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Significance of Hydrogen Evolution in the Carbon and Nitrogen Economy of Nodulated Cowpea[1]

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ABSTRACT

The carbon and nitrogen economies of a single cultivar of cowpea (*Vigna unguiculata* (L.) Walp. cv Caloona) nodulated with either a high H_2-evolving strain (176A27) or a low H_2-evolving strain (CB756) of *Rhizobium* were compared. The two symbioses did not differ in total dry matter production, seed yield, nitrogen fixed, the spectrum of nitrogenous solutes produced by nodules for export, or the partitioning of net photosynthate within the plant throughout the growth cycle. Detailed examination of the carbon and nitrogen economy of the nodules, however, showed a significant difference between the symbioses. Nodules formed with CB756 lost less CO_2 in respiration compared to the higher H_2-evolving symbioses and this could have been largely responsible for a 36% better economy of carbon use in CB756 nodules during the period of maximum H_2 evolution (48-76 days) and over the whole growth period (20-90 days), a 16% economy. In terms of overall net photosynthate generated by the plant, these economies were equivalent to 5% and 2% of the carbon utilized in the two periods, respectively. From the differences in H_2 evolution and CO_2 production by nodules of the two symbioses, the cost of H_2 evolution was found to be 3.83 ± 0.6 millimoles CO_2/millimoles H_2 for plants grown in sand culture and 1.69 ± 0.48 millimoles CO_2/millimoles H_2 for those in water culture. In both symbioses, the ratio of H_2 evolution to N_2 fixed varied markedly during ontogeny, indicating a significant variation in the relative efficiency and thus metabolic cost of N_2 fixation at different stages during development.

Concomitant with N_2 fixation in legume nodules the enzyme nitrogenase reduces protons to form H_2 (7, 8, 21). The ratio of H_2 produced to N_2 fixed has been suggested from studies of the isolated enzyme to occur with a minimum value of 1:1 (14) and it has been proposed that as much as 60% of the electron flow through nitrogenase *in vivo* may lead to proton reduction (8, 22). Since nodule functioning is apparently closely dependent on the availability of photosynthate (10), any diversion of electrons away from N_2 to protons would presumably result in a decreased energy supply for N_2 reduction, potentially reducing legume yield. Some *Rhizobium* strains contain a unidirectional hydrogenase capable of recycling some or all of the H_2 produced in nodules (3, 4, 6), thus serving to conserve energy and minimize the inefficiency of N_2 fixation which may result from H_2 evolution. Calculations based on a theoretical consideration of nodule functioning (19) indicate that complete H_2 recycling would recover 12.5% of energy expended by nitrogenase.

Significantly greater dry matter yield and N_2 fixation under growth cabinet conditions has been shown by a number of soybean symbioses formed with *Rhizobium* strains able to recycle H_2 compared to those formed with strains unable to oxidize H_2 (1). While the *Rhizobium* strains used could have differed in many traits as well as hydrogenase and, under field conditions, yield response to the character was not significant (9), these studies indicate that H_2 recycling in nodules may indeed enhance the efficiency of photosynthate use in legumes.

In this study, the detailed carbon and nitrogen economies of a single cowpea variety (*Vigna unguiculata* (L.) Walp. cv Caloona) nodulated either with a strain of *Rhizobium* resulting in a low level (CB756) or a high level (176A27) of H_2 evolution in the symbioses have been compared. Particular emphasis has been placed on the relative costs of N_2 fixation in the economy of carbon use in nodules of the two symbioses and in the possible significance of the difference in H_2 evolution to nodule functioning.

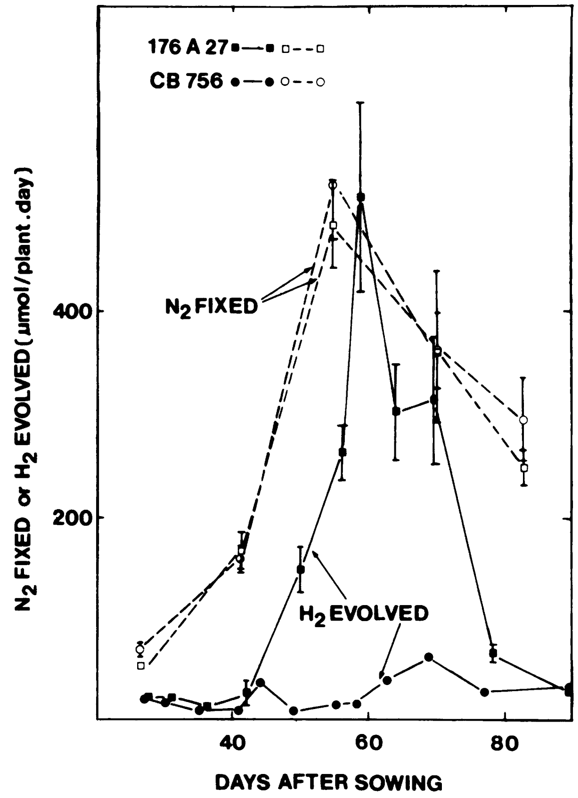

FIG. 1. Rates of N_2 fixation and H_2 evolution by cowpea plants nodulated with *Rhibozium* strain 176A27 or CB756 during growth. Bars are standard erros of means and where absent are within the diminsions of the symbols.

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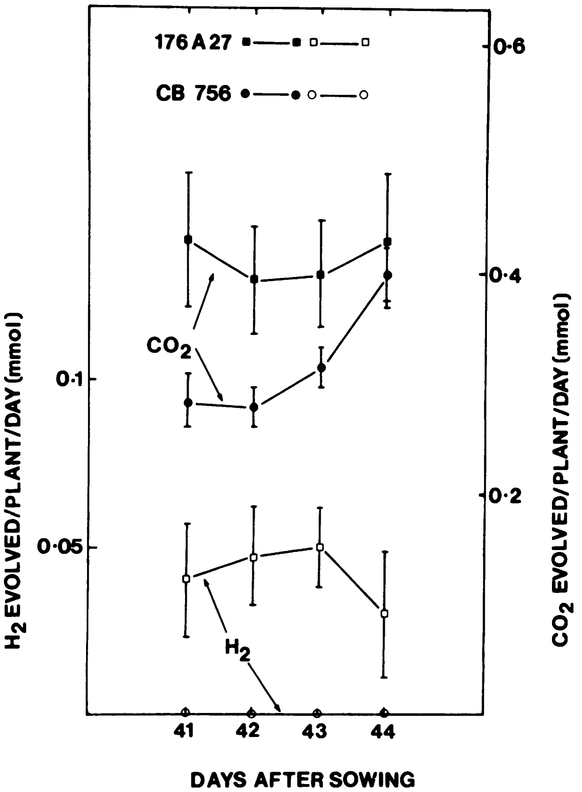

FIG. 2. Rates of H_2 evolution or CO_2 evolution from the nodules of cowpea plants nodulated with *Rhizobium* strain 176A27 or CB756. Plants were grown in liquid culture with cuvettes attached to the nodulated zone of the main root. Bars are the standard errors of the mean values for six separate plants. Values have been integrated for a 24-h period in each case.

MATERIALS AND METHODS

Plant Material. Effectively nodulated plants of cowpea (*Vigna unguiculata* (L.) Walp. cv Caloona) were grown with N-free nutrient in lidded 11-L containers (four plants/container) of stream-sterilized, quartz sand (11) or in N-free liquid culture (13) in a naturally lit glasshouse during November to March in Perth, Western Australia. Surface-sterilized seed was inoculated at sowing with either *Rhizobium* strain CB756 (CSIRO), Brisbane, Australia) or 176A27 (Nitragin Co.). Where incident light was reduced to 5% full sun, this was achieved by screening with a neutral mesh.

Inoculated plants of each symbiosis were also grown in 1 × 5-m field plots of a light sandy soil low in available N. Under these conditions, nodulation was profuse and effective.

Plant Harvest and Analysis. Dry weight carbon and N content were determined as previously (15, 16) in nodules and other plant parts following periodic sampling of five pots comprising 20 plants from the population.

Measurement of Gas Exchange in Sand Culture. Respiration of the intact nodulated root system was measured continuously from germination to pod maturation by connecting two Pettenkoffer tubes in series to each of 10 sealed pots to collect evolved CO_2 (11). Apportioning CO_2 loss between roots and nodules was based on the use of detached organs as detailed previously (11). H_2 production was measured in samples (0.5 ml) of gas taken from the effluent gas stream of the pot, before passage through the Pettenkoffer tubes, using a gas-liquid chromatograph equipped with a thermal conductivity detector (13). Samples for H_2 assay were taken at intervals on a diurnal basis throughout the study. Respiration of shoots at night was measured periodically during the study as described previously (11).

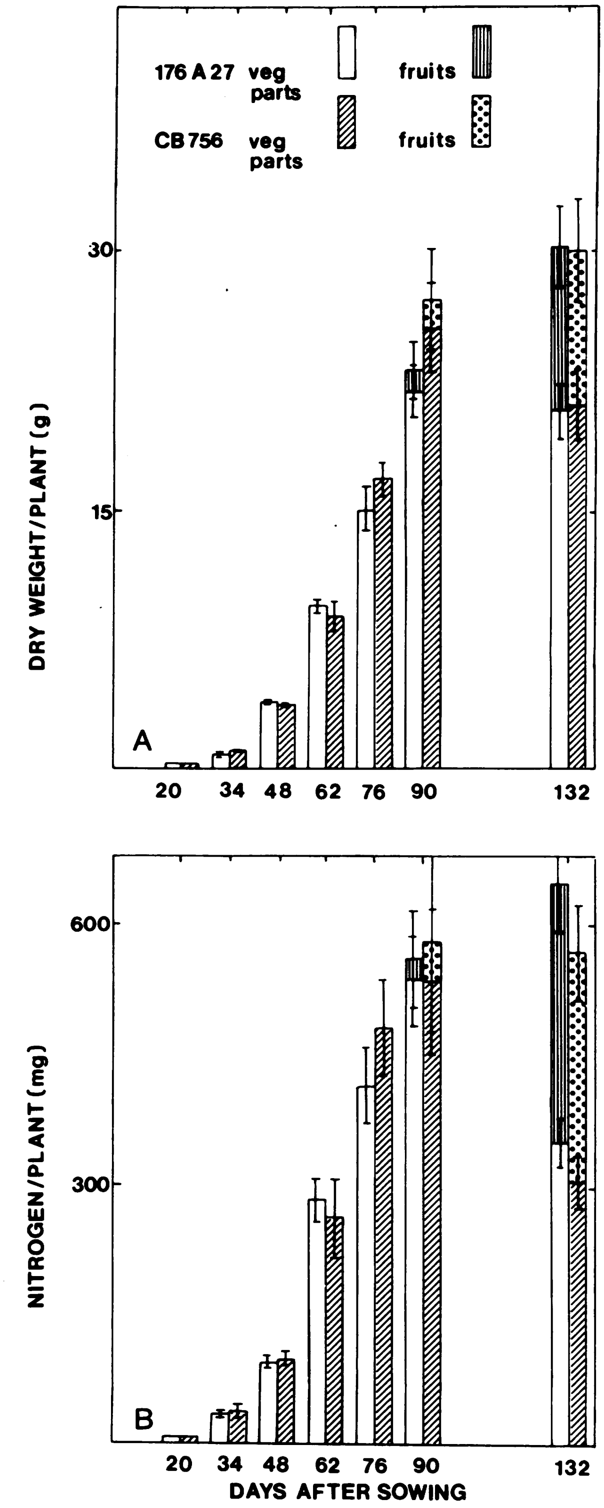

FIG. 3. Dry weight (A) and total N (B) of vegetative and reproductive parts of cowpea plants nodulated with *Rhizobium* strain 176A27 or CB756 during growth. Bars are the standard errors of mean values.

Measurements of Gas Exchange in Liquid Culture. The crown nodulated region of the main root of each of six plants was enclosed and sealed into small (10 cm^3 internal volume) plastic cuvettes (13) and CO_2 was measured sequentially (6-min sampling time) in each of the six effluent gas streams using a multiple gas-sampling device and an IR gas analyzer. After passage through the analyzer, a motorized gas sampling valve attached to a gas-

Table I. *Yield of Dry Matter and Total N of Fruits in Cowpea Plants*

Cowpea plants were grown in sand culture in a glasshouse or in field trials and nodulated with either a low H_2-evolving strain (CB756) or a high H_2-evolving strain (176A27) of *Rhizobium*. In sand culture, plants were exposed to full sunlight or were shaded to 5% full irradiance. Field plots were 1 × 5-m area on a sandy soil with low available N. In all cases, fruits were harvested at seed maturity.

	CB756		176A27	
	Dry matter/plant	N/plant	Dry matter/plant	N/plant
	g	*mg*	*g*	*mg*
Sand culture				
Full sunlight	5.2 ± 0.3[a]	158.0 ± 9.1	4.6 ± 0.5	175.1 ± 7.3
5% sunlight	0.91 ± 0.02	34.8 ± 4.3	1.01 ± 0.12	37.3 ± 4.1
Field trial	8.5	278.8	9.1	310.7

[a] Mean ± SE.

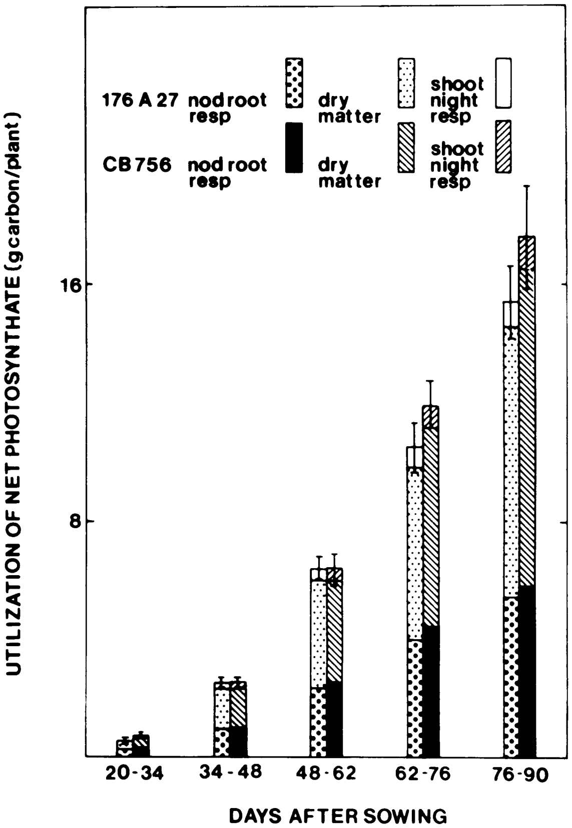

FIG. 4. Distribution of net photosynthate between dry matter, respiration of the nodulated root system, and respiration of the shoot at night during growth of cowpea plants nodulated with *Rhizobium* strain 176A27 or CB756. Bars are the standard errors of mean values.

liquid chromatograph automatically sampled each of the six effluent gas streams for H_2 measurements as above.

Analysis of Xylem Sap. Root bleeding sap was collected and analyzed for total N amino acid and ureide contents as described previously (2, 12, 17, 18).

RESULTS

Rates of N_2 Fixation and H_2 Evolution. The two symbioses showed similar patterns of N_2 fixation throughout the study period (20–90 d) with maximum rates between 62 and 76 d (Fig. 1). During this period, anthesis occurred and fruit development began. Patterns of H_2 evolution for the two symbioses differed markedly (Fig. 1). From 20 to 44 d, each showed about the same rate of H_2 efflux, from 44 to 76 d, the 176A27 symbioses evolved a significantly larger amount of H_2 than the association with CB756, while from 76 to 90 d, the rates of H_2 evolution for the two symbioses were again not signicantly different. Consequently, the relative efficiency of N_2 fixation, as measured by the ratio of the rates of N_2 fixed to H_2 evolved, varied throughout growth with minimum values occurring at the time of maximum fixation, and maximum relative efficiencies at times of lower rates of N_2 fixation (Fig. 1). The two symbioses also showed a marked difference in rates of H_2 efflux during a 4-d period in midvegetative growth of plants in liquid culture with nodulated root zones enclosed in cuvettes (Fig. 2).

Growth and Accumulation of Nitrogen and Dry Matter. There was no significant difference between the two symbioses in the amount of dry matter accumulated in both vegetative and reproductive parts throughout the life cycle (Fig. 3A). Similarly, the levels of N accumulated by the two symbioses did not differ, and the partitioning of N between the fruits and the vegetative parts was the same in each case (Fig. 3B). Yields of dry matter and N in the fruits of the two symbioses at final harvest were also not significantly different when the symbioses were cultured under severe light limitation or grown under field conditions in a light sandy soil (Table I).

Utilization of Net Photosynthate. The sum amount of net photosynthate fixed calculated as the sum of carbon incorporated into dry matter, carbon lost in respiration of the nodulated root system, and carbon lost in respiration by the shoot at night did not differ for the two symbioses (Fig. 4). In each case, approximately 40% of net photosynthate was consumed in nodulated root respiration, 55% in dry matter accumulation, and 5% in respiration of shoots at night.

Respiratory Losses by the Nodulated Root and Nodule. While respiratory losses of CO_2 from the whole root systems of the two symbioses were statistically not significantly different (Fig. 5), there was a trend for the low H_2-evolving strain (CB756) to have a lower rate of efflux from 60 to 90 d. Respiration of nodules alone, however, did differ significantly, with the high H_2-evolving strain (176A27) producing more CO_2 per plant than the low H_2 evolving nodules (Fig 5) after 44 d. A similar difference in the CO_2 efflux was also shown by the two symbioses grown in water culture with their nodulated root zones enclosed in cuvettes (Fig. 2).

Accumulation of Dry Matter in Nodules and Roots. The mass of nodules formed by the two symbioses did not differ over the study period (Fig. 6A). There was, however, a significant difference in root dry weight at 90 d (Fig. 6B), with the low H_2-evolving association (CB756) utilizing more carbon in forming root mass than the 176A27 symbioses.

Xylem Sap Composition. Analysis of root bleeding sap from the two symbioses throughout development showed a similar relative composition of nitrogenous solutes in xylem with the ureides, allantoin, and allantoic acid, predominating (Table II).

Table II. *Distribution of N between Solutes and C/N Weight Ratio of Xylem Sap Collected as Root Bleeding Exudate from Cowpea Plants Nodulated with Rhizobium Strain 176A27 or Strain CB756 and Grown in Sand Culture*

Days after Sowing	Nitrogen of Xylem Sap as				C:N
	Ureides	Asparagine	Glutamine	Other[a]	
	%				*mg/mg*
176A27					
20	82	2	8	8	1.1
48	62	10	15	13	1.4
62	61	7	16	16	1.4
76	59	9	10	22	1.4
90	55	14	11	20	1.4
CB756					
20	83	3	8	6	1.1
48	74	10	8	8	1.2
62	65	7	20	8	1.3
76	67	7	15	11	1.3
90	64	9	13	14	1.3

[a] The sum of aspartate, glutamate, alanine, valine, threonine, serine, isoleucine, phenylalanine, histidine, and lysine.

Table III. *Economy of Carbon and N in Nodules of Cowpea Grown in Sand Culture and Nodulated with Either a Low H_2-Evolving Rhizobium Strain (CB756) or a High H_2-Evolving Strain (176A27)*

Item	20–90 d		48–76 d	
	176A27	CB756	176A27	CB756
	mg/plant			
1. C incorporated in nodule mass	230 ± 24[a]	210 ± 25	189 ± 24	170 ± 12
2. C loss as CO_2 in respiration	1764 ±168	1480 ± 144	1000 ± 99	729 ± 80
3. C exported with fixed N in xylem[b]	683 ± 96	688 ± 90	385 ± 35	437 ± 69
4. Total C imported in phloem (1 + 2 + 3)	2677	2358	1574	1336
5. Total N fixed	560 ± 79	580 ± 78	321± 29	379 ± 60
6. N incorporated in nodule mass	50 ± 6	41 ± 4	46 ± 6	35 ± 3
7. N exported (5 − 6)	510	539	275	344
		mg/mg		
8. C imported/N fixed (4/5)	4.8	4.1	4.9	3.5
9. C (as CO_2) evolved/N fixed (2/5)	3.2	2.6	3.1	1.9

[a] Mean ± SE for experimentally derived data only.

[b] C:N (weight basis ratios of xylem sap used where mean values for the two periods for each symbiosis from Table II.

DISCUSSION

Significance of H_2 Evolution in the Carbon Economy of Nodulated Cowpea. The two symbioses showed a marked difference in the amount of H_2 evolved from their nodules (Figs. 1 and 2), in the carbon loss as CO_2 of their nodules (Figs. 2 and 5), and perhaps also in the growth of roots late in development (Fig. 6B). These differences did not result in differences in yield of dry matter or N by the whole plant or in the fruits at final harvest (Fig. 3, A and B) either at high light intensity or under conditions of severe photosynthetic limitation (Table I) and in a field trial (Table I).

Using data for CO_2 efflux by nodules (Fig. 5), changes in nodule dry matter (Fig. 6A) and nitrogen content (taken from data used in Fig. 3B), total N fixed (Fig. 3B), and C:N ratio of xylem sap (Table II), the economy of carbon and N of nodules of the two symbioses was constructed (Table III) for the whole period of growth studied (20–90 d) and for the period of maximum H_2 evolution (48–76 d; Fig. 1). Whereas each fixed a similar amount of N_2, somewhat more was utilized in nodule development in the 176A27 symbiosis with slightly more N being exported from the nodules of the CB756 symbiosis. The two associations also differed in total carbon imported by nodules via phloem, especially in the 48- to 76-d period, where those formed with the low H_2-evolving strain (CB756) utilized 1.34 g carbon compared to 1.5 g carbon in the 176A27 symbiosis. These differences were reflected in significantly lower values for the amount of carbon imported per N fixed and were due mainly to a significant difference in the loss of carbon as CO_2 per N fixed.

The average difference in the carbon economy of nodules from the two symbioses during the 48 to 76-day period was 1.4 mg carbon/mg N fixed, representing around 30% better economy of C used by CB756. This value was considerably greater than the 9.1% (9) and 12.5% (19) recovery of energy expenditure calculated from theoretical considerations of the likely benefit from complete hydrogen oxidation *in vivo*. Over the whole period of growth studied (20–90 d), the differences in carbon economy of nodules from the two symbioses was 0.7 mg carbon/mg N fixed (Table II) or approximately 16% greater economy of carbon use by nodules of the lower H_2-evolving symbioses (CB756). In terms of the overall net photosynthate generated by the plant (Fig. 4), the difference in C use by nodules was around 5% for the 48 to 76-d period and 2% over the growth cycle. Differences of this magnitude were unlikely to have been detected in overall yield.

The "extra" carbon substrate made available by reduced H_2

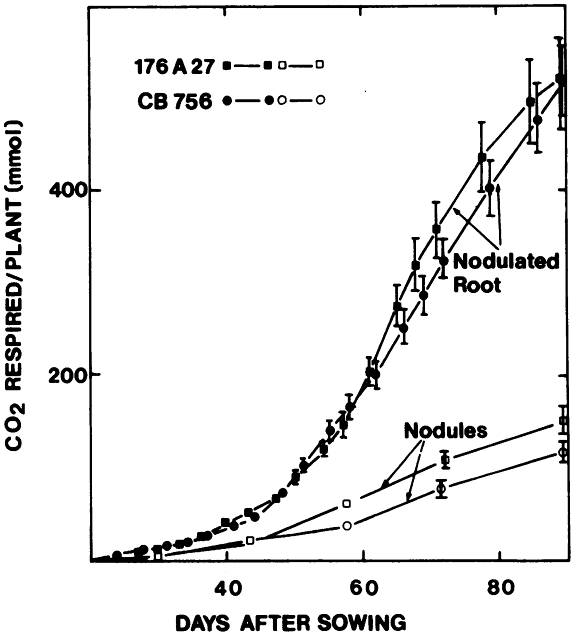

FIG. 5. Rates of efflux of CO_2 from the nodulated root system or the nodules of cowpea plants during growth, nodulated with *Rhizobium* strain 176A27 or CB756. Bars are the standard errors of mean values and where absent are within the dimensions of the symbols.

evolution in the CB756 symbiosis was apparently not utilized to fix more nitrogen. The significantly larger root system of this symbiosis compared with 176A27 (Fig. 6B) indicated that a change in the apportioning of C between roots and nodules may have occurred. Under pot culture conditions, differences in root system size may be of little significance but, in the field with water or nutrient limitation, they could result in increased yield by plants nodulated with low H_2-evolving *Rhizobium* strains. It is important to stress, however, that the comparison made in this study was between two unrelated *Rhizobium* strains nodulating a single cowpea cultivar and that the differences seen may not be representative of a wider range of bacterial strains and cowpea types.

Respiratory Cost of Hydrogen Evolution in Nodules. As nodules formed in the two symbioses were not significantly different in size (Fig. 6A), fixed N_2 at the same rate (Fig. 1), and produced the same mixture of exported nitrogenous solutes in xylem (Table II), it is reasonable to expect that respiration associated with the reduction of N_2 to ammonia, with the assimilation of ammonia to produce amino compounds and ureides and with nodule growth and maintenance would be the same in each. While processes other than H_2 evolution could also have differed in the two symbioses, and so contributed to the greater respiratory CO_2 efflux in nodules from the higher H_2-evolving strain, it is tempting to associate the increased respiration specifically with H_2 evolution. On the basis of such an assumption, in the period of 48 to 76 d, nodules of the 176A27 symbiosis grown in sand culture produced an 'extra' 2.8 mol CO_2/mmol N_2 fixed compared to those of the CB756 symbiosis (calculated from Table III). This was associated with 0.63 mmol extra H_2 evolved by the 176A27 symbiosis (from Fig. 2), giving a ratio of 4.4 mmol CO_2/mmol H_2. Similar calculations for the 41 to 44-d period studied in water-cultured plants (Fig. 2) indicated that the 176A27 symbiosis produced an extra 1.62 mmol CO_2 and 0.96 mmol H_2/mmol N_2 fixed compared to that with CB756, giving a value of 1.69 mmol CO_2/mmol H_2 evolved.

Based on the utilization of seven ATP eq/H_2 produced and assuming nodule oxidative phosphorylation to operate with a $P/2e^- = 3$ for the respiration of sugar, H_2 evolution theoretically costs 1.1 mmol CO_2/H_2. While the value estimated above using plants grown in water culture was closer to the theoretical estimate than that derived using sand culture plants, the somewhat higher CO_2 efflux found experimentally indicates that processes other than H_2 evolution could have contributed to the higher respiration in the 176A7 symbiosis or that the theoretical treatment of the energetics of respiration and electron transfer reactions in the nodule do not ultimately reflect the situation *in vivo*.

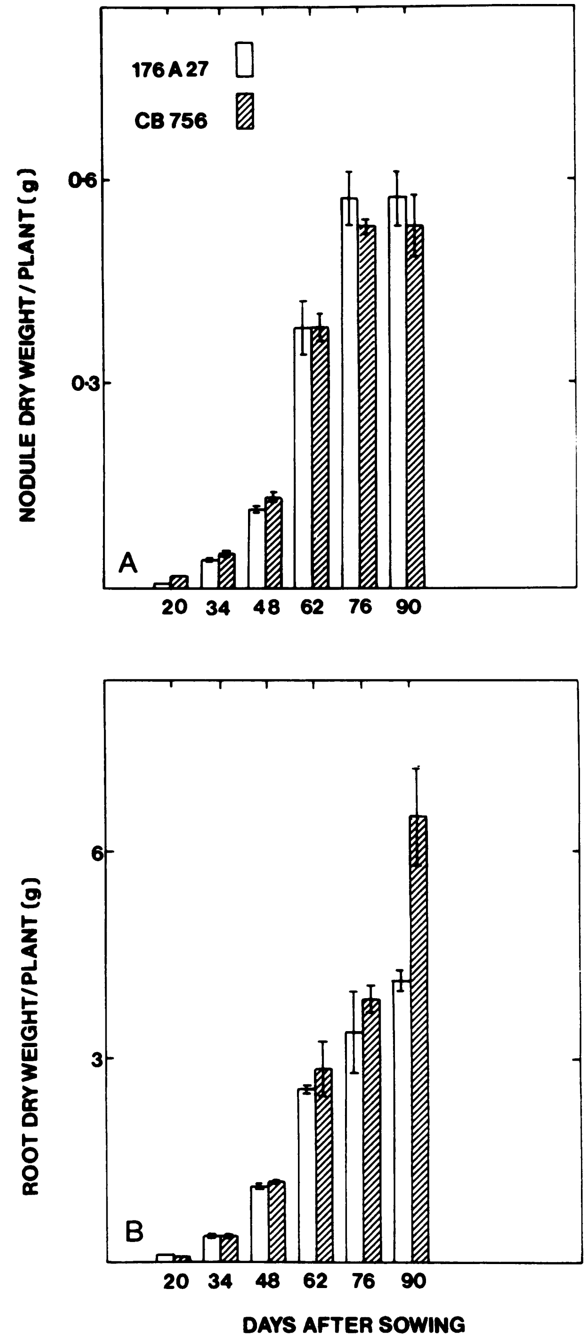

FIG. 6. Changes in the dry weight of nodules (A) or roots minus nodules (B) of cowpea plants during growth, nodulated with *Rhizobium* strain 176A27 or CB756. Bars are the standard errors of mean values.

Relationship between Hydrogen Evolution and Nitrogen Fixation. Whole plant studies with Alaska pea have shown that the relationship between H_2 evolution and total electron flow through nitrogenase varied with plant ontogeny (3) and with irradiance (4). These variations have been ascribed to both a shift in the partitioning of electrons between N_2 and proton reduction by nitrogenase and to changes in the activity of H_2 uptake hydrogenase (5). In the present study, it was not possible to assess the extent to which changes in electron partitioning and hydrogenase activity separately contributed to the complex relationship between H_2 evolved and N_2 fixed during growth (Fig. 1). However, in both symbioses, the relationship was not constant, indicating that the relative efficiency of N_2 fixation and the cost in terms of oxidizable substrate utilized could be expected to vary depending on the stage of growth and the rate of fixation.

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