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Transient Responses of Nitrogenase to Acetylene and Oxygen in Actinorhizal Nodules and Cultured *Frankia*[1]

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ABSTRACT

Nitrogenase activity in root nodules of four species of actinorhizal plants showed varying declines in response to exposure to acetylene (10% v/v). *Gymnostoma papuanum* (S. Moore) L. Johnson. and *Casuarina equisetifolia* L. nodules showed a small decline (5–15%) with little or no recovery over 15 minutes. *Myrica gale* L. nodules showed a sharp decline followed by a rapid return to peak activity. *Alnus incana* ssp. *rugosa* (Du Roi) Clausen. nodules usually showed varying degrees of decline followed by a slower return to peak or near-peak activity. We call these effects acetylene-induced transients. Rapid increases in oxygen tension also caused dramatic transient decreases in nitrogenase activity in all species. The magnitude of the transient decrease was related to the size of the O_2 partial pressure (pO_2) rise, to the proximity of the starting and ending oxygen tensions to the pO_2 optimum, and to the time for which the plant was exposed to the lower pO_2. Oxygen-induced transients, induced both by step jumps in pO_2 and by O_2 pulses, were also observed in cultures of *Frankia*. The effects seen in nodules are purely a response by the bacterium and not a nodule effect *per se*. Oxygen-induced nitrogenase transients in actinorhizal nodules from the plant genera tested here do not appear to be a result of changes in nodule diffusion resistance.

The acetylene reduction assay has been central to our understanding of the physiology, biochemistry, and genetics of many diazotrophic organisms (4). Recently, unwanted side effects of acetylene exposure have been reported that seriously limit the usefulness of the acetylene reduction assay in some cases. Nitrogenase activity in many legume nodules has been shown to be inhibited by acetylene, demonstrating what is now called the acetylene-induced decline (10). This sensitivity has been ascribed to an increase in diffusion resistance of the nodules induced by exposure to acetylene (22) resulting in an oxygen limitation of nitrogenase. In all cases so far reported, the acetylene decline is not spontaneously reverse (10, 11) but may be artificially reversed by increasing pO_2[4] (22).

Several species of actinorhizal plants have been tested for the acetylene-induced decline (17), and it is clear that the pattern of response to acetylene is complex and quite different from that in legume nodules. Nitrogenase activity declines to varying degrees following an initial rise and at varying speeds. In many cases, activity spontaneously returns to the initial, pre-decline rate. We prefer to call this effect an acetylene-induced transient, rather than an acetylene-induced decline. Given the differences in anatomy between legume and actinorhizal nodules and the likelihood that the endosymbiont *Frankia* plays an important role in regulating gas diffusion to the site of nitrogenase activity (18), it is not surprising that the two systems should differ in their response to an acetylene perturbation. The pattern and mechanism of changes in nitrogenase activity and the possible role of a diffusion barrier are not clear and more data are needed.

Transient changes in nitrogenase activity also follow other types of perturbation, including step changes in ambient oxygen concentration. The first observation of oxygen-induced nitrogenase transients was by Witty *et al.* (22) in nodulated pea plants submitted to stepwise increases in oxygen tension. The authors made no explicit mention of the transients which appeared in their results, and it was not until 1987 (7) that transient depressions in nitrogenase (measured as H_2 evolution) were explicitly studied. Hunt *et al.* (7) used soybean in their study and, while they were unable to ascribe a particular mechanism to the transient effects, they preferred an hypothesis that located the effects in the bacterium rather than the nodule tissue. Further work on soybean nodules (8) supported the idea that short-term biochemical changes in the state of nitrogenase, rather than changes in diffusion resistance, were responsible for oxygen transients in nitrogenase activity. Oxygen-induced transients have been observed in *Alnus rubra* nodules (L. Winship, unpublished observations) but have not been described in the literature.

Transient changes in nodule nitrogenase activity are important from at least two perspectives. First, anyone measuring nitrogenase activity on nodulated plants must take transients caused by perturbation into account. It would be useful to have general information about when and how transients are caused. Second, transients, particularly those caused by changes in oxygen conditions, may provide information about the homeostatic mechanisms regulating oxygen diffusion into root nodules. In the present paper, we present data which characterize the acetylene- and oxygen-induced transients in several untested genera of actinorhizal plants and point out the extremely dynamic nature of the response to oxygen shifts in root nodules. Our results highlight the great variation and

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[4] Abbreviation: pO_2, O_2 partial pressure.

plasticity that exists amongst the actinorhizal plants. As we have been able to look at responses both in intact nodulated plants and in cultured *Frankia*, it has been possible to assign specific physiological responses to either the bacterium or the nodule symbiosis and thus define more closely the site of the transient activity.

MATERIALS AND METHODS

Symbionts

The following host plants were used in this study. Fruits of *Myrica gale* L. were collected near Harvard Forest and germinated as previously described (19). Fruits of *Alnus incana* ssp. *rugosa* (Du Roi) Clausen. were collected in Pelham, Massachusetts, stored dry, and soaked overnight in distilled water prior to germination. *Casuarina equisetifolia* L. seed was obtained from Canberra, Australia, and sown directly onto sand covered with vermiculite. *Gymnostoma papuanum* (S. Moore) L. Johnson. was propagated from cuttings according to methods previously described (9).

Frankia isolates include: HFPCcI3 (catalog No. HFP020203), referred to as CcI3, isolated from root nodules of *Casuarina cunninghamiana* Miq. (23); HFPArI3 (catalog No. HFP013103), referred to as ArI3, isolated from *Alnus rubra* Bong. (1); and two as yet uncatalogued strains HFPGpI1 isolated by Ms. Suzanne Racette from *Gymnostoma papuanum*, and HFPM+gale isolated from spore plus nodules of *Myrica gale* by Z. Zhang. Isolates were maintained on BAP propionate medium (12) in 250-mL flasks containing 50 mL of culture medium on a shaker (90 rpm) at 26 °C. Induction of vesicles was initiated by transferring to nitrogen-free medium and maintained as above.

Inoculation

Seedlings or cuttings were transferred at 4 to 6 weeks after germination into minus nitrogen quarter-strength Hoagland solution and inoculated by injecting 0.1 mL of appropriate culture onto the roots of each plant. Plants were maintained in a growth cabinet at 280 E m^{-2} s^{-1} PAR, 16 h light at 26 °C, 8 h darkness at 19 °C. After nodule induction, plants were maintained in aerated water culture, sparged with air or an appropriate gas mixture. Water level was lowered so that the majority of nodules were in the gas phase but kept moist by the fine mist of nutrient solution created by breaking bubbles. Plants maintained at raised or lowered pO_2 were aerated in a closed loop in which a 200 L polyvinyl chloride gas reservoir was connected via a diaphragm pump to the sealed growth container. Oxygen levels were measured daily and adjusted to maintain pO_2 within 5% of the nominal value. Gas mixtures were prepared from commercial gas cylinders and tested by gas chromatography. For the open cuvette assay system, mixtures were made by volumetric transfer with a 1.5 L syringe into 10 L polyvinyl chloride beach balls fitted with septum seals and pipe work. Where appropriate, 10 kPa acetylene was added and oxygen values were kept within ±0.1 kPa of stated values during assays.

Nitrogenase Assays

Nitrogenase activity was measured by acetylene reduction with 0.1 mL samples injected into a Carle 9500 gas chromatograph fitted with 1.0 m Porapak T column and flame ionization detector. Acetylene was generated from calcium carbide and used at a rate-saturating concentration of 10 kPa.

All assays were conducted in an open flow cuvette based on the techniques described by Minchin *et al.* (10). The gas reservoirs were connected to a manifold of low volume so that various mixtures could be switched in rapid succession. Gas was pumped via a peristaltic pump (Cole-Parmer 7567-70) containing four heads capable of delivering from 1 to 400 mL·min^{-1} at constant rate. Flow rates in the range of 2 to 10 mL·min^{-1} were used for *Frankia* cultures and 10 to 90 mL·min^{-1} used for root systems of intact plants. The cuvette for whole plant studies consisted of a 60 mL plastic syringe with the head removed and a split rubber stopper inserted to take a plant. The plunger was retained and covered with wet filter paper, thus giving a variable volume cuvette. Whole plants were removed from water culture, and root systems installed in the cuvette with gas entry tube at the bottom and exit tube at the top. The plunger was adjusted to give a volume normally 30 to 50 mL, and flow rate was controlled to give at least one gas change per min. Plant roots were equilibrated in the flow gas without acetylene for 30 min then switched to an acetylene-containing gas stream containing the same pO_2. Great care was taken to ensure that the pO_2 of the equilibration gas supply was within ±0.1 kPa of the gas supply containing C_2H_2 during investigation of acetylene-induced decline. The cuvette was normally installed in a 25 °C water bath.

Frankia cultures were also assayed in an open flow cuvette by the techniques previously described (13). In addition to assaying cultures in stirred liquid, suspension cultures were assayed in a plastic, 25 mm Swinney syringe filter holder as a cuvette. In this case, two layers of either Whatman No. 1 filter paper or of 40 μm nylon mesh were installed in the filter; gas flow was started, and one mL of concentrated culture, injected into the gas stream above the filter, was lodged on the filter allowing the liquid medium to pass through to waste.

Ethylene production was measured on the gas exit stream of the above apparatus by taking repeated 0.1-mL samples of gas stream close to the exit port, and injecting directly into the gas chromatograph. This configuration allowed samples to be taken at 40 to 60 s intervals. More rapid sampling (10–30 s) was conducted on occasions by taking samples into syringes and storing them by forcing the needles into a rubber stopper for later analysis.

Whole plant assays were conducted in the lab without added lighting. All gas mixtures were water-saturated, and plants were kept in polyethylene bags to reduce transpirational water loss. Under these conditions, constant rates of acetylene reduction could be sustained for 4 to 8 h for whole plants and for liquid *Frankia* cultures, and up to 2 h for *Frankia* cultures on filter paper or nylon mesh. Oxygen levels were assayed on separate samples taken from the exit gas stream. In most cases, results are expressed in moles $C_2H_4 \cdot g^{-1}$ (dry weight)·

min^{-1}; however, where plants were not destructively harvested at the time, results are expressed on a per plant basis.

Diffusion resistance was measured in nodules by an acetylene-lag-time method similar to that of Davis (3). The rate of ethylene production by intact nodulated *Myrica* root systems was measured in a small cuvette and a high gas flow rate. Samples were taken at 10 to 30 s intervals, for 3 to 4 min after introduction of acetylene. The time required to a steady rate of ethylene production was taken as a measure of the time required for acetylene to diffuse to the site of nitrogenase and for ethylene to diffuse back out. This measure of diffusion resistance was relative, because nodule size and mass were not taken into account, but lag times could be validly compared between treatments applied to the same root system. Replicate treatments were highly repeatable.

RESULTS

Transient Responses of Nodulated Root Systems to Acetylene Exposure

Response to acetylene was tested on nodulated root systems of four different genera of actinorhizal plants. Two distinctly different responses were apparent as shown by representative results with individual plants from each genus (Fig. 1). *Casuarina* (Fig. 1A) and *Gymnostoma* (Fig. 1B) plants always showed a small acetylene-induced decline with negligible recovery in 15 min. All *Myrica* plants (Fig. 1C) and most *Alnus* plants (Fig. 1D, top trace) showed a very marked decline (27% and 41% of peak activity, respectively, for the two plants shown) followed by rapid recovery.

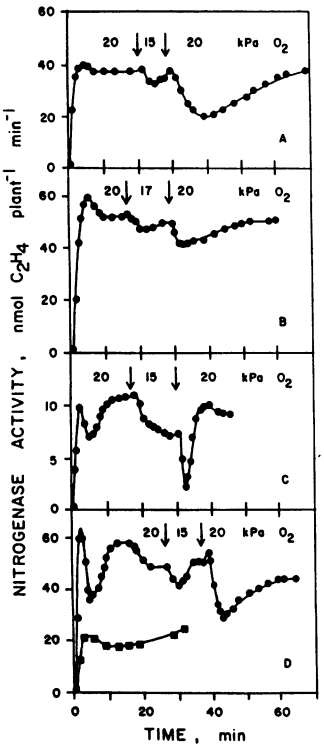

Figure 1. Typical nitrogenase responses to acetylene and oxygen by individual nodulated plants. All plants were equilibrated in a flowing gas phase of 20 kPa O_2 at 30 ml min^{-1} for 30 min, then switched to 10 kPa C_2H_2, 20 kPa O_2 gas stream at time zero. Graphs show the rise in acetylene reduction (as ethylene production) for four different species: A, *C. equisetifolia*; B, *G. papuanum*; C, *M. gale*; D, *A. incana* ssp. *rugosa*. Arrows mark the step changes in pO_2. Two separate *Alnus* plants illustrate the range in response of this species.

The above experiment was repeated many times with different *Myrica* and *Alnus* plants and the *Myrica* always recovered rapidly. The pattern of response was extremely repeatable for a given plant. We observed little variation among individual *Myrica* plants, while individual *Alnus* plants showed a range of patterns, falling roughly into fast and slow recovery categories. The two graphs in Figure 1D illustrate the two types of effects seen in *Alnus*; in one case (top trace) the rapid recovery was followed by a partial decline, while in the other a small decline was followed by a slow recovery (lower trace). The general and spontaneous recovery from acetylene inhibition, in at least some actinorhizal plants, leads us to rename this phenomenon an acetylene-induced transient.

Transient Responses of Nodulated Root Systems to Changes in pO_2

Nodulated root systems of all four genera showed transient declines in nitrogenase activity in response to decreases as well as to subsequent increases in ambient oxygen tension (Fig. 1). Plants were subjected to a change to 15 kPa O_2 (17 in case of *Gymnostoma*) following 20 min at 20 kPa, followed by a return to 20 kPa O_2 at about 30 min. In all cases, the drop to 15 kPa O_2 resulted in an immediate decrease in nitrogenase activity. The response of *Myrica* plants was always dramatic (33% in the instance illustrated in Fig. 1C) and was not associated with any recovery. Plants of the other three genera showed a small decline (10–14% in Fig. 1, A, C, and D) followed by some recovery. When the ambient pO_2 was returned to 20 kPa O_2, all plants again showed a precipitous drop in nitrogenase activity, which was occasionally preceded by a slight rise (*Alnus*, *Myrica*, and *Casuarina*). The sharp drop in activity was followed by recoveries of varying speeds to an activity close to, or exceeding, the former rate at 20 kPa O_2.

Myrica plants stand out in demonstrating very rapid transient responses to exposure to acetylene and to pO_2 shifts. For this reason, the majority of the results that follow pertain to this genus. In addition, responses of *Myrica* plants were highly repeatable. We found that transient responses to pO_2 shifts were observed only when oxygen shifts near to the optimum were made. Figure 2 shows a *Myrica* plant exposed to a series of sequential doublings of pO_2 from 2.0 to 4.5 to 9 to 19 kPa O_2 with a transient inhibition occurring only at the last shift. This result was further confirmed when oxygen shifts were studied on plants optimized to alternative pO_2 levels, *e.g.* a *Myrica* plant grown for 6 weeks at 40 kPa O_2 showed no nitrogenase transient when shifted from 5 to 10 kPa O_2, a small transient following a 10 to 20 kPa O_2 shift, and a dramatic transient from 20 to 36 kPa O_2 (Fig. 3).

The results presented above show nitrogenase transients of considerable variation with the most obvious sources of variation being the extent of the pO_2 shift and how close to the optimum pO_2 of the plant this shift is made. Another, perhaps

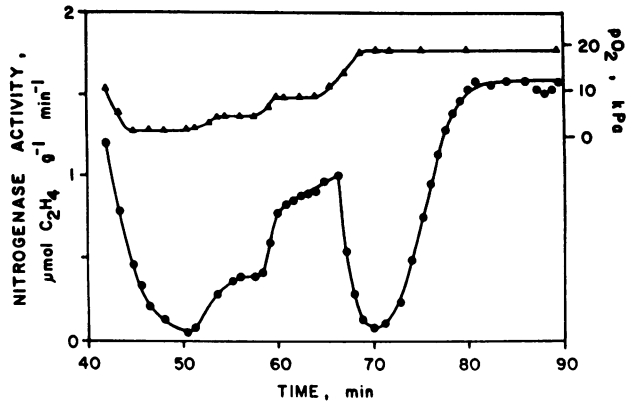

Figure 2. Nitrogenase response of *M. gale* plant grown in air to step changes in pO_2. Plant was transferred to 3 kPa O_2 at 40 min, then given oxygen step shifts of 5, 9, 19 kPa (▲——▲). Nitrogenase response (●——●) shows massive transient at the 9 to 19 kPa O_2 shift.

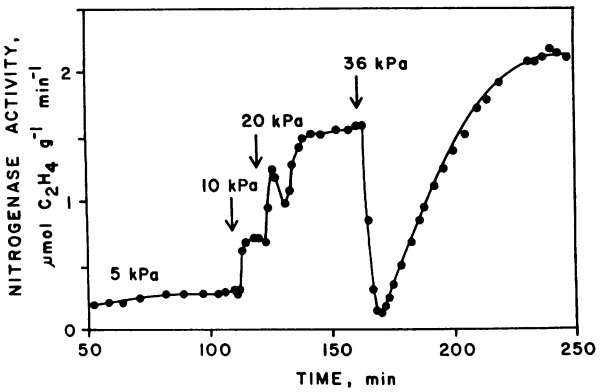

Figure 3. Nitrogenase response of *M. gale* plant grown at 40 kPa O_2 to step changes in pO_2. Plant was transferred to 5 kPa O_2 at 40 min.

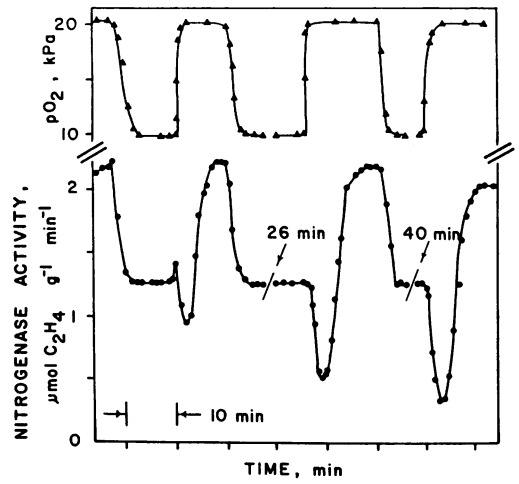

Figure 4. Nitrogenase response of *M. gale* plant grown in air. Plant was held for 10, 26, and 40 min at 9.3 kPa O_2 prior to change to 20 kPa O_2.

Table I. *Outline of Experimental Regime for Measuring Diffusion Resistance of M. gale Nodules during an O_2-Induced Nitrogenase Transient*

Root system was continuously flushed with gas, equilibrated at 14 kPa O_2 (called "14−") for 15 min prior to each treatment, then changed to 20 kPa O_2 minus acetylene (called "20−") for 1, 2, or 3 min followed by 20 kPa O_2 plus acetylene (called "20+") to follow ethylene rise. (See Figure 5 for results.)

Treatment	Phase		
	Equilibrating	Transient	Acetylene
Control	14−	14−	20+
1	14−	20−, 1 min	20+
2	14−	20−, 2 min	20+
3	14−	20−, 3 min	20+

more subtle effect, was the length of time that the root system was held at the lower pO_2. Figure 4 shows a typical response of a *Myrica* plant held at 9.3 kPa O_2 for 10, 26, and 40 min prior to returning to 20 kPa O_2. The extent of the transient drop in nitrogenase (25%, 60%, and 75%, respectively) was proportional to the time at the lower pO_2.

Effects of pO_2 Shifts on Nodule Diffusion Resistance

Recent work on legumes (7, 16) has indicated that there may be a variable diffusion resistance located in the nodule and that diffusion resistance may change in response to the presence of acetylene or to changes in pO_2. We tested whether the transients we observed might be due to rapid changes in nodule diffusion resistance by measuring diffusion resistance during a transient cycle. Again, we chose *Myrica* for these experiments because the time course of the transients in acetylene reduction activity induced by rapid shifts in pO_2 was predictable and repeatable. Our hypothesis in designing this experiment was, if the O_2-induced transient is due to a rapid increase in diffusion resistance resulting in decreased nitrogenase activity, then the equilibrium time for ethylene production (*i.e.* the time required for ethylene production to reach a maximum rate) following acetylene exposure (a measure of the diffusion path resistance to nitrogenase) should alter dramatically during the course of the transient. We would expect that transients of the extent shown for *Myrica* in Figure 1 would require at least a three-fold increase in resistance and for the transients shown in Figures 2 and 3 would require extremely high resistance at the time of maximum inhibition.

The experiment (Table I) was designed to cause a transient decline in nitrogenase activity similar to that shown in Figure 1C, *i.e.* a 15-min equilibrium time at 14 kPa O_2 followed by a rapid rise to 20 kPa O_2. It was assumed in this experiment that as the oxygen transient would be large compared with the acetylene transient (Fig. 1C) we would be observing primarily the O_2 transient. A *Myrica* plant grown in aerated water culture (pO_2 of 20 kPa) was placed in the assay cuvette and equilibrated at 14 kPa O_2 minus acetylene for 15 min. The root system was then exposed to 20 kPa O_2 (minus acetylene, to cause the oxygen shift transient) for a short time. The duration of the exposure to 20 kPa O_2 was varied from 0 to 3 min over the course of several separate trials with the

same plant (Table I). Following the timed exposure to 20 kPa O_2, the root system was exposed to a gas mixture containing 10 kPa acetylene as well as 20 kPa O_2. The time for C_2H_4 production to come to equilibrium during this acetylene exposure was used as a measure of nodule diffusion resistance (3).

As shown in Figure 5, ethylene production reached equilibrium in the same span of time (approximately 1.5 min) following each of the oxygen exposure treatments. While the specific rates of nitrogenase activity and the actual time to equilibrium varied between plants, the pattern of identical times to equilibrium across treatments was consistent and repeatable.

Responses in *Frankia*

The results above indicate that the transient drop in nitrogenase activity associated with a rapid pO_2 shift is not mediated by a change in diffusion resistance of the host tissue. Both the speed at which the transients occur and the magnitude of the decline suggest that the effect is at the level of the bacterium rather than the host tissue. Cultures of *Frankia* were therefore tested for both acetylene and O_2-shift induced transients in nitrogenase activity.

A variety of experiments on cultures of CcI3 and M+gale isolates failed to detect any C_2H_2-induced decline (Fig. 6). Both in cultures assayed in liquid and cultures suspended on filter paper or nylon mesh, a rapid rise to equilibrium and constant nitrogenase activity was always observed.

When liquid cultures were assayed at various pO_2 levels, it was also impossible to detect oxygen-induced transient nitrogenase activity. Cultures assayed this way showed a very slow response even when bubbled with the gas mixture and vigorously stirred with a magnetic stirrer (Fig. 7). It was obvious from the above result that the aqueous medium was damping the response of *Frankia* and, even under vigorous stirring, the solution time for oxygen and the diffusion rates of all gases through the medium was liable to effectively eliminate a rapid response. All further experiments were conducted with a Swinney filter holder as the cuvette. By this technique, the response time of the culture was very significantly reduced, and the cells remained active for at least 2 h.

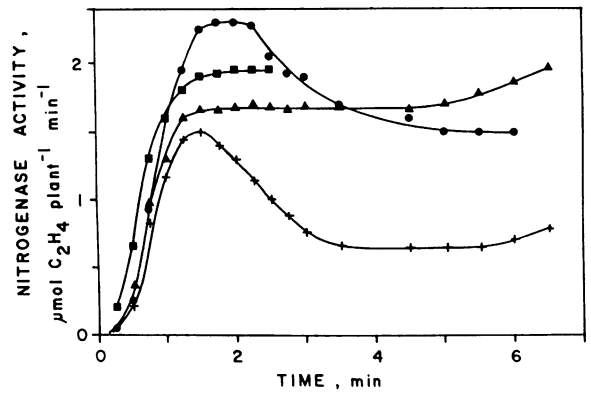

Figure 5. Measurement of diffusion resistance in *M. gale* nodules. Plants equilibrated in 14 kPa O_2 without C_2H_2, then exposed to 20 kPa O_2 for varying lengths of time prior to exposure to acetylene at time zero. For plan see Table I. Exposure to 20 kPa for 0 min (■——■) 1 min (+——+), 2 min (●——●), 3 min (▲——▲).

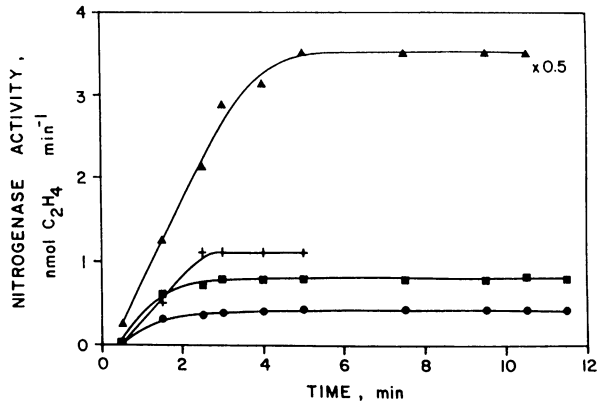

Figure 6. Induction of acetylene reduction in cultures of *Frankia*. Cultures were incubated in absence of acetylene then exposed to acetylene at time zero: CcI3 in liquid culture (▲——▲); CcI3 on filter paper (+——+); M+gale on nylon mesh (■——■); M+gale on filter paper (●——●).

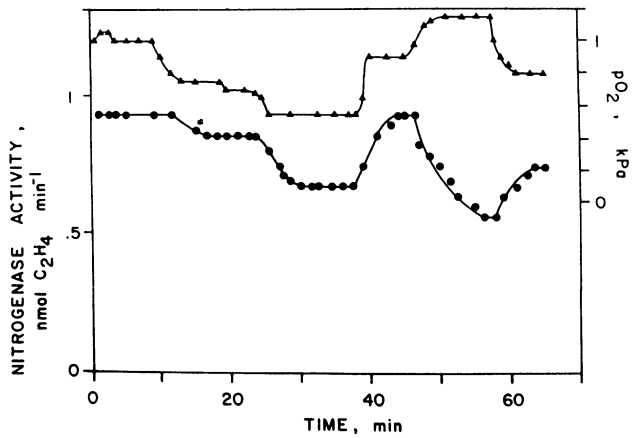

Figure 7. Effects of varying pO_2 on nitrogenase activity in aqueous culture of *Frankia* CcI3. Culture grown at 2 kPa O_2 before transfer to cuvette. Top curve is pO_2, lower curve is nitrogenase.

Two types of response were studied in *Frankia:* first, pulses of above-ambient oxygen were given, and the response of nitrogenase was followed; and second, the so-called transient response was followed in response to step-shifts in pO_2. The results are summarized in Figure 8, which gives a typical trace for *Frankia* strain M+gale. The culture was grown in air and suspended on nylon mesh. It is obvious that typical transient drops and responses to oxygen pulses are present. In both cases, it is significant that nitrogenase activity almost fully recovered after each drop and that the transient effect is proportional to the magnitude of the applied pO_2 pulse.

DISCUSSION

Oxygen-Induced Transients

Nitrogenase transients such as we have observed in acetylene reduction were first reported by Hunt *et al.* (7) for H_2 evolution in response to pO_2 shifts in soybean nodules. In a

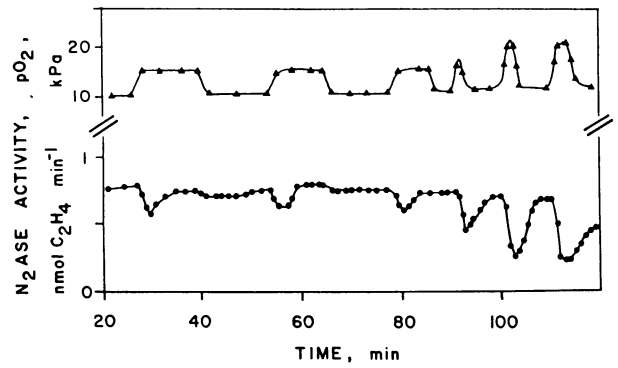

Figure 8. Effects of step changes and pulses of O_2 on nitrogenase activity in *Frankia* culture M+gale. Culture was grown in air, then suspended on nylon mesh in cuvette before exposure to varying pO_2.

subsequent paper (8), these same workers showed that the infected cell oxygen concentration increased during the decline in nitrogenase activity. The drop in activity was most likely due to an effect close to the bacterium, a mechanism akin to conformational protection. While we have no method for measuring the oxygen concentration within infected cells comparable to the method of King *et al.* (8), the time course and shape of the transients we have reported here is consistent with a transient, inhibitory increase in local oxygen concentration near nitrogenase.

Other observations also indicate that rapid changes in diffusion resistance are not responsible for oxygen-induced nitrogenase transients. In considering a possible role for variable diffusion resistance in explaining this phenomenon, it is important to differentiate between the short-term and medium-term effects. The short-term event covers the 2 to 10 min period of the transient itself, and Figure 1 shows that transients are very variable but in all cases are characterized by a steep drop in activity followed by a variable rate of recovery. In *Myrica*, the drop and recovery are both characteristically precipitous (Figs. 1 and 4), while in other species there may be an initial rapid recovery followed by a slower response. Using *Myrica* as perhaps the extreme example, we believe the results of Figure 5 conclusively demonstrate that variable resistance is not involved in the transient event in *Myrica*.

The medium-term effects involve the period during which the root system is exposed to below-ambient pO_2. This period of time correlated strongly with the magnitude of the transient (Fig. 4) in every experimental trial. One explanation could be that the nodule is adapting to lower pO_2 by reducing diffusion resistance over the 10 to 40 min period (Fig. 4) and thus becoming more predisposed to the resulting oxygen change. This conclusion is ruled out on two counts. First, *Myrica* is strongly oxygen-limited at below-ambient pO_2 levels, and if diffusion resistance was reducing during the time in subambient pO_2, then one would expect nitrogenase activity to increase during that time, which it does not (Fig. 4). Second, if diffusion resistance changed significantly during the prior exposure, nitrogenase activity at later exposures to 20 kPa O_2 would not be equal to activity prior to exposure to subambient pO_2. The data in Figure 4 show they are very nearly similar.

The magnitude of the transient nitrogenase response is controlled by the size of the oxygen step change, proximity to pO_2 optimum, and duration the plant is held at the lower oxygen, but the nature of the transient, its locus, and the metabolic processes involved are totally obscure. The virtually instantaneous nature of the response along with other evidence led us to postulate that this effect is not a nodule event *per se* but is a response of the bacterial cell. We have previously shown a rapid nitrogenase response by *Frankia* to pulses in oxygen (13) in cultures grown at very low oxygen and shaken very vigorously during assay, but we were unable to repeat this in stirred aqueous cultures (Fig. 7). However, the experiment shown in Figure 7 did show that *Frankia* recovers rapidly from oxygen shock (note recovery following exposure to 1.2 kPa O_2) and led us to believe that the diffusion resistance of the water was masking the event. When *Frankia* was suspended in a filter cuvette (Fig. 8), it showed exactly the same effects as whole plants, and we conclude that the transient events are expressions of the sensitivity of *Frankia* to changes in oxygen tensions. While identification of the site of this phenomenon is relatively simple, an understanding of the physiology of the process presents more problems.

As has been stressed many times, the oxygen environment of the N_2-fixing site within nodules is a fine balance between oxygen consumption within and oxygen diffusion across a resistance barrier(s). Respiration accounts for the majority of O_2 consumption, although H_2 oxidation in nodules with uptake hydrogenase may also account for a significant amount. It has been shown (20, 21) that for *Alnus rubra* respiration and nitrogenase activity show parallel increases over the range 3 to 20 kPa O_2. Thus, oxygen consumption as well as providing the energy required for nitrogenase also maintain the delicate oxygen balance. We believe that the step jump in oxygen tension, which initiates a transient response, produces a transient increase in oxygen tension at the fixing site, which has an effect either directly on nitrogenase or on the electron transport chain to nitrogenase. The increase in pO_2 results in an increase in respiration, as shown by Winship and Tjepkema (21), which rapidly reduces internal pO_2 and reestablishes the equilibrium low internal pO_2 level.

Rapid switches in nitrogenase activity in response to O_2 have been reported for *Azotobacter* and were given the generic term of conformational protection (2). These reactions are now believed to involve an association of the two nitrogenase units with a third protective FeS protein which renders the complex oxygen tolerant but inactive (14). Autoxidation of an electron donor such as flavodoxin hydroquinone is considered to be a possible oxygen sensor, and such a mechanism could account for the extremely rapid switch-off of nitrogenase (14).

Acetylene-Induced Transient

The effect of shutting off nitrogen fixation, either by acetylene addition or by removal of N_2, on initiating a decline in electron transfer through nitrogenase is now well documented for legumes (7, 10, 22). The effect has been studied by acetylene reduction and hydrogen evolution (7, 10) and has been reported for many but not all legume crop and forage plants. In a few reported cases, nitrogenase activity shows spontaneous recovery, sometimes followed by a second de-

cline (15). Full recovery of peak activity has not been observed in legumes. Our results, showing a wide range of responses to acetylene, are in agreement with those of Tjepkema *et al.* (17).

Acetylene decline occurs only in those legumes which also show tolerance to particularly high pO_2, while those legumes that are intolerant of high pO_2 show no decline (22). The initiation of the decline has now been extended to other perturbations such as root disturbance (11), defoliation (6, 11), and soil moisture (5). These decline phenomena are all accounted for by a rapid reduction in the diffusion resistance of the nodule (22) which, in the case of strongly oxygen-limited systems (*i.e.* those that have high pO_2 tolerance and high pO_2 optima), become even further oxygen limited.

The situation we report here bears little resemblance to the reported acetylene decline in most legumes. While the small declines shown for *Casuarina* and *Gymnostoma* (Fig. 1, A and B) may be superficially similar, the spontaneously recovered transients of *Myrica* and *Alnus* (Fig. 1, C and D) are far more similar to oxygen-induced transients, both in terms of kinetics and recovery characteristics. Further work, especially measurements of nodule oxygen consumption, is required before we can evaluate the role of diffusion resistance in the acetylene-induced transient of actinorhizal nodules.

The implication of O_2 in the physiological control of a wide variety of related perturbations, including temperature, moisture, disturbance, and acetylene, emphasize yet again the central role of this gas in nodule function. Over a very wide range of different plants, including legumes and nonlegumes involving very different bacteria and different physiologies, the mean rates of nitrogenase are remarkably similar (18), and the systems are remarkably fine-tuned to make best use of available oxygen. It is now becoming clear that control of nodule function by diffusion resistance and protection by a rapid switch-off mechanism are fundamental to all root nodules. The results included here confirm again the exceptionally dynamic responses of nitrogenase in symbiosis. We would like to underline the warnings given previously (7, 10, 13) regarding interpretation of acetylene reduction results which have been conducted in closed gas exchange systems.

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LITERATURE CITED

1. **Berry AM, Torrey JG** (1979) Isolation and characterization in vivo and in vitro of an actinomycetous endophyte from *Alnus rubra* Bong. *In* JC Gordon, CT Wheeler, DA Perry, eds, Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Oregon State University, Corvallis, OR, pp 69–93
2. **Dalton H, Postgate JR** (1969) Growth and physiology of *Azotobacter chroococcum* in continuous culture. J Gen Microbiol **56:** 307–319
3. **Davis LC** (1984) Diffusion of gases through plant tissues. Plant Physiol **76:** 854–857
4. **Dixon ROD, Wheeler CT** (1983) Biochemical, physiological and environmental aspects of symbiotic nitrogen fixation. *In* JC Gordon, CT Wheeler, eds, Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications. Nijhoff, The Hague, pp 107–171
5. **Durand J-L, Sheehy JE, Minchin FR** (1987) Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water deprivation. J Exp Bot **38:** 311–321
6. **Hartwig U, Boller B, Nosberger J** (1987) Oxygen supply limits nitrogenase activity of clover nodules after defoliation. Ann Bot **59:** 285–291
7. **Hunt S, King BJ, Canvin DT, Layzell DL** (1987) Steady and nonsteady state gas exchange characteristics of soybean nodules in relation to the oxygen diffusion barrier. Plant Physiol **84:** 164–172
8. **King BJ, Hunt S, Weagle G, Walsh KB, Pottier RH, Canvin DT, Layzell DL** (1988) Regulation of oxygen concentration in soybean nodules observed by *in situ* spectroscopic measurement of leghemoglobin concentration. Plant Physiol **87:** 296–299
9. **Lundquist R, Torrey JG** (1984) The propagation of *Casuarina* species from rooted stem cuttings. Bot Gaz **145:** 378–384
10. **Minchin FR, Witty JF, Sheehy JE, Muller M** (1983) A major error in the acetylene reduction assay: decrease in nodular nitrogenase activity under assay conditions. J Exp Bot **34:** 641–649
11. **Minchin FR, Sheehy JE, Witty JF** (1986) Further errors in the acetylene reduction assay: effects of plant disturbance. J Exp Bot **37:** 1581–1591
12. **Murry MA, Fontaine MS, Tjepkema JD** (1984) Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3. Arch Microbiol **139:** 162–166
13. **Parsons R, Silvester WB, Harris S, Gruijters WTM, Bullivant S** (1987) *Frankia* vesicles provide inducible and absolute protection for nitrogenase. Plant Physiol **83:**728–731
14. **Robson RL, Postgate JR** (1980) Oxygen and hydrogen in biological nitrogen fixation. Annu Rev Microbiol **34:** 183–207
15. **Schuller KA, Minchin FR, Gresshoff PM** (1988) Nitrogenase activity and oxygen diffusion in nodules of soybean cultivar Bragg and a supernodulating mutant: effects of nitrate. J Exp Bot **204:** 865–878
16. **Sheehy JE, Minchin FR, Witty JF** (1983) Biological control of resistance to oxygen flux in nodules. Ann Bot **52:** 565–571
17. **Tjepkema JD, Schwintzer CR, Monz CA** (1988) Time course of acetylene reduction in nodules of five actinorhizal genera. Plant Physiol **86:** 581–583
18. **Tjepkema JD, Schwintzer CR, Benson DR** (1986) Physiology of actinorhizal nodules. Annu Rev Plant Physiol **37:** 209–232
19. **VandenBosch KA, Torrey JG** (1983) Host endophyte interactions in effective and ineffective nodules induced by the endophyte of *Myrica gale.* Can J Bot **61:** 2898–2909
20. **Winship LJ, Tjepkema JD** (1983) The role of diffusion in oxygen protection of nitrogenase in nodules of *Alnus rubra.* Can J Bot **61:** 2930–2936
21. **Winship LJ, Tjepkema JD** (1985) Nitrogen fixation and respiration by root nodules of *Alnus rubra* Bong. Effects of temperature and oxygen concentration. Plant Soil **87:** 91–107
22. **Witty JF, Minchin FR, Sheehy JE, Mingues MI** (1984) Acetylene-induced changes in the oxygen diffusion resistance and nitrogenase activity of legume root nodules. Ann Bot **53:** 13–20
23. **Zhang Z, Lopez MF, Torrey JG** (1984) A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. Plant Soil **78:** 79–90