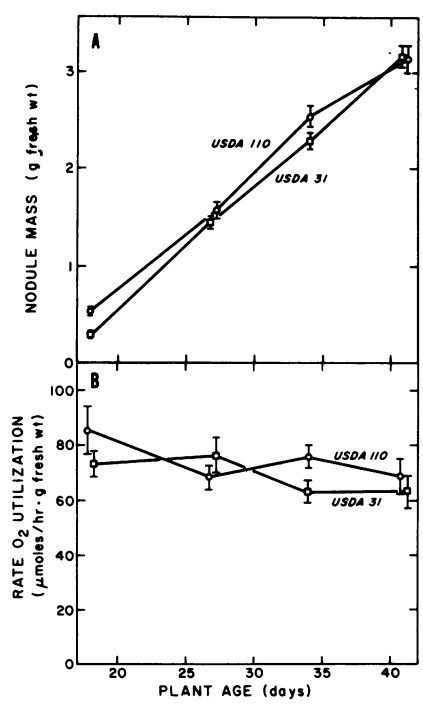

FIG. 1. Rates of nodule mass (A) and O_2 utilization (B) are given for soybean plants (cv. Anoka) inoculated with either USDA 31 (□) or USDA 110 (○). Values are means of determinations from 10 replicate samples ± the SEM. Rates of O_2 utilization were determined amperometrically on samples used for measurements of H_2 evolution. Total nodule mass was determined for each replicated pot culture.

Table I. Rates of C_2H_4 Formation and H_2 Evolution of Soybean Nodules as a Function of Plant Age and Rhizobium Strain

Plant Age	Rate C_2H_4 Formation[1]		Rate H_2 Evolution[1] in Ar:O_2:CO_2	
	Strain USDA 31[2]	Strain USDA 110	Strain USDA 31	Strain USDA 110
(days)				
18	9.0 ± 1.0b	16.4 ± 1.1c	9.6 ± 0.9b	4.4 ± 0.8a
27	15.0 ± 1.2c	10.9 ± 0.5b	15.3 ± 1.5c	2.4 ± 0.5a
34	17.4 ± 0.9b	15.9 ± 1.5b	15.9 ± 1.5b	5.2 ± 0.9a
41	7.4 ± 0.3b	7.8 ± 1.4b	9.6 ± 1.9b	4.0 ± 1.6a

[1]Results are means of determinations on ten replicate cultures ± the standard error of means (SEM) and are expressed in μmoles product formed per hour per g fresh wt of nodules. Values at each plant age followed by same letter are not significantly different using a Tukey's value at the 5% level of 2.5. Since both C_2H_2 reduction and H_2 evolution under Ar are measures of electron flow through nitrogenase the statistical treatment is applicable to both determinations.

[2]Nodules produced by strain USDA 31 evolved H_2 in air while those produced by strain USDA 110 did not.

different strains (Fig. 1B). At each harvest date (Table I) the rate of H_2 evolution in $Ar/O_2/CO_2$ and the rate of C_2H_2 reduction for nodules from plants inoculated with USDA 31 were not appreciably different. This observation is consistent with a previous report (14) showing that the rate of H_2 evolution in an atmosphere lacking N_2 and the rate of C_2H_2 reduction were approximately equivalent when no detectable H_2 recycling was taking place.

As shown in Table I, there was a marked difference between the rate of H_2 evolution under Ar and the rate of C_2H_2 reduction in the nodules from the Anoka cultivar inoculated with USDA 110. This suggests the involvement of a hydrogenase in H_2 recycling in this symbiont combination. There were, however, no consistently significant differences between the mean rates of C_2H_2 reduction of the Anoka-USDA 31 and Anoka-USDA 110 combinations for any of the four determinations. In contrast, the rates of H_2 evolution in air were markedly different for the two symbionts (Fig. 2A). Nodule samples from Anoka soybeans inoculated with USDA 31 exhibited net H_2 evolution in air. The proportion of total electron flow through nitrogenase in nodules from USDA 31 that is involved in N_2 reduction was relatively constant ranging from a relative efficiency value of 0.66 to 0.74 at all four sampling dates (Fig. 2B). This behavior may be contrasted with that of nodules from the Anoka cultivar inoculated with USDA 110 which evolved essentially no H_2 in air (Fig. 2A) and showed relative efficiency values near unity throughout the growth period.

Total dry matter production and total N_2 fixed were used as criteria in the evaluation of the over-all efficiency of the two symbiotic combinations. During the last three sampling dates in the experiment, the Anoka cultivar inoculated with USDA 110 (with hydrogenase) produced a significantly higher content of dry matter than the same cultivar inoculated with USDA 31 (Fig. 3A). Although there was no appreciable difference in N contents of plants inoculated with the two strains at the initial sampling date, after 18 days the total N_2 fixed by the Anoka-USDA 110 combination was significantly higher than the Anoka-USDA 31 combination (Fig. 3B). After 41 days of growth the Anoka cultivar inoculated with USDA 110, in comparison with the same cultivar inoculated with USDA 31, showed a 31% increase in N_2 fixed. From the experiments conducted so far, the only known difference between the two symbionts is the demonstrated capability of nodules form Anoka inoculated with USDA 110 to oxidize H_2 via a hydrogenase (Fig. 4).

Experiments with Cowpeas. From previous research it was determined that *Rhizobium* strain 176A27 in symbiosis with cowpea cv. Whippoorwill evolved H_2 from nodules while strain 176A28 in symbiosis with the same cultivar did not (15). These two strains were further evaluated in this experiment to determine effects on relative efficiencies, nodule fresh weight, plant dry weight, and total N_2 fixed during growth (Table II). Nodule fresh weight and plant dry weight did not differ appreciably between the two strains for the first three sampling periods. By the fourth

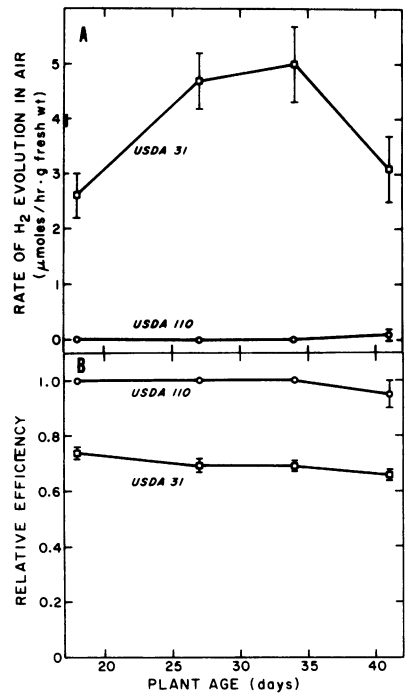

FIG. 2. Relative efficiencies and rates of H_2 evolution for excised nodules from soybeans (cv. Anoka) at various times during growth. Values of rates of H_2 evolution in air (A) and relative efficiencies (B) are given for nodules excised from soybean plants inoculated with either strain USDA 31 (□) or USDA 110 (○). Values shown are means of 10 replicates ± the SEM. If no error bar is shown, the SEM was less than 0.005 of the value presented.

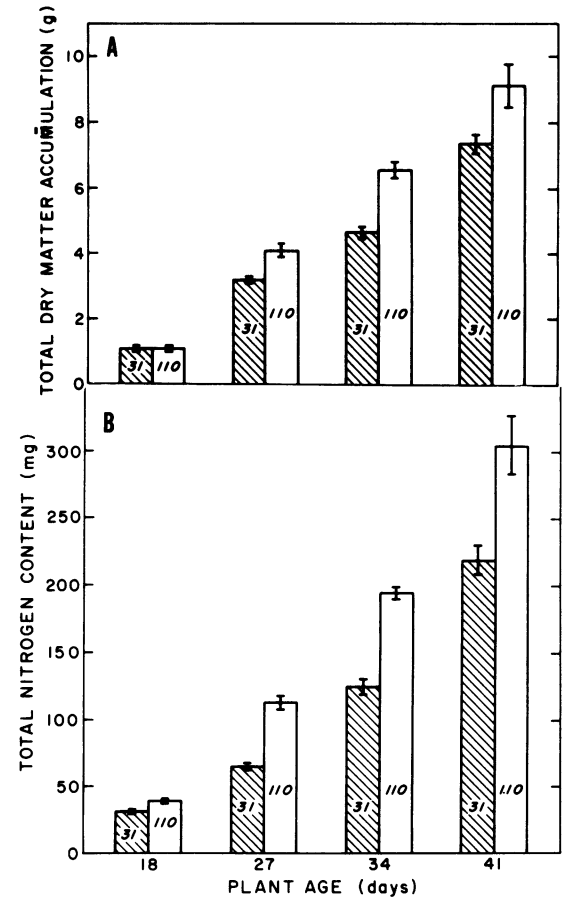

FIG. 3. Total dry matter accumulation (A) and total nitrogen (B) for soybean plants. Results are means of 10 replicated treatments ± the SEM for soybean plants harvested at various times during growth. Plants were grown without added combined nitrogen. Strains of *R. japonicum* used were USDA 31 and USDA 110. All differences in dry weight between strains were significant at the 5% level except at 18 days after planting. All differences in total nitrogen content between strains were significant at the 1% level. Initiation of flowering occurred between 34 and 41 days after planting.

sampling date, however, there was a significant increase in nodule weight for strain 176A28 as compared to 176A27. A marked plant dry weight difference in favor of strain 176A28 also was observed at this sampling date.

The most dramatic difference between the two symbionts is the total N_2 fixed and the relative efficiencies. Strain 176A28 in symbiosis with the Whippoorwill cultivar fixed more N_2 than strain 176A27 at 50 and 56 days of age (Table II). The relative efficiencies of nodules formed by strain 176A28 were consistently higher at each sampling date than nodules formed by strain 176A27. Relative efficiencies of nodules formed by strain 176A27 decreased with increasing plant age.

DISCUSSION

In this paper the yield and total N contents of a cultivar each of soybeans and cowpeas inoculated with a H_2-evolving and a H_2-recycling strain of *Rhizobium* have been compared. Most legumes lose 30% or more of the total energy supplied to the nitrogenase system through H_2 gas evolution, but a few legumes inoculated with selected *Rhizobium* strains have been found that produce nodules containing a hydrogenase (13–15). This provides a means for the utilization of the H_2 that is produced from the nitogenase reaction. In addition to the legumes and nonlegumes that have been examined some bacteria possess mechanisms that enable them to recycle H_2 from the nitogenase system (2, 5, 10, 12, 16). The magnitude of H_2 evolution with many legumes such as the Anoka cultivar of soybeans inoculated with USDA strain 31 amounts to 25 to 35% of the total nitrogenase activity. The capacity to recycle H_2 would be expected to increase the efficiency of energy utilization by an N_2-fixing organism especially under conditions where photosynthate or other energy sources are limited (2, 4, 6, 8, 9, 14, 16). The cultivar of soybean, Anoka, inoculated with USDA strain 110 and also Whippoorwill cowpeas inoculated with *Rhizobium* strain 176A28 produced nodules with

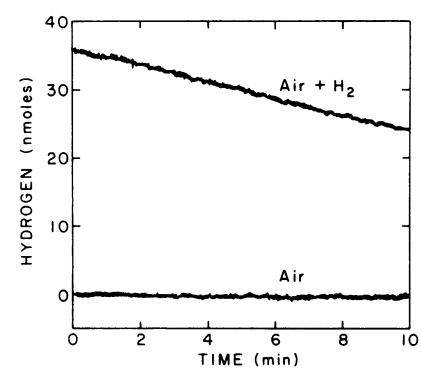

FIG. 4. Utilization of exogenous H_2 by excised nodules of soybean plants (cv. Anoka) inoculated with *R. japonicum* strain USDA 110 as a function of time. Nodules (0.2270 g) were excised from 41-day-old plants and the rates of H_2 evolution in air and H_2 utilization in air were monitored amperometrically. Tracings for both measurements were superimposed on the same time frame for convenience.

an active H_2 uptake mechanism and evolved little or no H_2 in air. The relative efficiency value which is an estimate of the fraction of the total energy supplied to nitrogenase that actually is used for N_2 reduction apparently approaches unity for symbionts of this type.

Table II. Growth, Nitrogen Fixation and Relative Nodule Efficiencies of Cowpeas as Influenced by Rhizobium Strain.

Results are means of determinations on ten replicate cultures ± the standard error of means (SEM).

Plant age	Nodule fresh weight		Plant dry weight		Total nitrogen		Relative efficiency[1]	
	strain[2] 176A27	strain[2] 176A28	strain 176A27	strain 176A28	strain 176A27	strain 176A28	strain 176A27	strain 176A28
(days)	(grams/culture)		(grams/culture)		(mg/culture)			
24	1.22 ± .07	1.13 ± .07	2.58 ± .07	2.95 ± .09	86.0 ± 3.7	95.5 ± 3.4	0.82 ± .01	0.98 ± .01
36	2.15 ± .08	2.22 ± .10	5.95 ± .57	5.64 ± .27	208.6 ± 12.2	221.6 ± 11.7	0.65 ± .04	1.00 ± .00
50	4.32 ± .18	4.49 ± .11	16.00 ± .94	17.72 ± .42	528.3 ± 36.0	608.2 ± 17.4	------	------
56	7.42 ± .25	8.47 ± .28	21.66 ± 1.29	24.15 ± .94	693.5 ± 43.7	798.2 ± 35.5	0.53 ± .12	1.00 ± .00
LSD[3] (0.05)	0.46		1.94		70.3		0.12	
(0.01)	0.62		2.57		93.4		0.33	

[1]Determined by 1 $\frac{\text{rate of } H_2 \text{ evolution in air}}{\text{rate of } C_2H_2 \text{ reduction}}$

[2]Nodules produced by strain 176A27 evolved H_2 while those produced by strain 176A28 did not. All plants were growing vegetatively.

[3]LSD values may be used for comparison of differences between strains at a given plant age.

Hypothetically the rate of N_2 fixation in an organism that does not evolve H_2 from the nitrogenase system (relative efficiency of 1) conceivably might be 50% higher than a comparable organism that loses 33% (relative efficiency of 0.67) of the electron flow through nitrogenase as H_2 evolution. This conclusion is based upon the assumption that the efficient organism either produces no H_2 from the nitrogenase reaction or is 100% efficient in reclaiming the energy by recycling the H_2 produced by the nitrogenase reaction. There are no known examples of isolated nitrogenase systems that fix N_2 without concomitant H_2 evolution. So far all non-H_2-evolving nodules that have been observed exhibit a capacity to utilize H_2 via a recycling process (13–15).

If it is assumed that 2 electrons and 4 molecules of ATP are required to form 1 molecule of H_2 (4, 6) and that 2 molecules of ATP, or one pair of electrons is derived from the oxidation of H_2 (6), then only one-third of the energy lost through H_2 evolution from the nitrogenase system is recovered by the recycling process. Under conditions where 30% of the energy supplied to nitrogenase in nodules is being lost as evolved H_2 only a 10% increase in N_2 fixed would be predicted as a result of H_2 recycling. This argument assumes that the energy conserved by the recycling process is used solely to alleviate an energy limitation for N_2 fixation. Alternatively, the energy conserved from H_2 recycling might decrease the demand for photosynthate which would be required to support N_2 fixation. This conserved energy might be expected to be available for increases in plant biomass with corresponding increases in total N_2 fixed (11).

Comparisons of the effects of H_2-evolving *versus* non-H_2-evolving strains of *Rhizobium* used for the inoculation of soybeans and cowpeas have shown that both legumes that were nodulated with the non-H_2-evolving strains produced increases in plant dry weight and total quantity of N_2 fixed. In the experiment with soybeans the percentage increase in total N by plants inoculated with the hydrogenase-producing strains was greater than the percentage increase in dry weight which suggests that the actual rate of N_2 fixation was higher for this symbiont. Increases in total N_2 fixation apparently are not associated with effects of the strains on total nodule mass or rates of nodule respiration. According to Dixon (6) the O_2 requirement for respiration of pea nodules that exhibited hydrogenase activity was actually less than the requirement for nodules lacking hydrogenase. No such trend has been observed in our experiments with soybeans and cowpeas. The only known difference in the two types of strains that we have examined is the capablity to produce a hydrogenase in nodules that apparently participates in a H_2 recycling process. Although the capacity to recycle H_2 seems to be correlated with increased yields and N_2 fixation in these limited tests, the possibility exists that the nodules formed by the H_2-evolving and hydrogenase-producing strains of *Rhizobium* differ in other unknown ways. Increases in yield and N_2 fixation possibly might be associated with a more efficient conversion of photosynthate into ATP and reductant within the nodules inoculated with the strains of *Rhizobium* that have a capacity to synthesize hydrogenase and recycle H_2. Definitive results regarding these questions must await the availability of strains of *Rhizobium* that are isogenic with the exception of hydrogenase. Alternately it may be possible to compare a large population of *Rhizobium* strains that have the capability of synthesizing hydrogenase in nodules with populations that lack this capacity. Conclusions then could be based upon the assumption that variability in characteristics other than the known differences in capacity to synthesize hydrogenase were random among both populations.

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