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UPTAKE AND EVOLUTION OF H_2 AND REDUCTION OF C_2H_2 BY ROOT NODULES AND NODULE HOMOGENATES OF *ALNUS GLUTINOSA*

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KEY WORDS

Acetylene reduction Actinomycetous symbiosis *Alnus glutinosa* Hydrogenase Hydrogen evolution Hydrogen uptake Nitrogen fixation Respiration Root nodules

SUMMARY

In the growing season no net H_2 evolution is detected when root nodules of *Alnus glutinosa* are incubated in air or in argon containing 20% O_2. Due to the hydrogenase activity, N_2-fixing root nodules consume added H_2 at a rate of about 1.4 μmoles H_2.g fresh nodule^{-1}.h^{-1}. The uptake of H_2 is only found in summer. At the end of the season, in autumn, nodules evolve significant quantities of H_2 although the nodules still continue to fix nitrogen.

In-vitro studies with fractionated homogenates of summer-harvested nodules show that the recovery of the hydrogenase is high when using methylene-blue or phenazine metasulfate as electron acceptors. No hydrogenase activity is detected in homogenates of autumn-harvested nodules.

The hydrogenase is localised in the microsymbiont.

INTRODUCTION

Free-living and symbiotically living N_2-fixing bacteria probably all possess an active hydrogenase. The H_2 which is evolved by nitrogenase can be reutilized by this hydrogenase. The amount of H_2 evolved is therefore dependent on the activities of both enzymes.

Most legumes that were tested lost significant amounts of H_2 *via* nitrogenase, probably due to incomplete uptake by hydrogenase. But a few legumes, *e.g.* cowpeas, and most non-legumes with actinomycetous nodules evolved no or only traces of H_2 when incubated in air[6,8,9,10].

The relationship between H_2 metabolism and N_2 fixation has been investigated mainly in leguminous plants. Actinomycetous root nodules received little attention. Recently, it was shown that H_2 was rapidly consumed by root nodules of *Alnus rubra*[10] and *A. glutinosa*[2]. In this study nodule homogenates were used to obtain more information about the localisation of the hydrogenase in the nodules and about its relation to nitrogen fixation in *A. glutinosa*.

MATERIALS AND METHODS

Plant material

Root nodules were collected from 3–4 year old *Alnus glutinosa* plants. Ten to fifteen g fresh weight of nodules were collected per tree.

Plants were raised from seeds (origin 'Lichtenvoorde', received from J. A. Verweij, Institute for Forestry and Landscape Planning 'De Dorschkamp', Wageningen). Seedlings were inoculated with suboptimum concentrations of a suspension of spore-rich nodules in order to obtain plants with low numbers of nodules. Nodules became larger when the number of nodules per plant was low (unpublished results). After precultivating the seedlings for one year on perlite with Hoagland solution without nitrogen, the trees were planted on an experimental plot (30 × 40 m^2) in a sandy soil low in nitrogen. Since no alder plants had been cultivated previously in this soil for at least 50 years, the soil was almost free from endophyte, and new nodules were not formed on the plants. This cultivation method enabled us to collect large quantities of nodule material from well-defined origin (Akkermans and Houwers, in preparation).

C_2H_2-reduction assay

Detached nodules were assayed for C_2H_2 reduction as described previously[3]. Homogenous samples of detached nodule lobes were used, free from root pieces. Assays of C_2H_2 reduction by detached nodule lobes and nodules attached to pieces of roots showed that the activity of the nodules decreased 25–30% due to detachment. The results with detached nodule lobes from field-grown plants can be used for interpretation of the situation *in vivo*.

O_2 consumption and H_2 metabolism by detached nodules

Aliquots of 2 g fresh weight of nodules were incubated in 14.6-ml Hungate tubes, with 0.7% H_2 in air. After preincubation for 15 min, the rates of the respiration (O_2-consumption) and net H_2-uptake or H_2-evolution were simultaneously assayed on a gas chromatograph.

Preparation of nodule homogenates

Root nodules were homogenized anaerobically in a Virtis homogenizer ('45' Hi-speed) for three minutes at 5,000 rpm in a P-buffer (pH 7.7), containing sucrose (0.3 *M*), soluble polyvinyl-pyrrolidone (4%) and dithioerythritol (5 m*M*). The homogenate was flushed with argon and passed through 100-μm and 20-μm filters, to separate the vesicle clusters of the microsymbiont from plant-cell debris[3]. The 20-μm residue (containing the vesicle clusters) was taken up in 6 ml of the P-buffer (1.5 g fresh weight of nodules.ml^{-1}). The 20-μm filtrate was centrifuged for 10 minutes (12,000 × *g*) and the pellet was taken up in the buffer solution (0.9 g fresh weight of nodulus.ml^{-1}).

In vitro assay of hydrogenase in nodule homogenates

Fractions of nodule homogenates (2 ml) were injected into 14.6-ml Hungate tubes together with 0.2 ml of a solution of an electron acceptor. The following electron acceptors were used: 2,6-dichlorophenolindophenol (DCPIP), methylene-blue (MB), phenazine metasulfate (PMS) and $K_3Fe(CN)_6$. The final concentration was 10 m*M*. The gas phase was argon with 0.7% H_2. Hydrogenase activity was assayed by measuring the H_2-uptake from the gas phase at 24°C after preincubation for 15 minutes. All values were expressed as μmoles H_2 consumed per g fresh nodule.

RESULTS

In preliminary experiments it was found that in accordance with earlier reports[6,8,9,10] root nodules of *Alnus glutinosa* in summer did not evolve H_2 when incubated either in air or in argon with 20% O_2. These results can be explained by the presence of an active hydrogenase, consuming the H_2 formed by the nitrogenase. This was confirmed by the experimental results in Table 1. Alder nodules consumed H_2 at a rate of about 1.4 µmoles H_2.g fresh nodule^{-1}.h^{-1} in summer.

The uptake of H_2 is O_2-dependent. No significant H_2 uptake was detected when nodules were incubated in argon with 0.7% H_2, with the exception of a small decrease in the H_2 concentration during the first 15 minutes of incubation. Additional experiments, not shown here, gave evidence that the little change in concentration was due to dissolving of H_2 into the nodule tissue. Possible interference of traces of O_2 still present in the intercellular spaces of the nodules was excluded by pre-evacuating the nodules[2].

The rates of both C_2H_2 reduction and H_2 uptake varied to some extent in summer. The ratio C_2H_2 reduced/H_2 consumed was about 4 (Table 1), while in previous experiments[2] with nodules from the same trees and collected under similar conditions it was almost 6. This variation in the ratio was due to a change in the activities of hydrogenase and nitrogenase.

An extreme situation occurred in autumn when soil temperature had decreased till 7–8°C. As shown in Table 2, autumn-harvested nodules which continued to fix N_2 at a low rate, showed a net H_2 evolution. This H_2-gas was produced by the nitrogenase as was demonstrated by the increased H_2 evolution when the nodules were incubated in argon with 20% O_2. Under these conditions nitrogenase reduces only protons and evolves H_2. Both C_2H_2 reduction and H_2 evolution were enhanced by increasing incubation temperature up to 20°C.

Table 1. H_2 uptake, O_2 consumption and C_2H_2 reduction by detached nodule lobes of *Alnus glutinosa* in summer

µMoles substrate.g fresh nodule.$^{-1}$.h^{-1}		
H_2 uptake*	O_2 consumption*	C_2H_2 reduction**
1.37	28.9	5.88

* Aliquots of 2–3 g fresh weight of nodules were incubated in 14.6 ml of air with 0.7% H_2.
** Aliquots of 3 g fresh weight of nodules were incubated in 320 ml of air with 10% C_2H_2.
After preincubation for 15 min, the rates of H_2 uptake, O_2 consumption and C_2H_2 reduction were constant for at least 1 h.

Table 2. H_2 evolution and C_2H_2 reduction by detached nodules lobes of *Alnus glutinosa* in autumn

Incubation temperature (°C)	Incubation time (h)	H_2 evolution**		C_2H_2 reduction***
		Air/H_2	Ar/O_2/H_2	
7*	0.5	0.08	0.21	0.52
	1.0	0.16	0.41	1.13
	1.5	0.22	0.60	1.72
20	0.5	0.19	0.61	1.45
	1.0	0.35	1.16	3.18
	1.5	0.41	1.33	5.18

* The ambient soil temperature.

** µMoles $H_2.g^{-1}$ fresh weight of nodules.

Aliquots of 2 g fresh weight of nodules were incubated in tubes (gas vol. 14 ml) with either air/H_2 (0.7%) or Ar/O_2 (20%)/H_2 (0.7%).

*** µMoles $C_2H_2.g^{-1}$ fresh weight of nodules.

Aliquots of 2.5 g fresh weight of nodules were incubated in 100-ml Erlenmeyer flasks with 10% C_2H_2 in air.

Each value denotes the average of two aliquots of nodules taken from two different trees.

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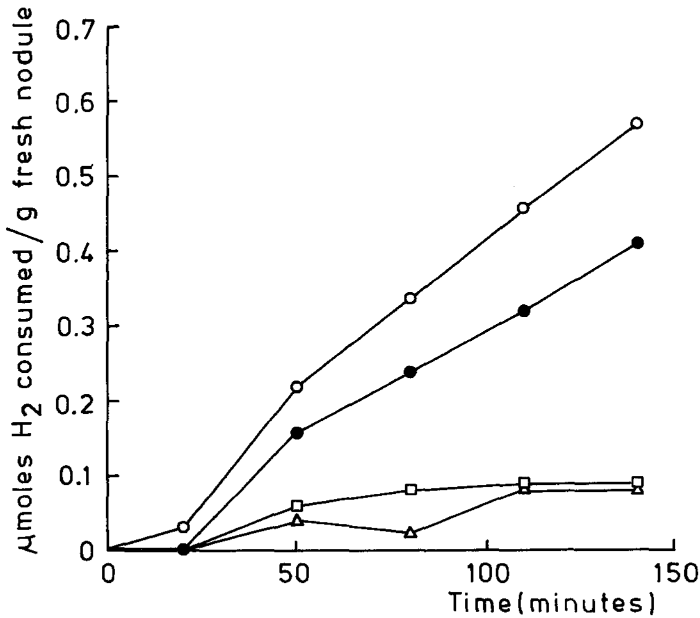

Fig. 1. Hydrogenase activity of the 100-µm filtrate of homogenates of summer-harvested Alnus nodules as measured with the following electron acceptors: PMS (○), MB (●), DCPIP (□) and $K_3Fe(CN)_6$ (△).

These results suggest that in summer, H_2 produced by nitrogenase is rapidly recycled by hydrogenase, resulting in a net H_2 uptake. However, in autumn the H_2 is incompletely recycled, due to low hydrogenase activity. This hypothesis is supported by *in-vitro* assays of hydrogenase in homogenates. In autumn, no hydrogenase activity was detectable, while in summer, nodule homogenates consumed rapidly H_2 in the presence of either MB or PMS (Fig. 1). Other electron acceptors, *viz* DCPIP and $K_3Fe(CN)_6$ seem to be unadequate. Attempts to use O_2 as a natural electron acceptor failed because nodule homogenates of *A. glutinosa* did not possess any respiratory activity[1].

The localisation of the hydrogenase in summer-harvested nodules was investigated by the *in-vitro* assay with MB as electron acceptor. As shown in Fig. 2, the 20-µm residue, containing the undamaged vesicle clusters of the microsym-

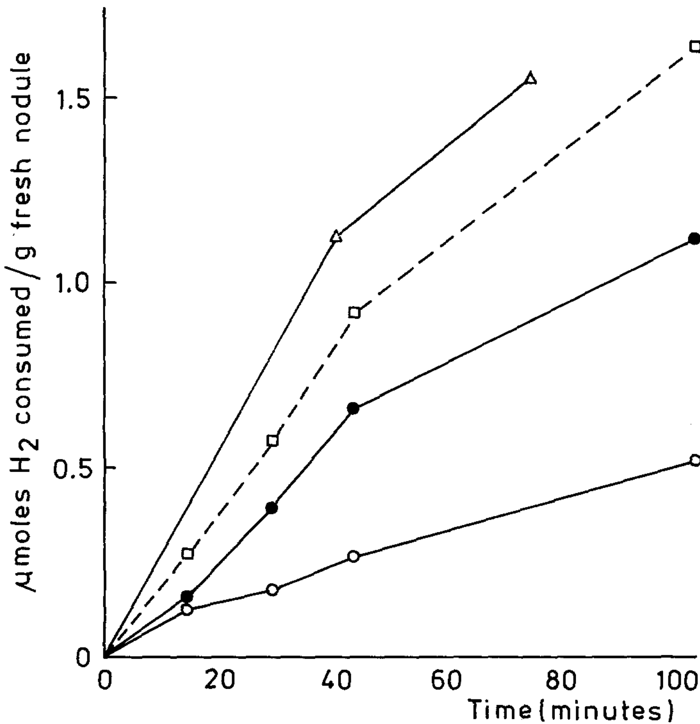

Fig. 2. H_2 uptake by intact Alnus nodules (△) and nodule homogenates in summer. The *dotted* line (□) is the sum of the activities of the 20-µm residue (○) and the 20-µm filtrate (●). Intact nodules were incubated in air with 0.7% H_2; fractions of nodule homogenates were incubated in N_2 with 0.7% H_2 and MB as electron acceptor.

biont, had a high hydrogenase activity. The hydrogenase activity of the 20-μm filtrate, containing fragments of broken vesicle clusters, seemed to be all particle-bound, since the supernatant (12,000 × g) had no detectable hydrogenase activity.

DISCUSSION

The results demonstrate that summer-harvested nodules of *A. glutinosa* can consume H_2 from the gas phase at a considerable rate. This phenomenon explains why alder nodules do not give net H_2 production, even when incubated in argon with 20% O_2.

From Fig. 2 it can be concluded that the *in-vitro* assay of hydrogenase activity approached the rate of H_2 uptake by intact nodules. Apparently, little or no enzyme activity is lost during the homogenization procedure. This method is suitable for further physiological and biochemical studies of the H_2 metabolism.

The distribution of hydrogenase activity over the 20-μm residue and the 20-μm filtrate of the nodule homogenate was similar to the distribution pattern of nitrogenase[1,12] and of glutamate dehydrogenase[4]. Since nitrogenase is a marker enzyme of the actinomycete, it can be concluded that presumably all hydrogenase activity originates from the actinomycete. The localisation of hydrogenase in the microsymbiont of alder nodules is in accordance with earlier observations by Dixon[7] on the location of the enzyme in bacteroids of pea nodules.

The functioning of the hydrogenase by the microsymbiont may be twofold. Firstly, it recycles H_2 which is evolved by nitrogenase. Secondly, it enables the nodule to take up H_2 from soil atmosphere. The latter possibility is likely since alder plants grow in mesotrophic, moist bogs. It is as yet unknown to what extent additional H_2 uptake by alder nodules can contribute to the symbiosis by providing energy and reducing equivalents for the growth of the microsymbiont as it occurs in facultatively chemo-autotrophic hydrogen bacteria[11]. A further possibility is that H_2 is used for nitrogen fixation as has been observed with *e.g.* a methane-oxidizing bacterium by de Bont[5].

The results show that the hydrogenase activity of alder nodules varied during the growing season. Summer-harvested nodules had a net uptake of H_2; autumn-harvested nodules a net H_2 evolution. Under these stress conditions the relative efficiency of energy utilization for N_2 fixation was decreased below the summer level of one. From Table 2, relative efficiencies were calculated based either on the ratio H_2 evolved in air/H_2 evolved in Ar + O_2 or on the ratio H_2 evolved in air/C_2H_2 reduced, according to Schubert and Evans[9,10]. At the ambient soil temperature (7°C), these values were 0.62 and 0.86, respectively. Higher values

were obtained when nodules were incubated at 20°C, *viz* 0.69 and 0.89, respectively. Although these figures can only be explained by a change in the hydrogenase activity in autumn, it is still questionable whether the enzyme activity is completely lost or is decreased till a low level. The discrepancy between H_2 evolution in Ar/O_2 and the C_2H_2 reduction in air (Table 2) suggests that a part of the hydrogenase is still functioning. However, no hydrogenase activity could be detected *in vitro*, at least in the particle-bound fraction of the homogenates.

The significance of the decreased hydrogenase activity in autumn is still unclear. We do not know yet whether this phenomenon is due to specific conditions in autumn or is a more general feature of stress conditions. Further studies are needed to estimate the importance of the H_2 metabolism in actinomycetous root nodules.

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