Nitrogenase Activity in *Alnus incana* Root Nodules. Responses to O_2 and Short-Term N_2 Deprivation[1]

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O_2 and host-microsymbiont interactions are key factors affecting the physiology of N_2-fixing symbioses. To determine the relationship among nitrogenase activity of *Frankia-Alnus incana* root nodules, O_2 concentration, and short-term N_2 deprivation, intact nodulated roots were exposed to various O_2 pressures (pO_2) and $Ar:O_2$ in a continuous flow-through system. Nitrogenase activity (H_2 production) occurred at a maximal rate at 20% O_2. Exposure to short-term N_2 deprivation in $Ar:O_2$ carried out at either 17%, 21%, or 25% O_2 caused a decline in the nitrogenase activity at 21% and 25% O_2 by 12% and 25%, respectively. At 21% O_2, nitrogenase activity recovered to initial activity within 60 min. The decline rate was correlated with the degree of inhibition of N_2 fixation. Respiration (net CO_2 evolution) decreased in response to the N_2 deprivation at all pO_2 values and did not recover during the time in $Ar:O_2$. Increasing the pO_2 from 21% to 25% and decreasing the pO_2 from 21% to 17% during the decline further decreased rather than stimulated nitrogenase activity, showing that the decline was not due to O_2 limitation. The decline was possibly due to a temporary disturbance in the supply of reductant to nitrogenase with a partial O_2 inhibition of nitrogenase at 25% O_2. These results are consistent with a fixed O_2 diffusion barrier in *A. incana* root nodules, and show that *A. incana* nodules differ from legume nodules in the response of the nitrogenase activity to O_2 and N_2 deprivation.

Biological N_2 fixation is inhibited by atmospheric levels of O_2 because of the O_2 sensitivity of nitrogenase. N_2-fixing organisms living in an aerobic environment therefore use various physiological and biochemical mechanisms to provide an acceptable O_2 concentration for nitrogenase and, simultaneously, to allow the use of O_2 in oxidative phosphorylation. Root nodules of many legumes such as soybean, alfalfa, lupin, and clover have an apparently variable barrier to gas diffusion that may function to regulate internal O_2 concentration and protect nitrogenase activity in the rhizobial microsymbiont (Hunt and Layzell, 1993). Exposure of those legume nodules to N_2 deprivation with an $Ar:O_2$ or a 10% acetylene treatment caused a decline in nitrogenase activity (Minchin et al., 1983). This decline was associated with an apparent decrease in nodule permeability that reduced the infected cell O_2 concentration, thereby making the nodule more O_2 limited (Hunt et al., 1987; King and Layzell, 1991; Diaz del Castillo et al., 1992; Kuzma et al., 1993). A similar reduction in the permeability to gas

diffusion also occurred during various stress conditions (Hunt and Layzell, 1993). The O_2 limitation of nitrogenase activity could for most types of stresses be reversed by gradually increasing the external O_2 pressure (pO_2).

In several actinorhizal N_2-fixing symbioses, symbioses between the actinomycete bacterium *Frankia* and a range of plant species including *Alnus incana*, nitrogenase activity also declined shortly after exposure to acetylene in continuous flow assay systems (Rosendahl and Huss-Danell, 1988; Tjepkema et al., 1988; Silvester and Harris, 1989; Tjepkema and Murry, 1989; Silvester and Winship, 1990; Harris and Silvester, 1994). The magnitude and time course of the decline varied among the symbioses and, in contrast to legume symbioses, for some species showed an almost complete recovery to initial activity during the assay. The mechanism explaining the decline in nitrogenase activity remains to be demonstrated.

Among actinorhizal root nodules there is a diversity in structural organization in the host part as well as in the microsymbiont, and their physiologies relative to O_2 are apparently also diverse (Silvester et al., 1990). For *Myrica gale*, acetylene reduction activity typically declines but recovers partially within 70 min to a steady-state level (Zeng and Tjepkema, 1995). In response to changes in pO_2, nitrogenase activity did not show any short-term adaptation or recovery that would be consistent with a variable diffusion resistance (Zeng and Tjepkema, 1995). The diffusion resistance did, however, decrease with increasing temperature and was suggested to be located in the wall of the infected plant cells (Zeng and Tjepkema, 1994). In *Coriaria arborea* nodules, acetylene reduction activity declined to a stable activity with no recovery (Harris and Silvester, 1994). In response to an $Ar:O_2$ treatment, respiration and nodule permeability to gas diffusion also decreased, suggesting the presence of a variable diffusion resistance mechanism in this species.

A. incana nodules are considered well ventilated, having gas diffusion pathways into the zone of infected cells (Tjepkema, 1979). The thick-walled, multilaminated envelope of the vesicle, the bacterial cell-type expressing nitrogenase, is thought to be the most important diffusion barrier that matches O_2 supply to its consumption within the vesicle so that O_2 does not accumulate to inhibitory levels (Winship and Silvester, 1989; Silvester et al., 1990). Nevertheless, the fact that *Frankia* can live within a plant cell with respiratory O_2 consumption by plant mitochondria suggests the possibility that the plant cell could affect the O_2 environment

of *Frankia*. Growing *A. incana* nodules at elevated pO_2 caused the *Frankia* vesicles to produce more lipid layers in their envelopes (Silvester et al., 1988; Abeysekera et al., 1990), although not completely with the same clear response as that of vesicles of cultured *Frankia* (Parsons et al., 1987; Harris and Silvester, 1992). The increase in numbers of lipid laminae in response to increased growth pO_2 was calculated to be insufficient to act as the exclusive barrier to O_2 diffusion for protection of nitrogenase in *A. incana* nodules (Abeysekera et al., 1990). Anatomical changes in the plant part of the nodule were also found (Silvester et al., 1988; Abeysekera et al., 1990), which could imply that there are additional barriers or processes that aid in providing a suitable O_2 environment for nitrogenase.

Metabolic interactions between the plant host and the microsymbiont clearly occur, since the host supplies *Frankia* with reductant in some form. Also, the assimilation of NH_4^+ in *A. incana* root nodules is likely to be carried out by the infected plant cell rather than in *Frankia*, since neither of *Frankia's* two forms of Gln synthetases are expressed in the symbiotic stage (Lundquist and Huss-Danell, 1992). By preventing NH_4^+ formation in Ar:O_2, the ATP demand of the nodule and thereby the nodule respiration may be reduced. Studies on N_2 deprivation could therefore indicate metabolic interactions between the plant cell and the microsymbiont and reveal interactions affecting the O_2 environment of nitrogenase.

The goals of this study were: (a) to characterize the optimum pO_2 of nitrogenase activity for this *Frankia-A. incana* symbiosis, and (b) to address the questions of whether Ar:O_2 treatment causes a decline in nitrogenase activity and respiration, and if such a decline affected by external pO_2?

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cuttings of a clone of gray alder (*Alnus incana* [L.] Moench) were rooted and inoculated with "the local source of *Frankia*" lacking uptake hydrogenase activity (Sellstedt et al., 1986; Huss-Danell, 1991). The cuttings were planted in pots with gravel (approximately 3 mm in diameter) of granite rock as support but without water-holding capacity. Nutrient solution was supplied with tubing in an air-lift system with recirculating nutrient solution (Lundquist and Huss-Danell, 1991). Plants were grown in a climate chamber with 17 h of light at 25°C and 7 h of darkness at 15°C, relative humidity of 70%, and a photosynthetic photon flux density (PPFD) of about 250 μmol m^{-2} s^{-1} from metal halogen lamps (Power Star HQI-T 400W/DH, Osram, Germany). After 4 weeks, the PPFD was raised to about 600 μmol m^{-2} s^{-1}. The plants were used 12 ± 0.5 (mean ± SE, $n = 58$) weeks after inoculation and were 70 ± 3 (mean ± SE) cm high.

Gas Exchange Measurements

Nitrogenase activity was measured as H_2 evolution and respiration as net CO_2 evolution in a gas exchange system

generally adapted from a system described previously (Layzell et al., 1989). Specifically, H_2 was measured using a semiconductor detector (model TGS-821, Figaro Engineering, Osaka). CO_2 was measured with an infrared gas analyzer (IRGA) (type 225 Mk3, ADC BioScientific, Hoddesdon, UK) operated in the absolute mode with CO_2-free air flushing through the reference cell. Two electrochemical fuel cells sensitive to O_2 (model KE-25, Figaro Engineering) were used to measure pO_2 in the system. One was connected upstream of the cuvette and one at the outlet close to the H_2 sensor. Gas flows were controlled by mass flow controllers (Bronkhorst High-Tech B.V., Ruurlo, The Netherlands).

The H_2 sensors, O_2 sensors, IRGA, and mass flow controllers were connected to a Macintosh IIfx computer via an analog to digital interface board. Outputs and data collection were operated through the program Workbench (Strawberry Tree, Sunnyvale, CA), which was programmed so that any desired partial pressure of N_2, Ar, and O_2 could be achieved without affecting the total flow. The signals were averaged over 10 s and recorded every 20 s. Changes in outputs to flow controllers were programmed in Workbench when possible to improve reproducibility in gas composition and timing between experiments. Two plants were measured simultaneously. The total flows were controlled by the mass flow controllers and the flows to each cuvette were manually adjusted to 0.8 L min^{-1} via needle valves and flow meters. Before entering the cuvette, the gas passed through a humidifier at 25°C. The gas volume of the cuvettes were approximately 0.25 L when they contained roots and gravel. The effluent gas from each cuvette was led into a water trap on an ice bath, where gas was sampled at 0.4 L min^{-1} with a diaphragm pump through a drying column containing magnesium perchlorate crystals, through the IRGA, through a water condenser maintained at −78°C in a dry ice-ethanol mix, and finally to the H_2 and O_2 sensors. One of the two parallel measuring systems lacked the IRGA and the outlet O_2 sensor, but made it possible to measure H_2 evolution from two plants at the same time. Similar results were obtained in the two systems.

During the experiments the plants were kept in a chamber covered with plastic and containing a temperature-controlled water bath to provide a stable cuvette temperature. The chamber was kept humid by spraying water on the white absorbent paper covering the inside walls. A second water bath acted as a heat trap below the metal halogen lamp. The PPFD at 40 cm above the cuvette was approximately 450 μmol m^{-2} s^{-1}. Before each experiment the plants were brought to the chamber and the pots were watered with approximately 0.25 L of fresh nutrient solution with a temperature of 23°C, and were then left to drain for 25 to 35 min in the chamber before the measurement. All experiments were conducted on nodulated root systems of intact plants, in which the root system was left in the pot and the pot was sealed with two lids and used as a cuvette. The space around the stem was sealed with a piece of styrofoam and some sealing putty (Terostat IX, Teroson GmbH, Heidelberg). The tubing was attached and the cu-

vette put into the water bath maintained at 23°C. The cuvettes were left with a flow of N_2:O_2 at a pO_2 of 21%.

The H_2 analyzers were calibrated using a standard gas of H_2 (1,787 μL L^{-1} in N_2; AGA Gas AB, Stockholm) diluted into the gas stream in the range 4 to 160 μL L^{-1}. Calibrations were done in N_2:O_2 and Ar:O_2 at 13%, 17%, 21%, and 25% O_2. The O_2 sensors were calibrated against dry air. The IRGA was operated in the absolute mode and calibrated against a standard gas containing 355 μL L^{-1} CO_2 in air (AGA Gas AB). Since the IRGA showed different sensitivities in N_2:O_2 and Ar:O_2, it was also calibrated by diluting a standard gas (1.78% CO_2 in air) in the range 100 to 700 μL L^{-1} in N_2:O_2 and Ar:O_2 at 21% O_2. Linear responses were obtained and used for correcting the differences in sensitivity in N_2:O_2 and Ar:O_2.

Responses to Changes in pO_2

Two experiments were performed to characterize the responses of H_2 evolution and CO_2 evolution to pO_2. In experiment 1, in which the response in N_2:O_2 was investigated, the nodulated root systems were first kept with a flow of N_2:O_2 at 21% O_2 for 25 to 30 min. The rates had then been stable for 10 to 15 min and a short shift from N_2:O_2 to Ar:O_2 and back was done for 3 min to measure the total nitrogenase activity. The pO_2 was then kept at 21% for 15 min and then changed in steps of 1% every 5 min for different plants ending either at 17% or 25% O_2, which was kept for the remaining part of the experiment. The gas composition was either kept as N_2:O_2 up to 75 min, or changed from N_2:O_2 to Ar:O_2 15 min after reaching the final pO_2, as described for studies of responses to N_2 deprivation.

In the second experiment, the nodulated root systems were kept in the gas exchange system for 50 min at 21% O_2. The pO_2 was then altered in steps of 2% O_2 every 10 min, first down to 15% O_2, then up to 25% O_2, and finally back to 21% O_2. During the 10-min period at each pO_2, the gas composition was first N_2:O_2 for 4 min followed by Ar:O_2 for 2.5 min and then N_2:O_2 for 3.5 min. The plotted values are from the end of the 4-min N_2:O_2 period and from the end of the Ar:O_2 period. The switch to Ar:O_2 was necessary to measure the total electron flux through nitrogenase, since no N_2 is reduced in Ar:O_2 and all electrons are used for H_2 production. The electron allocation coefficient (EAC) of nitrogenase was calculated according to the method of Edie and Phillips (1983) as EAC = 1 − ([H_2 evolution in N_2][H_2 evolution in Ar]$^{-1}$) using values for each individual plant.

Responses to N_2 Deprivation

To test the effect of a short period of N_2 deprivation on H_2 evolution and on the remaining H_2 evolution in N_2:O_2 afterward, nodulated root systems were pretreated by keeping them in N_2:O_2 at 21% O_2 for 50 min. The gas composition was then changed from N_2:O_2 to Ar:O_2, kept for 15 min, and then changed back to N_2:O_2.

The effect of a longer period of N_2 deprivation on H_2 evolution and CO_2 evolution was tested by exposing nod-

ulated root systems to Ar:O_2 for 60 min at different pO_2 values. This was done with the plants used in experiment 1 in the study of responses to changes in pO_2 described above. The exposure to Ar:O_2 was carried out at either 17% or 25% O_2 starting 15 min after reaching that pO_2, or at 21% after the same total time had elapsed. After the Ar:O_2 period, the gas composition was changed back to N_2:O_2 for an additional 15 min.

To determine whether O_2 could stimulate or further inhibit H_2 evolution during the Ar-induced decline, the pO_2 was either increased to 25% O_2 or decreased to 17% starting 15 min after the change from N_2:O_2 to Ar:O_2. This pO_2 was kept for 15 min and then returned to 21%. Finally, the gas composition was changed from Ar:O_2 to N_2:O_2. The pretreatment for these plants was the same as for the experiments on N_2 deprivation described above.

To determine whether a complete elimination of N_2 fixation was necessary until a decline occurred, the nodulated root systems were exposed to various external N_2 pressures (pN_2) balanced by Ar at 21% O_2. Root systems were initially kept in N_2:O_2 at 21% O_2 for 30 min. The gas composition was then changed to 20%/59%/21% (Ar:N_2:O_2) for 10 min, followed by 20 min in N_2:O_2. Using this protocol, changes in gas composition followed, which sequentially exposed the root systems to 39.5%, 20%, 10%, and 0% N_2. The H_2 and CO_2 evolution rates at the various pN_2s were normalized as percentages of the maximum H_2 evolution at 0% N_2 (Ar:O_2). The rates at which the rates of H_2 evolution and CO_2 evolution declined were calculated (linear regression) as the percent change per minute on the data obtained during 5 min starting 5 min after the change from N_2:O_2 to Ar:N_2:O_2. A coefficient of inhibition of N_2 fixation was calculated as: (H_2 evolution in N_2:Ar:O_2 − H_2 evolution in N_2:O_2)(H_2 evolution in Ar:O_2 − H_2 evolution in N_2:O_2)$^{-1}$. The inhibition coefficient was calculated from the same experiment as the decline rates but on data obtained during 2 to 4 min after the change from N_2:O_2 to Ar:N_2:O_2 to avoid any disturbance from later responses in the mixtures of Ar, N_2, and O_2. It describes the degree of reduction of electron allocation to N_2 fixation at the various pN_2s.

RESULTS

Responses to Changes in pO_2

The maximum H_2 evolution rate occurred at about 20% O_2 (Figs. 1 and 2A) when pO_2 was either reduced or increased from 21% O_2. During the time at low pO_2, the H_2 evolution adapted and increased to be about 8% higher the second time at 19% O_2 compared with the first time (Fig. 2A). At 25% pO_2, the H_2 evolution rate decreased by 36% but increased slightly upon return to 21% O_2 (Fig. 2A). The H_2 evolution recovered by a few percent during 10 min at 21% O_2.

CO_2 evolution also responded to changes in pO_2. In N_2:O_2 (Fig. 1), the CO_2 evolution rate decreased by 5% when pO_2 was reduced from 21% to 17% O_2, and increased by 3% when pO_2 was increased from 21% to 25% O_2. Below 20% O_2, the CO_2 evolution was linearly correlated to the
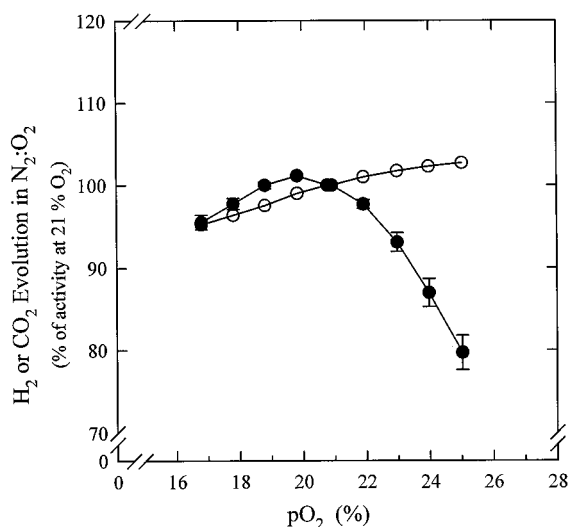
Figure 1. The responses to pO_2 of H_2 evolution (●) and CO_2 evolution (○) rates in intact nodulated *A. incana* root systems. The gas composition was $N_2:O_2$ throughout the experiment. The pO_2 was first 21% O_2 and then changed for different plants to either 17% or 25% in steps of 1% O_2 every 5 min. Values are means ± SE. Error bars are smaller than the symbol when not displayed. Average 100% activities were 31.4 ± 4.4, $n = 10$ (17% O_2) and 31.0 ± 3.2, $n = 8$ (25% O_2) μmol H_2 plant^{-1} h^{-1}, and 619 ± 110, $n = 5$ (17% O_2) and 534 ± 107, $n = 4$ (25% O_2) μmol CO_2 plant^{-1} h^{-1}.

H_2 evolution. A smaller but similar adaptation at low pO_2 as described above for H_2 evolution also occurred (Figs. 1 and 2B).

Responses to N_2 Deprivation

Changing from N_2 to Ar as balancing gas gave an immediate increase in H_2 evolution (Figs. 3, 4A, and 5A), as expected for nitrogenase activity since removal of N_2 allows allocation of all reducing equivalents to proton reduction. The H_2 evolution increased on average to 252% (Figs. 3, 4A, and 5A). The average EAC of several plants used in different experiments and calculated from the first short shift to Ar:O_2 in the pretreatment was 0.60 ± 0.01 (mean ± SE, $n = 28$). In the experiment where pO_2 was varied (Fig. 2A), EAC was 0.60% at 21% O_2 and showed a statistically significant lower value only at 25% O_2. This was apparently because the value in Ar:O_2 was recorded a few minutes after the value in $N_2:O_2$, when inactivation of nitrogenase at high pO_2 had proceeded further.

During the exposure to N_2 deprivation in Ar:O_2 (79:21), the total nitrogenase activity declined, as seen by the decline in H_2 evolution (Fig. 3). The declined H_2 evolution remained when the gas composition was changed back to $N_2:O_2$ after 15 min in Ar:O_2 (Fig. 3), which is about when the activity was at its lowest point in Ar:O_2 (Fig. 4A). The remaining activity in $N_2:O_2$ was 87% of the initial activity in $N_2:O_2$ and therefore had decreased as much as the activity in Ar:O_2 had decreased compared with the initial peak activity in Ar:O_2 (Fig. 3).

The decline in H_2 evolution was related to the pO_2 at which the N_2 deprivation treatment was carried out. The

change from $N_2:O_2$ to Ar:O_2 caused a decline in H_2 evolution within a few minutes when the change was carried out at 21% or 25% O_2 (Fig. 4A). The activity declined to a minimum at 88% of the peak activity after 17 min at 21% O_2 and to 75% of the peak activity after 24 min at 25% O_2. Following the decline, the activity increased and after 60 min in Ar:O_2, it had recovered almost completely at 21% O_2, but only partially at 25% O_2. In contrast, at 17% O_2 there was no decline in H_2 evolution following the change to Ar:O_2, and over the 60-min period in Ar:O_2 the activity increased to 113% of initial activity in Ar:O_2 (Fig. 4A). After the change back to $N_2:O_2$ the differences in H_2 evolution between the treatments remained.

H_2 evolution of plants kept as controls at 17% or 25% O_2 for 75 min without exposing them to Ar, increased gradually by 12% and 8%, respectively, over the 60-min period
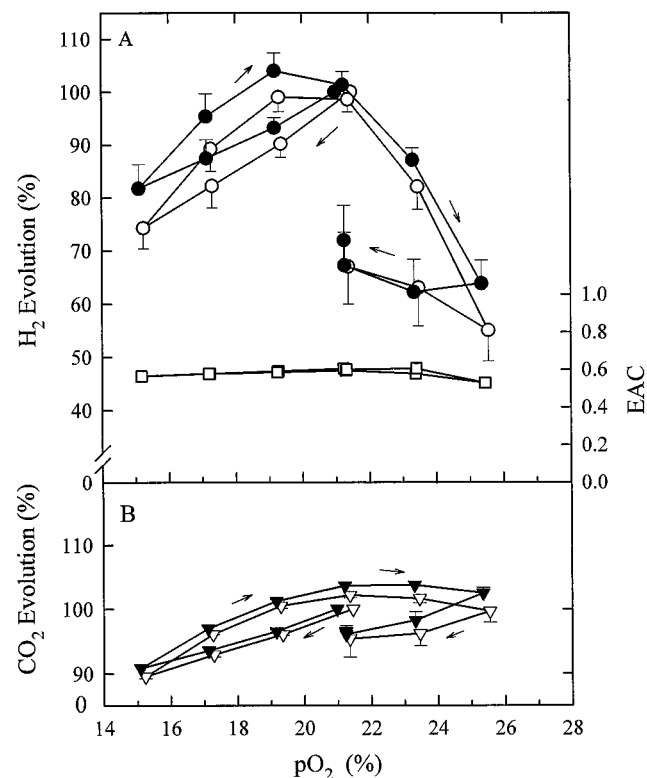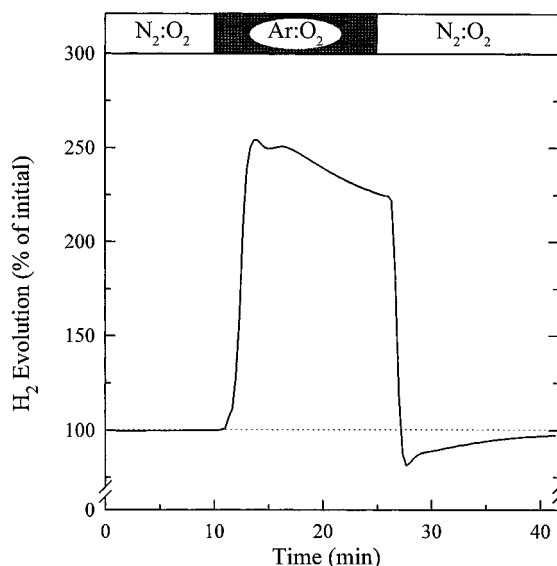

Figure 2. The responses to pO_2 of H_2 evolution rate and EAC (A) and CO_2 evolution rate (B) in intact nodulated *A. incana* root systems in $N_2:O_2$ and Ar:O_2. The pO_2 was first 21% and then altered in steps of 2% O_2 every 10 min first down to 15% O_2, then up to 25% O_2, and finally back to 21% O_2 as indicated by the arrows. During the 10-min period at each pO_2, the gas composition was first $N_2:O_2$ for 4 min, followed by Ar:O_2 for 2.5 min and then $N_2:O_2$ for 3.5 min. The plotted values and the values used for calculating EAC are from the end of the 4-min $N_2:O_2$ period and from the end of the Ar:O_2 period. The values of H_2 evolution are expressed as percentages of activity at 21% O_2. Error bars (SE) are in the positive direction for $N_2:O_2$, in the negative direction for Ar:O_2, and not displayed when smaller than the symbol. Values are means ± SE for six (H_2 and EAC) or three (CO_2) plants. Average 100% activities ± SE were 48.4 ± 10.8 (N_2) and 122.6 ± 27.2 (Ar) μmol H_2 plant^{-1} h^{-1}, and 739 ± 11 (N_2) and 726 ± 15 (Ar) μmol CO_2 plant^{-1} h^{-1}. In A, ●, in $N_2:O_2$; ○, in Ar:O_2; □, EAC. In B, ▼, in $N_2:O_2$; ▽, in Ar:O_2.
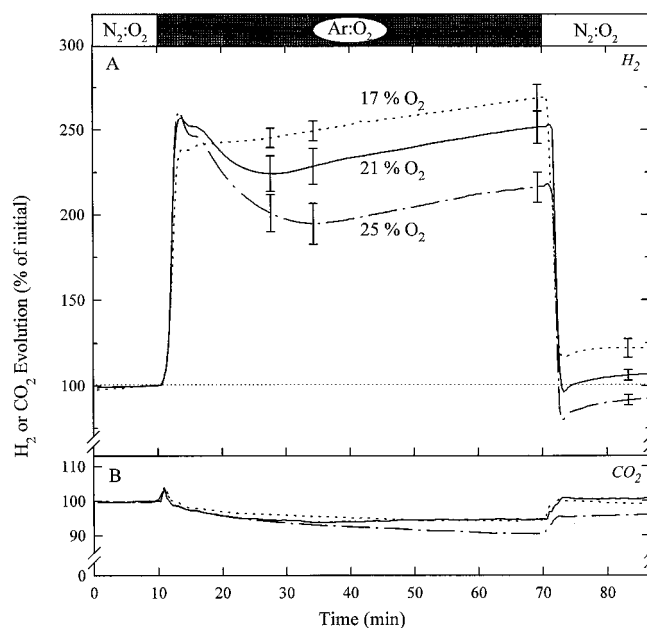
that corresponded to the Ar treatment (data not shown). To determine whether the decline in activity during N_2 deprivation was an effect of the increased gas flow through the root system during the assay, two plants were grown for 2 and 3 weeks in the regular growth conditions with an air flow through the pot at the same rate as during the gas exchange measurements. These plants showed a similar decline and recovery at 21% O_2 as in Figure 4A (data not shown).

A large part of the CO_2 evolution came from the nodules because only 50% of the nodulated root CO_2 evolution remained after removing the nodules from the nodulated roots. CO_2 evolution from the nodulated root systems decreased during N_2 deprivation (Figs. 4B and 5B). During the 60-min period in Ar:O_2 at 25% O_2, the CO_2 evolution rate decreased by about 10% of the initial CO_2 evolution and was still 5% lower after the change back to N_2:O_2, thus showing the same pattern as for H_2 evolution. In contrast, the CO_2 evolution rate of the root systems kept at 17% and 21% O_2 in Ar:O_2 only decreased by 5% and returned to the initial rate upon the change back to N_2. For nodulated root systems not exposed to Ar:O_2 and kept at either 17% or 25% O_2, the CO_2 evolution rate increased by 3% and 7%, respectively, during the corresponding 60-min period (data not shown). No significant effects on respiration of changing from N_2:O_2 to Ar:O_2 were found on root systems in which the nodules had been removed (data not shown).

To further investigate the relationship between O_2 and the Ar-induced decline in H_2 evolution, the pO_2 was changed 15 min after the change to Ar:O_2 (Fig. 5). The decline could not be reversed by increasing the pO_2. The pO_2 was either increased to 25% or decreased to 17% and gave in both cases a further decrease in activity from 227%
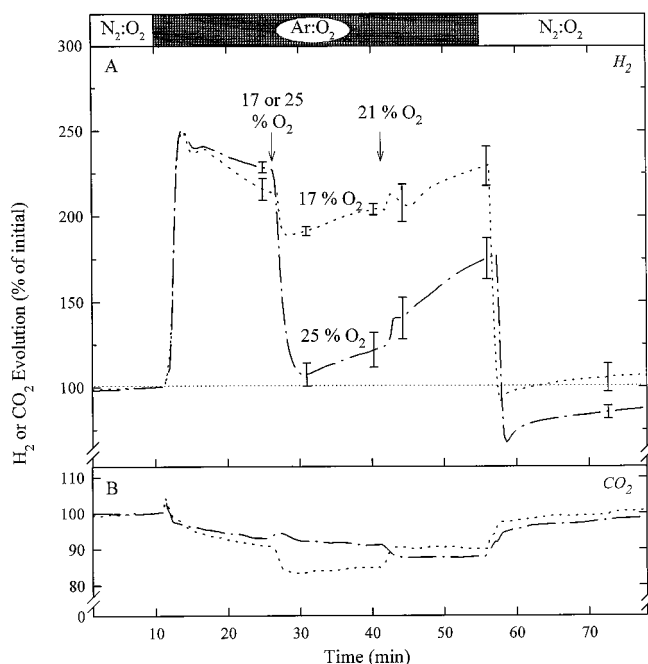

Figure 4. Time course of changes in H_2 evolution rate (A) and CO_2 evolution rate (B) in intact nodulated *A. incana* root systems during exposure to Ar:O_2 at 17% (dashed line), 21% (solid line), and 25% (broken line) O_2. Prior to the experiments the plants were kept at 21% O_2 for 25 to 30 min. The pO_2 was then changed with 1% every 5 min until the final pO_2 was reached and kept for another 15 min before changing to Ar:O_2. The gas composition during the experiment was changed from N_2:O_2 to Ar:O_2 and back, as indicated by the top panel. The activities in N_2:O_2 at the time just before the change to Ar:O_2 were set to 100%. The lines represent averages of all data, and error bars (SE) are displayed at selected time points. Average 100% activities ± SE were 26.7 to 31.2 μmol H_2 plant^{-1} h^{-1} (n = 5–7) in A, and 622 to 640 μmol CO_2 plant^{-1} h^{-1} (n = 3–4) in B.

to 107% and from 214% to 189% of initial in N_2:O_2, respectively. In both cases H_2 evolution recovered gradually at the new pO_2. After 15 min at either 17% or 25% O_2, the pO_2 was changed back to 21%. A small drop in activity followed by a recovery occurred after the change from 17% to 21% O_2. After an additional 15-min period, when Ar was replaced by N_2, the activity was close to the initial rate for the plants temporarily exposed to 17% O_2, but only 72% of the initial rate for the plants temporarily exposed to 25% O_2. These latter plants recovered their activity to 88% of initial values within 15 min.

The rate of the Ar-induced decline at different pN_2s was not linearly correlated to pN_2 (Fig. 6A). The decline occurred at pN_2 lower than 20%, where the inhibition coefficient of N_2 fixation indicated that N_2 fixation was significantly reduced (Fig. 6B). The decline rate showed a linear correlation to the inhibition coefficient of N_2 fixation ($P < 0.05$).

Figure 3. Time course of changes in the H_2 evolution rate of intact nodulated *A. incana* root systems during exposure to Ar:O_2 at 21% O_2. The gas composition was changed from N_2:O_2 to Ar:O_2, as indicated by the top panel, maintained for 15 min, and then changed back to N_2:O_2. The line represents the average of all data. Average 100% activity ± SE was 38.0 ± 4.1 μmol H_2 plant^{-1} h^{-1} (n = 7).

DISCUSSION

The experiments demonstrated the following major characteristics of nitrogenase activity in *Frankia-A. incana* root nodules. Nitrogenase activity has a sharp pO_2 optimum. In response to N_2 deprivation at pO_2s above optimum, the

Figure 5. Time course of H_2 evolution rate (A) and CO_2 evolution rate (B) in intact nodulated *A. incana* root systems in response to changes in pO_2 during exposure to $Ar:O_2$. The gas composition was $N_2:O_2$ (21% O_2) at the start and was changed to $Ar:O_2$ as indicated by the top panel. After 15 min in $Ar:O_2$ the pO_2 was changed to either 17% (dashed line) or 25% O_2 (broken line) and after 15 min back to 21% O_2, as indicated by the arrows. After an additional 15 min, the gas composition was finally changed from $Ar:O_2$ to $N_2:O_2$. The lines represent averages of all data, and error bars (SE) are displayed at selected time points. Average 100% activities ± SE were 45.8 ± 4.8 ($n = 5$) and 23.8 ± 2.3 ($n = 4$) μmol H_2 plant^{-1} h^{-1} at 17% and 25% O_2, respectively, and 516 ± 109 ($n = 3$) and 397 ± 132 ($n = 2$) μmol CO_2 plant^{-1} h^{-1} at 17% and 25% O_2, respectively.

nitrogenase activity declined followed by some recovery. In addition, the decline was not reversed by increased or decreased pO_2.

Responses to pO_2

The pO_2 giving maximum nitrogenase activity was 20% (Figs. 1 and 2A). Below 20% O_2, nitrogenase activity seemed limited by O_2 supply, since the activity decreased with decreasing pO_2 and was stimulated by a return to higher pO_2. Above 20% O_2, nitrogenase activity became inactivated (Figs. 1 and 2A), which was partly irreversible since the activity remained lower when the pO_2 was returned to 21%.

The optimum at 20% O_2, close to the pO_2 of the growth conditions, is consistent with earlier results for *A. incana* nodules (Winship and Tjepkema, 1985; Silvester et al., 1988) and free-living *Frankia* grown in cultures (Parsons et al., 1987). In the present study it was possible to investigate the nitrogenase activity under conditions that did not inhibit N_2 fixation, as in the acetylene reduction assay used in earlier studies, which thus avoided the decline in nitrogenase activity in root nodules as discussed below.

Respiration, measured as CO_2 evolution, increased with increasing pO_2 (Fig. 1). Below 21% O_2 the change in respiration closely followed nitrogenase activity (Figs. 1 and 2). Some adaptation of nitrogenase activity and respiration occurred during the short period at pO_2s lower than optimum (Fig. 2) and also during periods of 75 min at 17% and 25% O_2 in $N_2:O_2$. However, the linear relationship between respiration and nitrogenase activity below optimum pO_2 and the inhibition of nitrogenase activity above optimum are consistent with the presence of a fixed diffusion barrier to create a suitable internal O_2 concentration for nitrogenase.

Responses to N_2 Deprivation

The immediate rise in H_2 evolution (Fig. 3) showed that N_2 fixation was eliminated by the change from $N_2:O_2$ to $Ar:O_2$ due to removal of the substrate N_2. The EAC of approximately 0.6 found in the present study is close to the results from intact root systems of legumes (Hunt et al., 1987, 1989; Diaz del Castillo et al., 1992) and less then 0.73
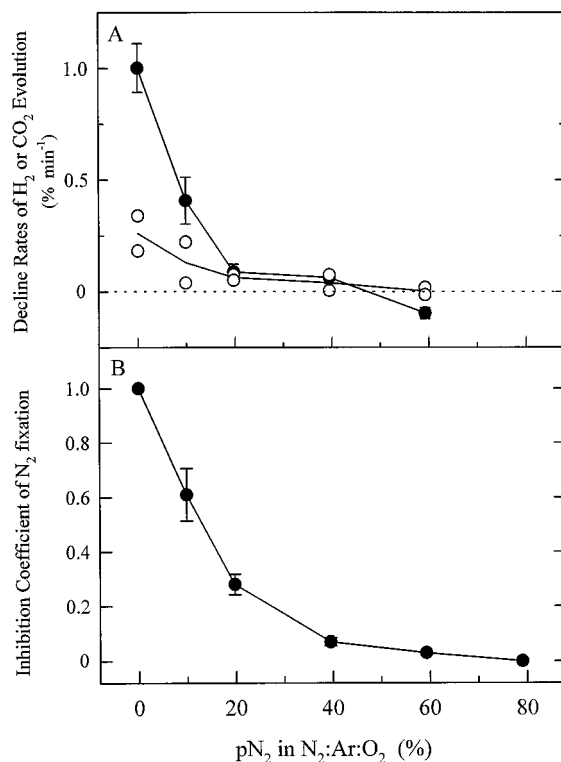
Figure 6. The responses of decline rates of H_2 evolution (●) and CO_2 evolution (○) rates (A) and the inhibition coefficient of N_2 fixation (B) to external pN_2 at 21% O_2. The intact nodulated root systems of *A. incana* were exposed to mixtures of Ar, N_2, and O_2, as described in "Materials and Methods." The H_2 and CO_2 evolution rates obtained during 5 min starting 5 min after the change from $N_2:O_2$ to $Ar:N_2:O_2$ were normalized as percentages of the maximum rates in $Ar:O_2$, and the rates at which the decline of the H_2 evolution and CO_2 evolution rates occurred were calculated. The inhibition coefficient describes the degree of reduction of electron allocation to N_2 fixation at the various pN_2 values. Mean values ± SE for four plants (H_2 and inhibition coefficient) or measured values for two plants (CO_2) are shown. Error bars were smaller than the symbol when not shown.

for purified nitrogenase measured in vitro (Simpson and Burris, 1984).

The occurrence of a decline in nitrogenase activity following the change from $N_2:O_2$ to $Ar:O_2$ (Fig. 3 and 4) resembles the acetylene-induced decline in nitrogenase activity of *Alnus* spp. nodules during the acetylene reduction assay (Rosendahl and Huss-Danell, 1988; Tjepkema et al., 1988; Silvester and Winship, 1990) and an Ar-induced decline of *Myrica gale* nodules (Tjepkema and Schwintzer, 1992). Exposure to acetylene (10%) and to $Ar:O_2$ both lead to cessation of N_2 reduction, which could explain the similarities in response. The primary cause of the decline in nitrogenase activity in response to the change to $Ar:O_2$ (Fig. 4A) is clearly an effect of cessation of NH_4^+ production. However, that O_2 also plays a role during the decline in the nitrogenase activity is supported by the big decline at 25% O_2, the smaller decline at 21% O_2, and the absence of a decline at 17% O_2 (Fig. 4A). Three different hypotheses that relate to these results and try to explain the decline of nitrogenase activity at 21% O_2 in particular are provided below.

First, the decline in nitrogenase activity could be caused by an increased diffusion resistance for O_2 in the nodule. In some legume plants the nitrogenase activity declines following exposure to $Ar:O_2$ (e.g. Hunt et al., 1987; King and Layzell, 1991; Diaz del Castillo et al., 1992), and this has been attributed to O_2 limitation of nodule metabolism caused by an increased diffusion resistance of a variable diffusion barrier in the inner cortex (Layzell and Hunt, 1990; King and Layzell, 1991). However, in the present study on *A. incana*, this seems unlikely because increasing the pO_2 from 21% to 25% during the decline further inhibited rather than stimulated nitrogenase activity (Fig. 5). Another difference to the legumes was that in the *A. incana* nodules there was also a recovery phase during the time in $Ar:O_2$ following the decline (Fig. 4A).

Second, the decline in nitrogenase activity could be caused by a limitation in a supply of reductant to nitrogenase. Cessation of NH_4^+ production and assimilation in $Ar:O_2$ could cause a disturbance in a supply of metabolites from the plant to *Frankia* that supports nitrogenase activity (compare with Fig. 7). The metabolism yielding reductant for nitrogenase in *Frankia* has not been elucidated. However, the overall high nitrogenase activity after 1 h with N_2 deprivation at 17% and 21% O_2 (Fig. 4A) suggests that there is not a simple short metabolic link between the production of NH_4^+ through N_2 fixation and sustenance of nitrogenase activity as H_2 evolution. Nevertheless, the decline in $Ar:O_2$ at 21% O_2, where nitrogenase activity operated more or less at its optimum pO_2, since neither an increase or a decrease in pO_2 stimulated nitrogenase activity (Fig. 5A), supports that nitrogenase activity declined due to decreasing amounts of reductant, ATP, or a specific metabolite. The decrease in CO_2 evolution during the decline in $Ar:O_2$ (Figs. 4B and 5B) could be interpreted as a decrease in an NH_4^+ assimilation-linked respiration. Also, the fact that a complete cessation of N_2 fixation was not necessary for an Ar-induced decline to occur, but rather that the rate of the Ar-induced decline correlated to the degree of inhibition of NH_4^+ production (Fig. 6) supports
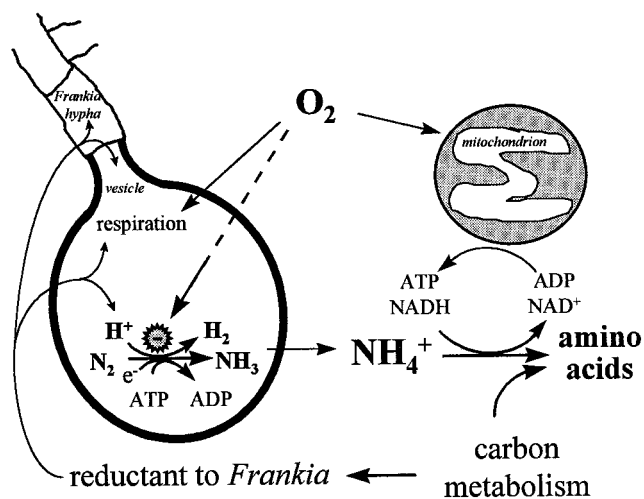
Figure 7. Schematic drawing of metabolic interactions in an *A. incana* root nodule cell containing N_2-fixing *Frankia*.

the hypothesis that disturbances in a process linked to NH_4^+ assimilation such as plant carbon metabolism could be important and involved in the response of nitrogenase activity.

The effect of O_2 on the occurrence of the Ar-induced decline (Fig. 4) in this context could be explained as follows. At the higher pO_2 the total respiratory demand increased (Figs. 1 and 2). As a result, the respiratory system would have fewer metabolite reserves and therefore less ability to immediately compensate for the disturbance caused by Ar on a link between amino acid synthesis and carbon flow to *Frankia*. Also, at the lower pO_2, nitrogenase activity becomes more O_2 limited, so any changes in reductant supply may have less effect on nitrogenase activity.

Nitrogenase activity and vesicle respiration could possibly be competing for reductant. If respiration is more successful at the higher pO_2, it is possible that reductant limitation could cause the decline in nitrogenase activity. However, simply decreasing the pO_2 from 21% to 17% after 15 min in $Ar:O_2$ did not relieve any competition from respiration but, rather, decreased nitrogenase activity further, which is why this explanation does not seem likely.

Third, the decline of nitrogenase activity in $Ar:O_2$ could be caused by inactivation of nitrogenase by O_2. The bigger decline at 25% O_2 compared with at 21% (Fig. 4A), and the high sensitivity to increasing pO_2 during the decline in $Ar:O_2$ (Fig. 5A) suggest that the decline is due to O_2 inactivation. The incomplete recovery of nitrogenase activity during the hour in $Ar:O_2$ (Fig. 4A) and the remaining inhibition of nitrogenase after the change back to $N_2:O_2$ (Figs. 3 and 4A) are further support for the idea that a partial inactivation of nitrogenase causes the Ar-induced decline of nitrogenase activity, in particular at 25% O_2. A simple calculation of the internal O_2 concentration according to Fick's first law of diffusion and assuming fixed diffusion resistances (Sheehy et al., 1983) would suggest that at an external O_2 concentration of 25%, the internal O_2 concentration would be substantially higher compared with at an external O_2 concentration of 21% and would be severely inactivating for nitrogenase.

An increase in vesicle O_2 concentration in response to short-term N_2 deprivation at 25% O_2, where *Frankia* vesicle respiration may be operating closer to saturation, would therefore take a longer time for the respiration to consume and consequently cause greater damage to nitrogenase. At 17% O_2, it would be easier for the vesicle respiration to respond and consume any extra O_2. The change from 21% to 17% O_2 (Fig. 5A) would be expected to give an increase rather then a further decrease in nitrogenase activity if changing from 21% to 17% O_2 relieved any O_2 inhibition, and this result argues against a substantial O_2 inhibition during the decline at 21% O_2. The observed response to decreasing the pO_2 from 21% to 17% O_2 is consistent with nitrogenase becoming O_2 limited, because of a limitation in factors such as ATP supply. Any possible relief of O_2 inhibition may therefore be obscured by these factors. Some degree of O_2 inhibition of nitrogenase at 25% O_2 is possible, since there is very strong inhibition even before the Ar:O_2 treatment begins.

There are two possibilities through which the O_2 concentration at the site of nitrogenase could increase. One explanation could be that the eliminated NH_4^+ production in Ar:O_2 could disturb metabolite supply from the plant to *Frankia* in a similar way as discussed for the second hypothesis and lead to a decrease in the O_2 consumption by the *Frankia* vesicle respiration due to substrate limitation. An alternative is that an increase in vesicle O_2 concentration could be due to an increase in O_2 concentration originating external to *Frankia*. Since NH_4^+ is assimilated in the plant host cells, exposure to Ar:O_2 deprives the NH_4^+ assimilation metabolism in the host cell surrounding *Frankia* of its substrate. The elimination of NH_4^+ production and subsequent assimilation could therefore inhibit plant metabolism and mitochondrial respiratory O_2 consumption through reduced turnover of ATP and NADH. This could lead to a temporary increase in O_2 concentration in the plant cytoplasm and subsequently in the vesicle, which would inactivate nitrogenase or its electron donors. The decrease in CO_2 evolution during the decline in Ar:O_2 (Figs. 4B and 5B), which could be due to a decrease in NH_4^+ assimilation-linked respiration, and the correlation between the rate of the Ar-induced decline and the degree of inhibition of NH_4^+ production (Fig. 6) both suggest that plant metabolism is affected by Ar:O_2.

A decline and recovery in nitrogenase activity in response to Ar:O_2 has also been demonstrated for detached nodules of the *Frankia-M. gale* symbiosis (Tjepkema and Schwintzer, 1992) when a low partial pressure (0.25%) of acetylene was used in the acetylene reduction assay. In that case the decline in nitrogenase activity was found at pO_2s above and far below the optimum pO_2. In contrast to the results presented here for *A. incana* (Fig. 5), the nitrogenase activity of *M. gale* increased when pO_2 was reduced during an acetylene-induced decline (Tjepkema and Schwintzer, 1992). The authors concluded that the decline was either due to O_2 toxicity or to competition between respiration and nitrogenase for reductant. This suggests that there are different physiological mechanisms operating in these two actinorhizal root nodule symbioses, although as a response to the same cessation of NH_4^+ production. The differences

in nodule anatomy between *M. gale* and *A. incana* and the higher hemoglobin content of *M. gale* nodules (Silvester et al., 1990) may explain differences in nodule physiology. Moreover, it has been suggested that the walls of the infected cells in *M. gale* nodules could be the major diffusion barrier (Zeng and Tjepkema, 1994). For root nodules of *Coriaria arborea*, results have been presented that are consistent with the presence of a variable diffusion resistance mechanism (Harris and Silvester, 1994), which further points out the heterogeneity of root nodule physiology among actinorhizal symbioses.

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