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Growth, nitrogen fixation and relative efficiency of nitrogenase in *Alnus incana* grown in different cultivation systems

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Key words *Alnus incana* C_2H_2-reduction Cultivation systems *Frankia* H_2-evolution in air Hydrogenase Nitrogen content Relative efficiency

Summary Three cultivation systems were compared. In one system the alders were grown hydroponically. In the two other systems the alders were planted in gravel and either given water and nutrients at intervals or the nutrient solution was continuously supplied. Alders continuously supplied with nutrients and water showed a significantly more rapid growth, higher biomass production and higher nitrogen content than did alders given nutrients and water at intervals or alders hydroponically grown. Alders continuously supplied with water and nutrients had a constant RE (relative efficiency of nitrogenase) of about 0.80 throughout the experimental period while alders supplied with water and nutrients at intervals showed a slight decrease in RE at the end of the experimental period. No strict relationship was found between RE and nitrogen content or between RE and plant productivity.

Introduction

For studies on physiological aspects of nitrogen fixation in higher plants, *e.g.* energy relationships and photosynthetic supply, it is important to have a cultivation system where neither water nor nutrients are limiting or fluctuating. Such cultivation systems are already described[2,10,13,17]. But, none of these systems was designed to permit repeated measurements of nitrogen fixation in intact plants, which is greatly needed in many kinds of physiological studies on nitrogen fixation. Growing the plants in a hydroponic system as done by Imsande and Ralston[17], fulfilled all the requirements stated, but had the disadvantage of disturbing the plants when the nitrogenase activity was measured.

Nitrogen fixation is always accompanied with a reduction of H^+ to H_2. The uptake of H_2 by a hydrogenase occurs frequently occurring in nitrogen-fixing organisms, but is lacking in some strains of *Rhizobium*[9].

The two hydrogen reactions occur also in actinomycete nodulated plants, but the H_2-evolution in air is reported to be lower in these symbioses than in legumes[22,23,25]. Recent work on leguminous plants revealed that plant ontogeny[4,11] altered the relative efficiency of nitrogenase (RE; electrons used for nitrogen reduction as part of total electron flux available for nitrogenase activity). Data on *Alnus glutinosa* indicate that there are fluctuations in H_2-evolution in air during the year[22].

The aim of this work on the *Alnus incana* – *Frankia* symbiosis was (1) to find a cultivation system suitable for studies on energy demand of nitrogen fixation and (2) to study how growth conditions and plant development affected the relative efficiency of nitrogenase. Three cultivation systems were evaluated for growth, nitrogen fixation and nitrogen content of the alders. Two of these cultivation systems were also evaluated for relative efficiency of nitrogenase.

Materials and methods

Plant material and growth chamber conditions

Green cuttings of one clone of *Alnus incana* (L.) Moench were rooted for 21 days in an aerated, diluted nutrient solution[14] complemented with 0.358 m*M* NH_4NO_3[15]. The rooting solution was not renewed during the rooting period. The rooted cuttings were inoculated with a water suspension of crushed nodules from alders of the same clone. The inoculum consisted of 0.1 g (fresh weight nodules) per ml distilled water, and 1 ml was given to each cutting immediately after transferring them to the different cultivation systems. During rooting as well as during growth the alders were in a controlled environment growth chamber with 17 h light, a thermoperiod of 17/7 h of 25/15°C and a relative air humidity of 75%. The light source was Osram HQI 400 W-70 halogen lamps giving a photon flux density of 200 μmol m^{-2} s^{-1} (Lambda Quantummeter LI-185 A).

Cultivation systems

In all cultivation systems the above-mentioned nutrient solution (pH *ca.* 7) was used. It was complemented with 0.358 m*M* NH_4NO_3 until root nodules were visible. Three cultivation systems were compared. In one system the plants were grown hydroponically in black 1-litre polystyrene pots with one plant per pot and the nutrient solution diluted to 1/10 of full strength. The nutrient solution was renewed three times a week when also the pH was measured. In the two other systems the rooted cuttings were planted singly in pots with gravel. The bottom of the pots was covered with a fine mesh net to prevent the roots from growing through the drainage holes. One group of plants, hereafter called the traditional system, received water twice a day and full strength nutrient solution once a day[14]. The other group of plants was continuously supplied with aerated, circulating, diluted nutrient solution. For this purpose an air-lift technique was used. The potted plants were kept above a tank with nutrient solution. For every pot there were two glass tubes, which had the lower end down in the nutrient solution and the upper end emerging into the pot. A smaller tube was inserted into the lower end of the glass tube. A stream of air was pumped (RENA 301 aquarium pump) through the smaller tube, and the air pressed the nutrient solution up through the glass tube and into the pot. A flow of 0.6 l h^{-1} was delivered to each pot and the drainage was collected in the tank, which contained 10 l of nutrient solution. Five plants were kept over each tank. The nutrient solution was renewed three times a week, when also the pH of the solution was measured. Two strengths of the nutrient solution were tested, viz. 1/4 and 1/10 of full strength.

The system with circulating nutrient solution was later on modified. In the modified continuous flow system the pots were kept over a black catchment tank. The nutrient solution was aerated with the aquarium pump and lifted (Iwasaki magnet pump) to the reservoir, which was placed higher than the plants. The solution was pumped to the upper reservoir faster than it could flow out through the distribution system and thereby a constant level of nutrient solution was kept in the upper reservoir. The outlet of each distribution tube was fitted with a removable syringe needle[13]. The needle served as a restricted outlet and gave back pressure so that all outlets delivered equal amounts of nutrient solution (0.6 l h^{-1} to each pot)[12]. The solution was complemented with only 0.072 m*M* NH_4NO_3 until root nodules were visible.

Measurements

C_2H_2 reduction Nitrogenase activity was measured on intact plants[14] as C_2H_2-dependent C_2H_4-production. Ten % (v/v) of the air in the incubation chamber was withdrawn and replaced with C_2H_2. During the incubation period (1.5 h) gas samples of 0.5 ml were taken at intervals and immediately determined by gas chromatography[14]. The plants were kept in the growth chamber during the C_2H_2-incubations which were always made the same time of the day. The measurements were made on each plant once a week, except for the hydroponically grown plants, whose C_2H_2-reduction was measured only at the end of the experimental period.

Relative efficiency of nitrogenase Measurements were made repeatedly and always at the same time of the day on intact plants during the experimental period. The plants were kept in the climate chamber during the incubations. H_2-evolution in air was measured in gas samples (0.2 ml) taken at intervals during the incubation period (1.5 h). H_2 was determined in a Varian 3700 gas chromatograph with a thermal conductivity detector and a 2 m stainless steel column (inner diameter 3.2 mm) containing Molecular Sieve 5 A (80–100 mesh). The carrier gas was N_2 at a flow rate of 0.33 ml s^{-1}, the column temperature was 80°C, the detector temperature 130°C and the filament temperature 250°C. The amount of H_2 was calculated by comparison with a standard mixture of H_2 in N_2 (AGA Specialgas, Lidingö, Sweden). H_2-evolution was measured on plants grown in the traditional and in the modified continuously circulating system. The H_2-evolution had a constant rate for at least 1 h. After measurements of H_2-evolution in air C_2H_2-dependent C_2H_4-production was measured with 10% (v/v) C_2H_2 in air as described above. Relative efficiency of nitrogenase (RE) was calculated as $1 - \left(\frac{H_2\text{-evolution in air}}{C_2H_2\text{-reduction}}\right)$ according to Schubert and Evans[23].

H_2 uptake H_2 uptake was measured on intact plants as well as in nodule homogenates[22]. Intact plants were incubated in 10% (v/v) C_2H_2[5] and either 0.1, 0.7[22], 1.7, 2.0[8] or 2.4% (v/v) H_2 in air. For nodule homogenates methylene blue as well as phenazine methosulphate were tried as electron acceptors[22] and the H_2-concentration was 1.3% (v/v). Gas chromatography was as described above but Ar served as carrier gas and the filament temperature was 280°C.

Growth and biomass production In general only one shoot developed on each plant and growth was therefore estimated as shoot length. Leaf areas were measured at harvest on detached leaves in a leaf area meter (Lambda LI-COR 3000) with a transparent belt conveyor accessory. The plants were harvested 45–47 days after planting and biomass was measured as dry weight (70°C, 24 h) separately for leaves, stem, roots and nodules.

Nitrogen content The dried plant organs were ground in a ball mill (Retsch Schwingmühle MM) and redried (70°C, 1 h). Samples of 150 mg were analysed for content of Kjeldahl-nitrogen using Cu and Se as catalysts in the digestion and colorimetric determination[6,19] of the ammonia.

Statistical treatments

Significant differences were evaluated by using the Mann-Whitney U-test according to Siegel[26], with $P < 0.05$ as significance level.

Results

Nutrient solution in the continuously circulating system

Two strengths of the nutrient solution were tested, *viz.* 1/4 and 1/10 of full strength. Of these concentrations the less diluted nutrient solution resulted in significantly higher shoot lengths and leaf areas (Table 1). The biomass production (Table 1) was also greater in alders

Table 1. Growth, nitrogenase activity and nitrogen content of *A. incana* grown in a continuously circulating system with two different strengths of nutrient solutions. The values are from the end of the growth period (45 days after planting). $\bar{x} \pm SE$; $n = 4$

Plant characteristic	Strength of nutrient solution	
	diluted to 1/4	diluted to 1/10
Shoot length (cm)	54.6 ± 3.1	41.6 ± 2.3
Leaf area (cm^2)	662.6 ± 65.4	391.8 ± 49.9
Dry weight (g): root system	0.951 ± 0.120	0.651 ± 0.057
shoot	4.300 ± 0.668	2.558 ± 0.331
whole plant	5.251 ± 0.783	3.209 ± 0.381
Nitrogenase activity (μmol C_2H_4 $plant^{-1}$ h^{-1})	64.72 ± 4.73	41.96 ± 7.72
Nitrogenase activity (μmol C_2H_4 g (dry wt nodule)$^{-1}$ h^{-1})	311.9 ± 21.9	317.6 ± 27.7
Nitrogen content of total plant (mg)	125.8 ± 17.1	74.1 ± 10.1
Nitrogen content of total plant (% of dry weight)	2.41 ± 0.08	2.30 ± 0.10

supplied with 1/4 of the full strength nutrient solution, though the difference was not statistically significant. The nitrogenase activity measured as C_2H_4-production per plant and hour showed a 54% greater value for plants in the less diluted solution but when the nitrogenase activity was related to the dry weight of the nodules there was no difference (Table 1). The nitrogen content was significantly higher in leaves, stem and roots as well as in the whole plant in the nutrient solution diluted to 1/4. However, there was no significant difference when the nitrogen content was related to the dry weight (Table 1). The nutrient solution diluted to 1/4 of full strength was used hereafter.

Comparison of cultivation systems

Alders grown in the continuously circulating systems were visibly nodulated after 14 days, *i.e.* about five days earlier than alders in the other cultivation systems. Alders grown in the hydroponical and in the continuously circulating systems showed no lag phase in growth, as traditionally grown alders did (Fig. 1). The hydroponically grown alders developed deficiency symptoms. The leaves became all yellow except for the outer edges which became brownish. At the end of the growth period the plants recovered somewhat and developed new green leaves. The root nodules had plenty of callus growth through the lenticels.

The alders in the other cultivation systems held green leaves during the whole experimental period. Alders continuously supplied with nutrient solution grew much faster than hydroponically or traditionally grown alders. At the end of the experimental period the alders in the continuously circulating system had developed stem branches and had

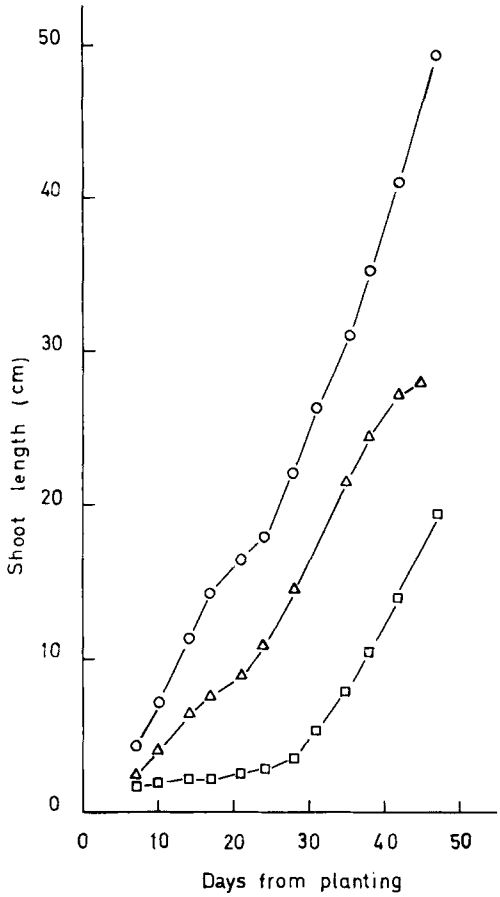

Fig. 1. Development of shoot length in *A. incana* grown in three different cultivation systems. $\bar{x}$, n = 5, SE was 5 to 26% of $\bar{x}$, with the highest SE values on day 7.
○ continuously circulating system
□ traditional system
△ hydroponic system

shoot lengths (main shoot) of 49.2 cm, *i.e.* about twice as long shoots as the other alders (Fig. 1). Leaf areas showed values of 796.4 cm^2 for alders in the continuously circulating system compared to only 209.5 cm^2 and 152.6 cm^2 for alders in the hydroponics and the traditional growth system, respectively (Table 2).

Also, the biomass production was significantly higher in all plant organs in alders grown in the continuously circulating system (Table 2). Although all rooted cuttings were inoculated with the same amount of *Frankia* (root nodules) the dry weight of the nodules was about five times higher in the continuously circulating system than in the traditional system (Table 2). The plants grown in the continuously

Table 2. Dry weight, leaf area, nitrogenase activity and nitrogen content of *A. incana* grown in three different cultivation systems. The values are from the end of the growth period (45–47 days after planting). $\bar{x} \pm SE$; $n = 5$

Plant characteristic	Cultivation system		
	Hydroponics	Traditional	Continuously circulating
Dry weight (g): shoot	1.473 ± 0.196	1.082 ± 0.279	5.351 ± 0.928
root	0.425 ± 0.040	0.283 ± 0.040	0.801 ± 0.109
nodules	0.047 ± 0.007	0.056 ± 0.020	0.313 ± 0.030
plant	1.946 ± 0.234	1.422 ± 0.338	0.685 ± 0.120
Root/shoot ratio	0.34 ± 0.03	0.35 ± 0.03	0.22 ± 0.02
Leaf area (cm^2)	209.5 ± 27.0	152.6 ± 41.7	796.4 ± 110.1
Nitrogenase activity (μmol C_2H_4 $plant^{-1}$ h^{-1})	0.20 ± 0.06	16.92 ± 7.14	75.35 ± 10.18
Nitrogenase activity (μmol C_2H_4 g(dry wt nodule)$^{-1}$ h^{-1}	6.0 ± 2.6	281.4 ± 19.5	254.6 ± 12.0
Nitrogen content (mg):			
Total plant	27.00 ± 2.68	26.21 ± 8.98	131.4 ± 22.6
Leaves	11.41 ± 2.06	14.48 ± 5.57	82.18 ± 15.94
Roots	7.36 ± 0.51	4.47 ± 0.71	13.19 ± 1.57
Nodules	1.63 ± 0.33	2.09 ± 0.84	11.38 ± 1.65
Nitrogen content (% of dry weight):			
Total plant	1.44 ± 0.16	1.67 ± 0.18	1.95 ± 0.16
Leaves	1.33 ± 0.28	1.84 ± 0.27	3.29 ± 0.53
Roots	1.75 ± 0.07	1.57 ± 0.04	1.66 ± 0.03
Nodules	3.34 ± 0.21	3.51 ± 0.15	3.87 ± 0.07

circulating system developed a lower root/shoot ratio than the plants grown in the traditional and the hydroponical system did (Table 2). The nodule percentage (dry weight of nodules as per cent of dry weight of the total plant) of the alders continuously supplied with nutrients was 4.5 ± 0.4 compared to 3.5 ± 0.6 for the traditionally grown and 2.4 ± 0.2 for the hydroponics ($n = 5$ in all cases). The values were significantly higher for alders continuously supplied with nutrients compared to the hydroponics, otherwise the differences were not significant.

The nitrogenase activity measured per plant and hour was significantly higher in alders continuously supplied with nutrients than in those grown hydroponically or traditionally (Fig. 2). When nitrogenase activity was expressed on a nodule dry weight basis, plants from the continuously circulating and the traditional system showed similar values, indicating a close relation between nodule dry weight and nitrogenase activity (Table 2). The hydroponically grown alders differed much with their very low nitrogenase activity, only 6.0 μmol C_2H_4 g^{-1} (dry wt nodule)$\cdot h^{-1}$. They were apparently disturbed by the movement from the growth conditions to the measurement conditions in air.

The amount of nitrogen in plants from the continuously circulating

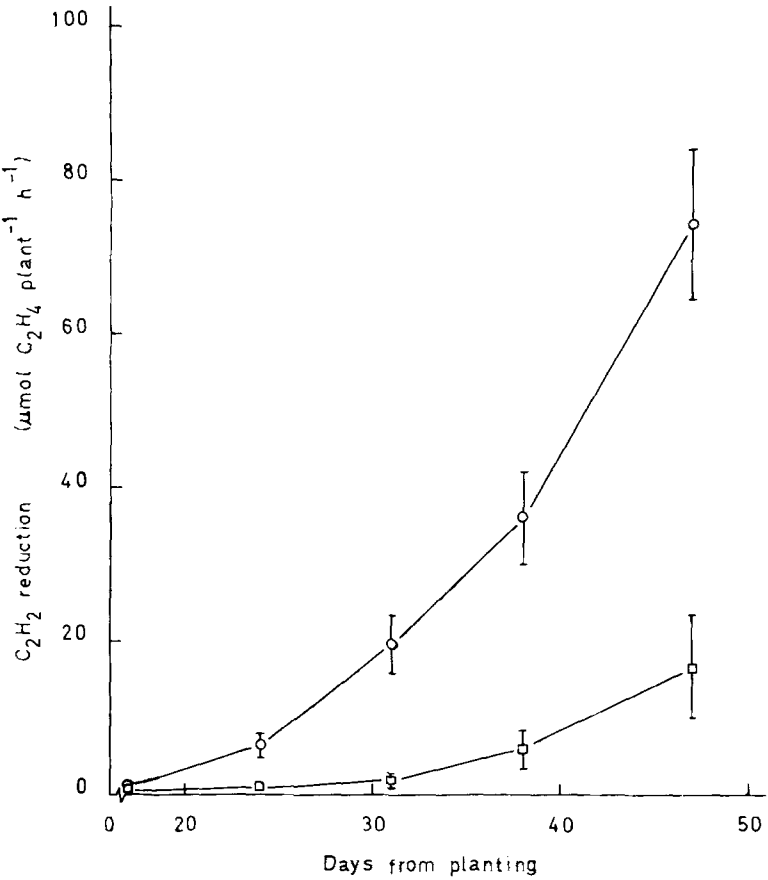

Fig. 2. Development of nitrogenase activity (C_2H_2-reduction) in *A. incana* grown in two different cultivation systems. $\bar{x} \pm$ SE; n = 5.
○ continuously circulating system
□ traditional system

system exceeded that of plants from the two other cultivation systems in all organs (Table 2). For example, the nitrogen content in the leaves was 82.18 mg compared to only 14.48 and 11.41 mg for the continuously circulating system, the traditional and the hydroponics system, respectively (Table 2). There was significantly more nitrogen per dry weight in the nodules of the alders continuously supplied with nutrients than in the nodules of the traditionally and hydroponically grown alders (Table 2). There was also more nitrogen per dry weight in the leaves of the continuously supplied alders (Table 2), although the difference was not statistically significant.

The modified continuously circulating system was more easily operated than the continuously circulating system. Alders grown in the modified system still showed significantly higher values of biomass,

shoot length, nitrogen content and nitrogenase activity than traditionally grown alders, but not as high values as alders in the continuously circulating system (Table 3).

Relative efficiency of nitrogenase

When C_2H_2-reduction increased also H_2-evolution in air increased (Fig. 3B). In alders grown in the modified continuously circulating system RE was fairly constant and ranged only from 0.75 to 0.83 (Fig. 3A). In the traditionally grown alders RE decreased slightly from 0.88 to 0.67 (Fig. 3A).

Neither in intact plants nor in nodule homogenates could any biological H_2-uptake (hydrogenase activity) be demonstrated. The decrease in H_2 in the gas phase was of the same rate in incubation tubes with only buffer and electron acceptor as in tubes with nodule homogenate and electron acceptor. The nodule homogenates were capable to reduce C_2H_2 *in vitro* (data not shown).

Discussion

The continuously circulating systems were developed in an attempt to obtain unstressed nitrogen-fixing alders for studies on the energy demand of the nitrogen fixation process. Such studies also requires a cultivation system where repeated measurements of C_2H_2-reduction can be made and this requirement was also met in the continuously circulating systems.

Alders continuously supplied with nutrients and water were visibly nodulated 5 days earlier than alders in the two other cultivation systems. This earlier nodule formation means an earlier start of nitrogen fixation and can also explain the absent lag phase in shoot growth

Table 3. Shoot length, dry weight, leaf area, nitrogenase activity and nitrogen content in two different cultivation systems. The values are from the end of the growth period (47 days from planting). $\bar{x} \pm$ SE, n = 4–6

Plant characteristics	Cultivation systems	
	Traditional	Modified continuously circulating system
Shoot length	21.7 ± 4.9	39.0 ± 2.6
Leaf area (cm^2)	184.3 ± 58.3	383.8 ± 30.1
Dry weight (g): total plant	1.552 ± 0.486	3.686 ± 0.328
Root/shoot ratio	0.36 ± 0.02	0.28 ± 0.01
Nitrogenase activity (μmol C_2H_4 $plant^{-1}h^{-1}$)	19.61 ± 7.21	42.03 ± 4.02
Nitrogen content (mg): total plant	37.8 ± 12.9	75.3 ± 8.0

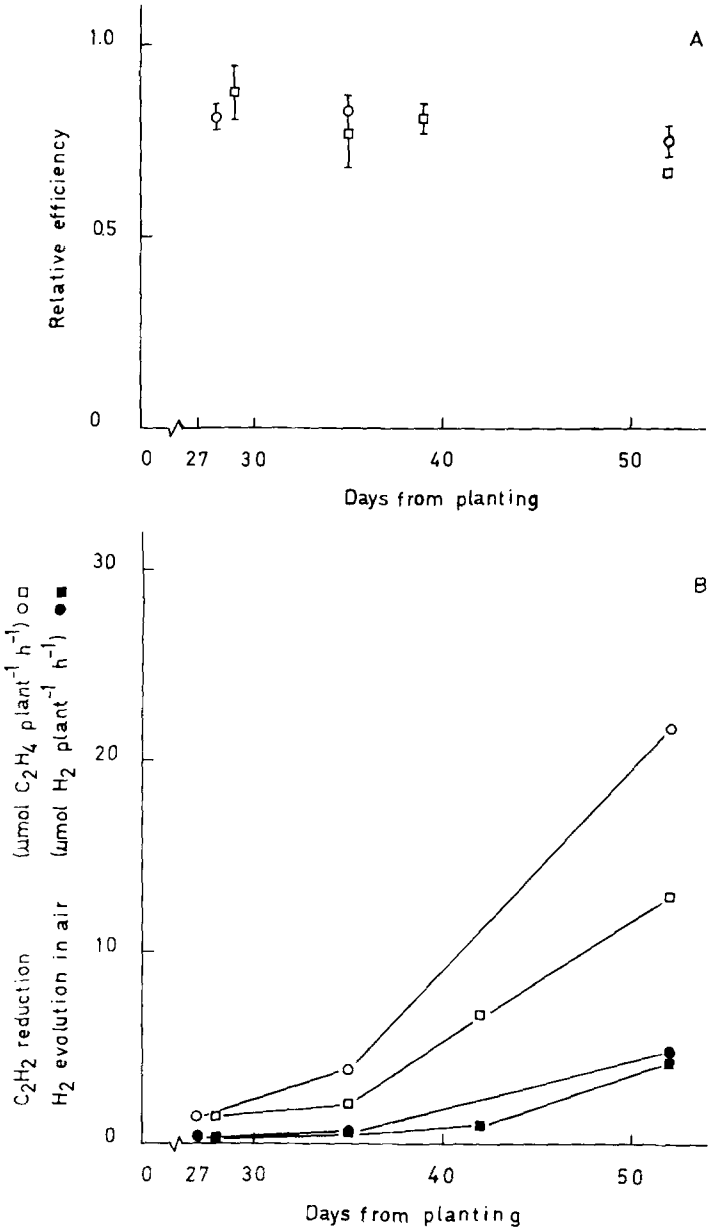

Fig. 3A. Development of relative efficiency of nitrogenase in *A. incana* grown in two different cultivation systems. $\bar{x} \pm$ SE, n = 4. Bars indicate SE, unless SE is smaller than the dimension of the symbol.

○ continuously circulating system

□ traditional system

B. Development of C_2H_2-reduction (○,□) and H_2 evolution (●,■) in air in *A. incana* grown in two different cultivation systems. $\bar{x}$, n = 4, SE was 5 to 31% of $\bar{x}$, with the highest SE values in the traditional system.

○ continuously circulating system

□ traditional system

(Fig. 1), as nitrogen content and growth are strongly correlated[18]. Alders grown in the continuously circulating system developed stem branches at the end of the experimental period. Therefore, the biomass or the leaf area gives a better description of the plant development than the shoot length does at this stage. The root/shoot ratio of the alders in the continuously circulating system indicates easily available nutrients (Table 2). Branched shoots is also a response to good nutrient status[7].

Thus, the continuous supply of water and nutrients permitted these plants to grow faster[13] and to have more nitrogen than plants in the two other cultivation systems studied. Plants in the traditional system received a limited supply of nutrients, like *e.g.* phosphorus, a nutrient of great important for nitrogen fixation. Together with the later onset of nitrogen fixation this led to a lower content of nitrogen, and a lower growth rate. In spite of a continuous supply of water and nutrients, the hydroponic system was less successful. The continuously circulating system provided a well aerated environment for the root system. The aeration of the solution in the hydroponic system was not sufficient, as indicated by the large amount of callus growth through the nodule lenticels.

The relative efficiency of nitrogenase in the *A. incana-Frankia* symbiosis ranged from 0.67 to 0.88. RE did not change during the part of ontogeny studied in the alders continuously supplied with nutrients and water. A slight decrease was observed in the traditionally grown alders. However, this decrease was smaller than the variation in RE reported for pea by Bethlenfalvay[4]. Such differences may well occur when symbioses with *Frankia* and *Rhizobium* are compared. The period studied is a limited part of the ontogeny in *Alnus,* while the studies made on legumes covered several ontogenic stages.

This whole plant study on the *Alnus-Frankia* symbiosis showed that irrespective of C_2H_2-reduction rate RE held a constant value during the growth period studied in the modified continuously circulating system (Fig. 3B). The results on traditionally grown alders were more like data on cowpea for which Rainbird *et al.*[21] reported maximum relative efficiency at low rates of N_2-fixation. It was not possible in the study by Rainbird *et al.*[21] to separate the extent to which changes in electron allocation by nitrogenase from N_2-reduction to H^+-reduction or changes in hydrogenase activity were contributing to the change in RE. In this study no hydrogenase activity was detectable and the changes in RE are therefore considered due to changes in electron allocations from N_2-reduction to H^+-reduction.

The relative efficiency differed between the two cultivation systems studied, showing a slight decrease in RE in the traditionally grown

alders while RE was constant in alders in the continuously circulating system. This indicates that the growth conditions affect the efficiency of nitrogen fixation. No strict relationship was found between RE and nitrogen content in this study, which is in contrast to value presented in a study of soybean[1,24] and cowpea[24]. If RE would have been the same in alders in the continuously circulating system and in the traditional system, the nitrogen content of the traditionally grown alders would have been about 6% greater. The value is estimated from the total C_2H_2-reduction integrated over the whole growth period. This nitrogen content is calculated from C_2H_2 reduction and H_2 evolution in air by the formula (C_2H_2 reduced/1.5) x RE. RE is easily overestimated when an uptake hydrogenase is present. But, in studies like the present one, where no hydrogenase was detected, RE seems to be a good tool for estimation of nitrogen fixation from C_2H_2-reduction measurements. Thus, the almost half as high nitrogen content (given by the analyses of Kjeldahl-N) of the traditionally grown alders compared to those in the continuously circulating system cannot be explained by the difference in RE. The amount of *Frankia* and nitrogenase is a more likely explanation to the difference in nitrogen content.

Growth, biomass production and nitrogen content are generally correlated. RE and nitrogen content were not closely related in this study. We conclude that the difference in RE between alders in the traditional and the modified continuously circulating system was too small to explain the obtained difference in biomass in these plants. Therefore, it is not expected that RE and plant productivity should be closely related to each other. Such results are in accordance with studies on pea[3,20]. RE might also give a better description of electron allocation to N_2 or H^+ than it describes the capacity of the symbiosis to fix nitrogen.

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