Adaptation of nitrogenase to varying oxygen tension and the role of the vesicle in root nodules of *Alnus incana* ssp. *rugosa*

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Growth of *Alnus incana* ssp. *rugosa* plants with root systems at Po_2 levels of 5, 21, and 40 kPa showed no significant differences among treatments over a 6-week period. Nitrogenase activity of attached nodulated root systems run in an open-cuvette continuous-flow system generally was responsive to Po_2 over a broad range around the optimum. Plants expressed acetylene-induced and oxygen-induced transient declines in nitrogenase activity, from which they spontaneously recovered. Nitrogenase activity was seldom stable at any one Po_2 during assay with apparent adaptation to both above- and below-ambient Po_2. Nodule morphology showed quantitative decreases in aeration pathways as ambient Po_2 was increased, with air spaces in the cortex and infected tissue being significantly affected. The major change in response to Po_2 was the change in vesicle structure. Vesicles from nodules at low Po_2 showed a vanishingly thin vesicle envelope under dark-field microscopy, while at high Po_2 vesicles appeared very bright and apparently thickened. The results suggest that the major barrier to O_2 diffusion in *Alnus* nodules is the vesicle envelope of the bacterium.

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La croissance d'*Alnus incana* ssp. *rugosa*, dont le système racinaire fut soumis aux niveaux 5, 21 et 40 kPa de Po_2 n'a pas montré de différences significatives entre ces traitements au cours d'une période de 6 semaines. L'activité de la nitrogénase des racines nodulées placées en cuvette ouverte et à flot continu a, en général, répondu aux niveaux de Po_2 sur une gamme étendue au voisinage de la pression optimale. Les plantes ont montré des diminutions transitoires d'activité de la nitrogénase, induites par l'acétylène et par l'oxygène, mais elles ont récupéré spontanément. L'activité de la nitrogénase était rarement stable à chacun des niveau de Po_2 durant l'essai, une adaptation aux Po_2 au-dessus et au-dessous de la pression ambiante est apparente. La morphologie des nodules a révélé des diminutions quantitatives des voies d'aération avec l'augmentation de Po_2 ambiante, les espaces d'air dans le cortex et le tissu infecté étant alors affectés de façon significative. Le changement principal en réponse aux Po_2 fut observé dans la structure des vésicules. Les vésicules des nodules à faible Po_2, examinées au microscope sous champ sombre, montraient une enveloppe vésiculaire très mince tendant à disparaître. À Po_2 élevée, les vésicules apparaissaient très brillantes et manifestement épaissies. Ces résultats suggèrent que la barrière principale à la diffusion d'O_2 dans les nodules d'*Alnus* est l'enveloppe des vésicules de la bactérie.

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Introduction

Nitrogenase is extremely sensitive to oxygen, and the high metabolic activity associated with nitrogen fixation is sustained in root nodules by a high flux rate of oxygen at a very low concentration. In legume nodules a diffusion-resistant layer of cells surrounding the bacterial zone is found, within which oxygen transport is facilitated by leghaemoglobin (Bergersen 1980). In actinorhizal nodules the situation appears to be quite different. Haemoglobin occurs in high concentration in some species but is in low concentrations or absent in others (Tjepkema 1983). The nodules appear to be well aerated internally and show a marked Po_2 optimum for nitrogenase at or near atmospheric levels of oxygen.

The endosymbiont of actinorhizal nodules is the filamentous actinomycete *Frankia*, which develops spherical vesicles when isolated and grown in culture medium lacking combined nitrogen. The vesicle has been identified as the site of nitrogenase in cultured *Frankia* (Tjepkema *et al.* 1980). In the case of the *Alnus* symbiosis vesicle production and nitrogenase activity of root nodules are closely correlated (Becking 1977; Mian and Bond 1978). *Frankia* has the unique capacity, shared only with the heterocystous cyanobacteria, of being able to fix nitrogen actively at atmospheric Po_2 (Tjepkema *et al.* 1980) and this activity has been related to the thickened multilaminate envelope of the vesicle (Torrey and Callaham 1982). *Frankia* can also adapt to a wide range of oxygen levels and retain active nitrogenase (Murry *et al.* 1984; Parsons *et al.* 1987) with acetylene reduction activity correlated with the number of lipid-like layers laid down in the vesicle envelope (Parsons *et al.* 1987).

Alnus nodules have been studied in considerable detail, including both structure and function. MacConnell (1959) grew *Alnus glutinosa* at oxygen levels from 1 to 20 kPa O_2 and showed that plants were severely limited in growth at 1 kPa O_2 and about 50% reduction in dry weight and nitrogen content occurred at 5 kPa O_2. Under normal atmospheres alder root nodules form numerous lenticels (Bond 1974) and an interlacing network of air spaces (Wheeler *et al.* 1979), which has been shown by India ink infiltration to be connected continuously to the soil atmosphere via the lenticels (Tjepkema 1979). Nitrogenase activity in *Alnus rubra* shows a broad optimum response to Po_2, being independent of oxygen concentration over the range 15–30 kPa O_2 but very limited by Po_2 outside that range (Winship and Tjepkema 1985). It is apparent that in contrast to legume nodules, the interior of *Alnus* nodules is very well ventilated, although there is a more restricted aeration pathway consisting of smaller air spaces in the infected cell zone, which may provide some diffusion resistance (Wheeler *et al.* 1979).

Protection of *Frankia* from excess oxygen in *A. rubra*

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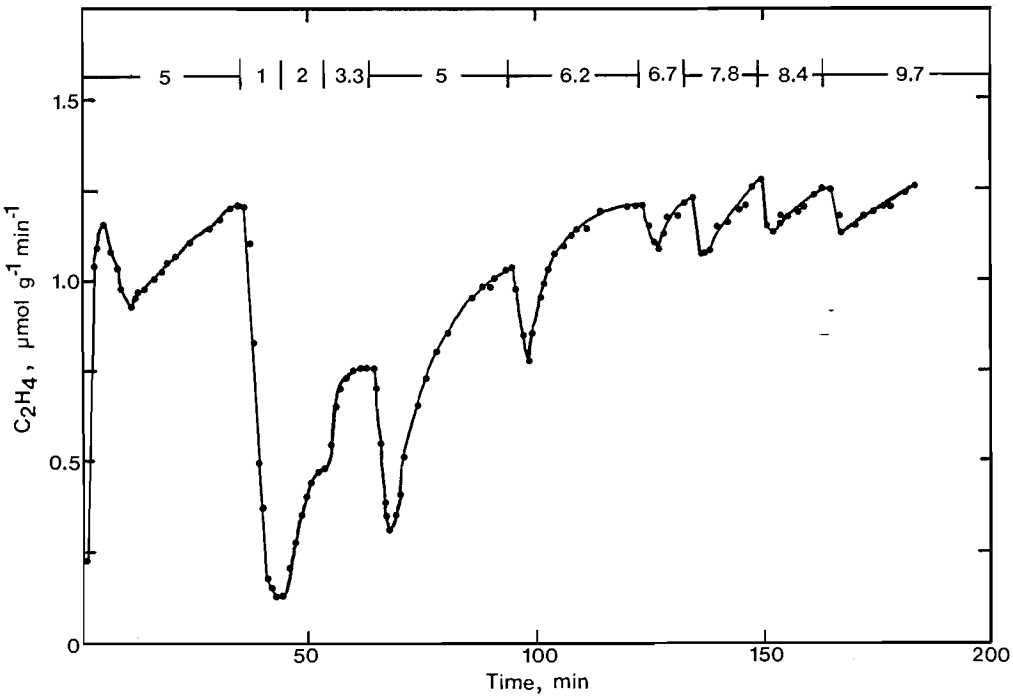


FIG. 1. Nitrogenase response curve for *A. incana* ssp. *rugosa* plant grown with its root system at 5 kPa O_2 then subjected to stepwise changes in Po_2 as indicated by the values shown across the top of the figure. The root system of the plant was equilibrated at 5 kPa O_2 then submitted to acetylene at 10 kPa in 5 kPa O_2 at time zero. Acetylene reduction activity was measured at each point on the curve.

nodules has been shown to result from a combination of diffusion limitation coupled with a high rate of oxygen-sensitive respiration (Winship and Tjepkema 1983). While this model adequately explains the observed physiological responses, the actual site(s) of diffusion resistance has not been identified in actinorhizal nodules. We applied the strategy, used before for cultured *Frankia* (Parsons *et al.* 1987) and for root nodules of *Myrica* (Silvester *et al.* 1988), of varying the ambient Po_2 around the root systems of growing plants to observe physiological adaptation and associated morphological changes.

Materials and methods

Plant growth

Fruits of *Alnus incana* ssp. *rugosa* (Du Roi) Clausen were collected near the Harvard Forest, stored dry, and germinated in wet sand with light. Six weeks after germination, plants were transferred to minus nitrogen, quarter-strength Hoagland's nutrient solution and inoculated by injecting 0.1 mL of *Frankia* culture onto the roots of each seedling. The *Frankia* isolate used was HFP ArI3 (catalog no. HFP 013103, referred to as ArI3) isolated from *Alnus rubra* Bong. (Berry and Torrey 1979). Plants were maintained in a growth cabinet at 280 $\mu E\ m^{-2}\ s^{-1}$ photosynthetically active radiation, 16 h light : 8 h dark at 26:19°C (light:dark). After nodule induction, plants were transferred to modified aeroponics water culture, in which the solution level was lowered so that most nodules were in the gas phase but were kept moist by the aerosol created by breaking bubbles.

Analytical techniques

Plants were transferred to recirculating gas systems, which were maintained at 5, 21, and 40 kPa O_2. Gas supply and analytical techniques for measuring Po_2 content of gases and for open-cuvette acetylene reduction assay were as previously described (Silvester and Winship 1988).

Microscopy

Nodule lobes were removed at harvest and sectioned for light microscopy as described by VandenBosch and Torrey (1983). Additional nodules were dissected and observations made on vesicle morphology of symbiotic *Frankia*, using dark-field microscopy. Nodules were mounted under a stereo dissecting microscope and the periderm and outer cortex gently peeled off, then small cubes of infected tissue were removed from the interior of a nodule and gently crushed in a drop of water under the microscope. During crushing the large individual infected cells could be seen to break free and the compact *Frankia* mass within single cells released. Large cell debris was removed and the remaining material squashed under a cover slip. This process produces considerable fine debris, including many starch grains, which interfere with subsequent dark-field microscopy. To clear the slide, a drop of glycerol was placed on one side of the cover slip and drawn through beneath the cover slip by taking up the water with filter paper. This procedure removes most fine debris from the field and leaves a clean preparation, which after further squashing is suitable for dark-field microscopy. Material was viewed under dark-field to visualize vesicle structure as previously described (Parsons *et al.* 1987), except that a Leitz Ortholux microscope was used fitted with a dry dark-field condenser (D 0.8) and a Zeiss 40×, numerical aperature (NA) 1.0 oil-immersion objective, set at NA 0.6. Photographs were taken with Kodak T-Max 100 film and care was taken to standardize all times and conditions so that final images were truly comparable.

Results

Plant growth

Plants grown with root systems at 5, 21, and 40 kPa O_2 apparently quickly adapted to these conditions and after 6 weeks the plants showed no significant differences in size or vigor.

Nitrogenase activity

Figures 1, 2, and 3 show the nitrogenase responses of representative plants, grown with root systems at 5, 21, and 40 kPa O_2, respectively, and then exposed to changing Po_2. Plants were transferred to the cuvette by using a glove bag for the 5 and 40 kPa O_2 treatments and conditions were maintained at

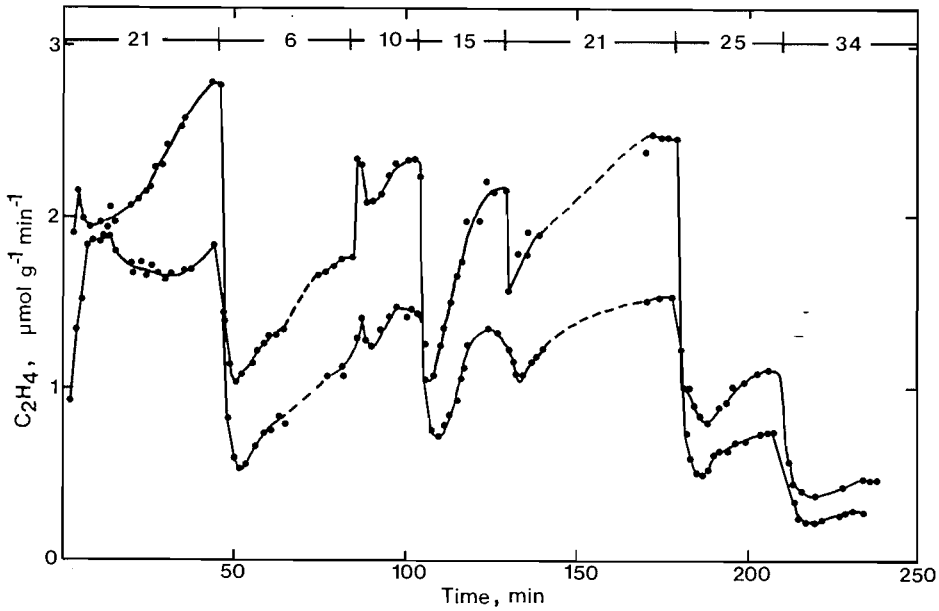


FIG. 2. Nitrogenase response curves for two separate plants of *A. incana* ssp. *rugosa* grown with their root systems in air (21 kPa O_2) then subjected to stepwise changes in Po_2 as shown across the top of the figure (see text for details).

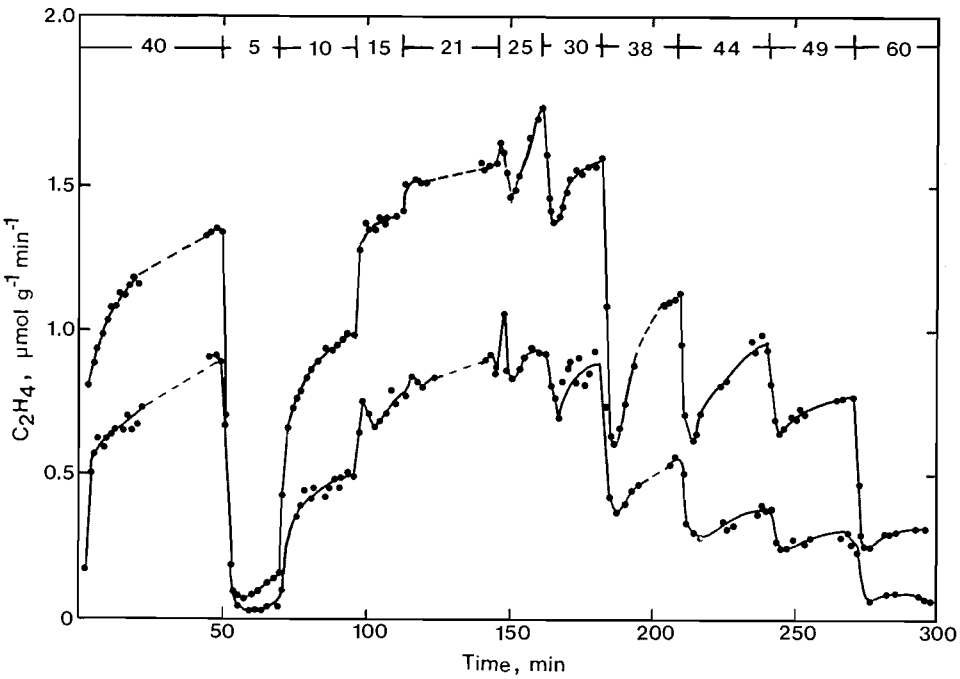
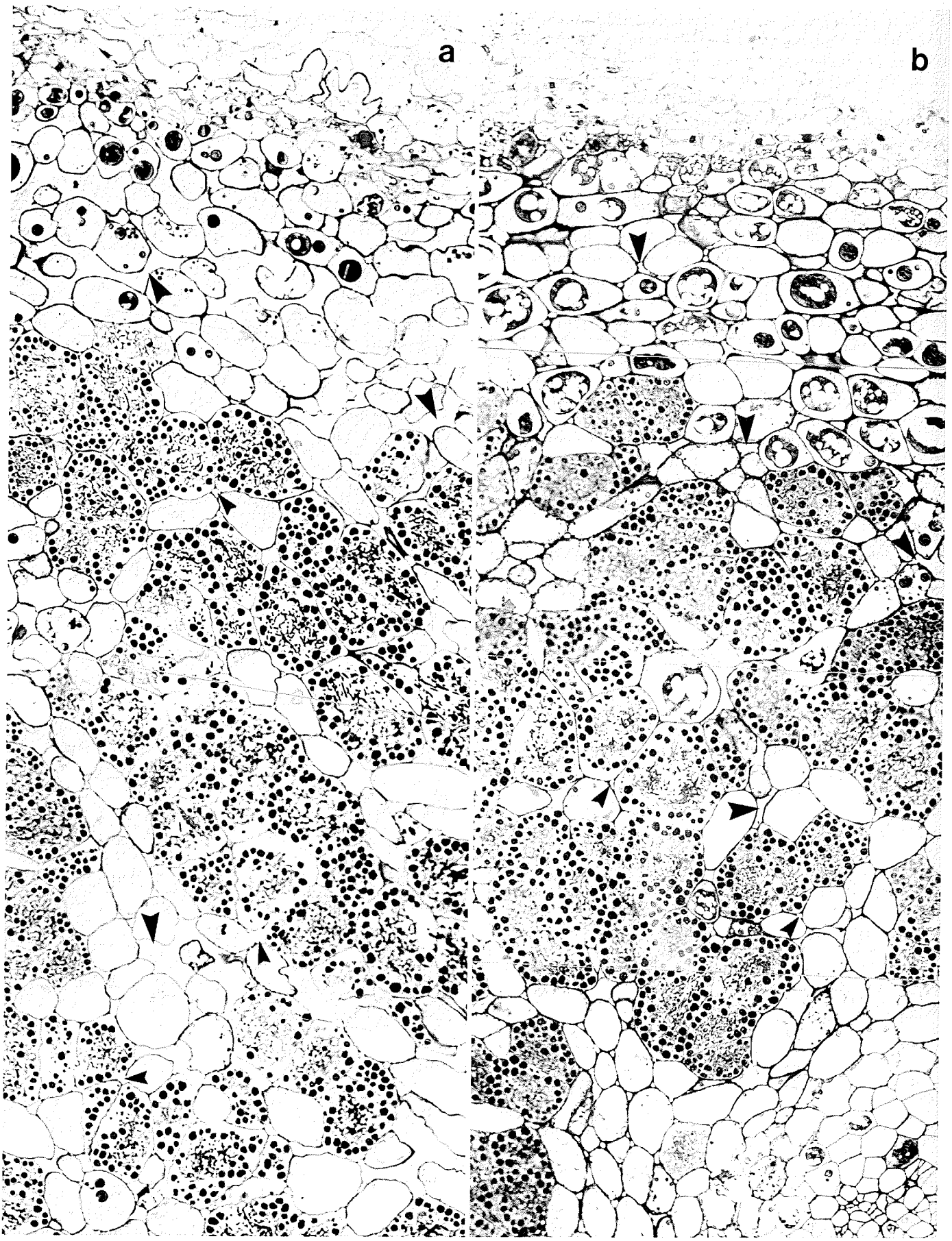


FIG. 3. Nitrogenase response curves for two plants of *A. incana* ssp. *rugosa* grown with their root systems at 40 kPa O_2 then subjected to stepwise changes in Po_2 as shown across the top of the figure (see text for details).

the experimental level until the experiment started (cf. Silvester *et al.* 1988). Plants were then equilibrated in the cuvette at that Po_2 without C_2H_2 and at time zero were switched to a flow gas with added C_2H_2 and maintained at precisely the same Po_2 as the equilibration gas. The apparent rise in nitrogenase activity beginning at time zero is the time required for acetylene reduction activity to commence. In two cases (Figs. 1 and 2) the rapid rise is followed by a decline from which recovery is spontaneous. Results from duplicate plants are presented in Figs. 2 and 3 and illustrate the very close agreement of any two plants that have been treated the same way. Although the curves have data points in common, the curves do not cross over at any time. Nitrogenase activity shows oxygen-induced transient responses when submitted to stepwise shifts in Po_2,

FIG. 4. Cross sections of mature root nodules of *A. incana* ssp. *rugosa* plants with root systems subjected continuously to 5 (*a*) or 40 kPa O_2 (*b*). Large intercellular spaces in both the cortex and in the parenchyma channels in infected tissue are indicated by large arrowheads. Smaller air spaces that occur immediately adjacent to infected cells are indicated by small arrowheads. ×450.



which are generally recovered spontaneously within 10 min. These transient responses have been described in detail elsewhere (W. B. Silvester and L. J. Winship, unpublished). In contrast to similar experiments with *Myrica gale* (Silvester *et al.* 1988) nitrogenase activity in *A. incana* ssp. *rugosa* consistently failed to achieve steady-state values in the cuvette. It is probable that, if any condition had been held for long enough, an equilibrium rate would have been obtained, but to achieve the desired changes in Po_2, this stability could not be achieved.

In general, the results show that *A. incana* ssp. *rugosa* plants did adapt nitrogenase activity to the Po_2 at which the plants were grown but that an optimum activity was achieved over a wide range of oxygen concentrations. It is possible that nodules were undergoing adaptation during the assay and this was particularly apparent for the plant grown with roots at 5 kPa O_2 (Fig. 1), which had very similar nitrogenase activity over the range 5−9.7 kPa O_2. Broad optimal activity was also seen for plants grown with roots at 21 (Fig. 2, optimum 10−21) and at 40 kPa O_2 (Fig. 3, optimum 15−30). A good example of apparent short-term adaptation to low Po_2 was shown by both of the plants grown in air, when submitted to 6 kPa O_2 (Fig. 2). From 50 to 85 min both plants showed a doubling in nitrogenase activity. Specific activity of nitrogenase at the optimum Po_2 ranged from 1.2 μmol C_2H_4 g^{-1} min^{-1} for nodules at 5 kPa O_2 to 2.8 μmol C_2H_4 g^{-1} min^{-1} for nodules at 21 kPa O_2.

Nodule morphology

Root nodules of *A. incana* ssp. *rugosa* grown aeroponically at normal atmosphere usually display large white lenticels. This appearance was true for plants at 5 and 21 kPa O_2, but plants at 40 kPa O_2 had nodules with relatively smooth surfaces and small lenticels. In general, nodule distribution was similar in the different oxygen levels, with most nodules in the gas phase above the water.

Anatomical investigation of the nodules showed significant quantitative differences in the degree of aeration of cortex and infected tissues (Fig. 4). For clarity, only the two extreme treatments of 5 and 40 kPa O_2 are presented; nodules in air showed an intermediate anatomy. In both cases the periderm cells are thickened and tightly packed but interrupted by lenticels, which lead into a cortex, which is well aerated at 5 and less so at 40 kPa O_2. The infected tissue is uniformly dissected by parenchyma channels that have large air spaces at low Po_2 and smaller air spaces at high Po_2. Infected cells generally have very small air spaces between them, but even in nodules grown at 40 kPa O_2 the infected cells may lie adjacent to an air space. At higher magnification (Fig. 5) differences in aeration are more apparent. At low Po_2 large air spaces adjoin the infected cells (Figs. 5*a*, 5*b*) and at high Po_2 the air spaces are still present but are very small (Figs. 5*c*, 5*d*). Cells from a nodule growing under water at 5 kPa O_2 are shown (Fig. 5*a*) and this example presumably adds an even lower Po_2 treat-

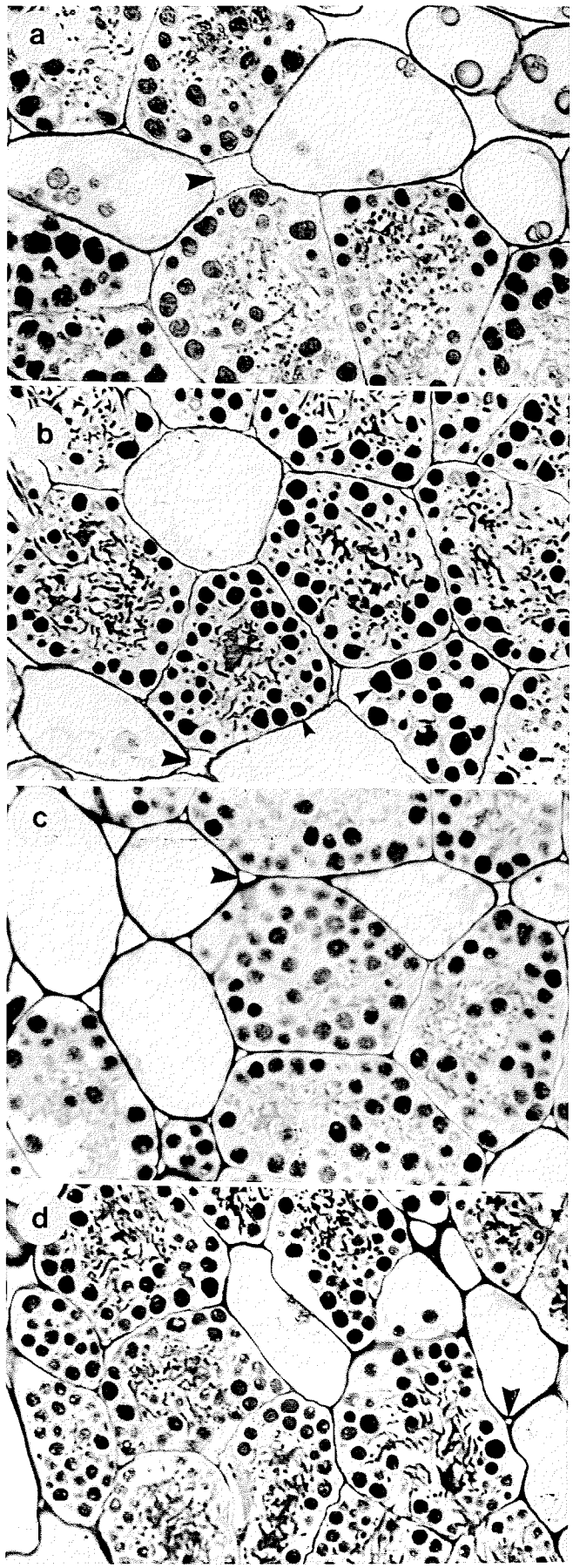


FIG. 5. Cross sections of mature root nodules of *A. incana* ssp. *rugosa* showing at higher magnification structural details of infected cells and associated parenchyma and intercellular spaces. (*a*) Nodules were subjected to 5 kPa O_2 and submerged in the nutrient solution. (*b*) Nodules were exposed to 5 kPa O_2 in the gaseous environment of the culture container. (*c*) Nodules exposed to 21 kPa O_2 in the gas phase. (*d*) Nodules exposed to 40 kPa O_2. Large arrowheads indicate intercellular air spaces adjacent to infected cells. Small arrowheads (Fig. 5*b*) show *Frankia* vesicles with "void areas" especially evident. ×1000.

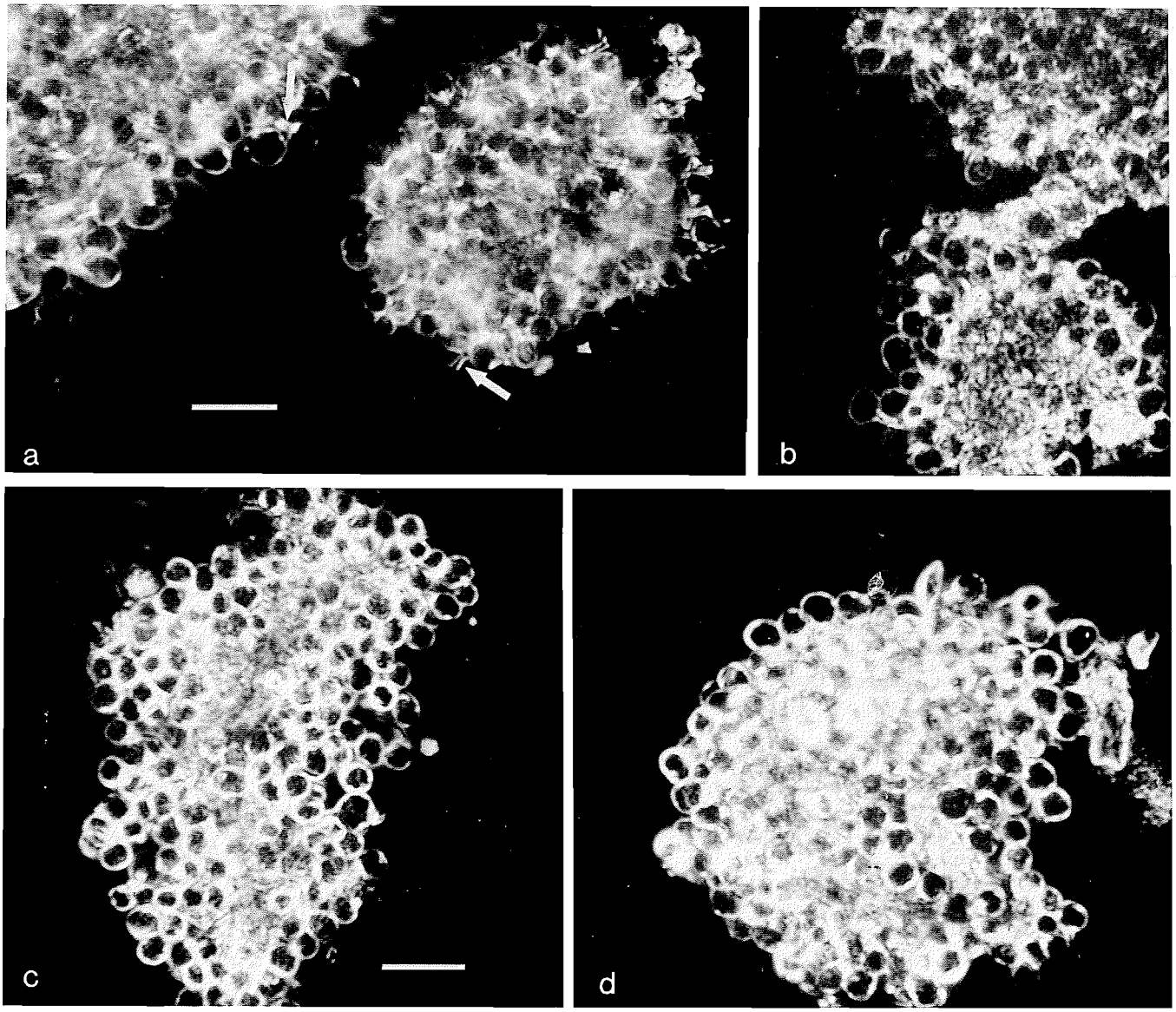


FIG. 6. Dark-field photomicrographs of *Frankia* vesicle clusters from root nodules of *A. incana* ssp. *rugosa* grown at different oxygen concentrations. (*a* and *b*) Replicate squashes from nodules grown at 5 kPa O_2. Note that the thin vesicle envelopes are especially clear at the edge of the cell mass. Arrows in Fig. 6*a* show that the vesicle stalks are thicker than the envelope. (*c* and *d*) Replicate squashes from nodules grown at 40 kPa O_2 with thicker, brighter vesicle envelopes. Scale bar = 10 μm.

ment to the series. Dark-stained vesicles show up well in *A. incana* ssp. *rugosa* sections and each vesicle is normally surrounded by a clear halo called a "void area" (Lalonde *et al.* 1976), which has been variously interpreted as a shrinkage artifact or the unstained envelope of the vesicle.

Dark-field microscopy

In viewing dissected nodules and vesicle clusters great care was taken to standardize all procedures because the images produced by dark-field microscopy are artifacts of light scattering and can be artificially modified by changing photographic exposure and development times. The resulting dark-field views (Fig. 6) show a clear difference in brightness (interpreted as thickness) of the vesicle envelope between Po_2 treatments. Differences are best seen at the peripheries of clusters. At low oxygen the vesicles were difficult to see under dark field, the envelopes often vanishingly thin, while at high Po_2, the envelopes were often brilliantly bright and apparently very much thicker.

Discussion

Wheeler *et al.* (1979) and Winship and Tjepkema (1983, 1985) studied the effect of Po_2 on acetylene reduction in *Alnus* spp. and noted that there is a broad optimum range between 10 and 30 kPa O_2. Our results confirm this broad optimum range but in contrast to Winship and Tjepkema (1985), who stated that acetylene reduction was stable over the time span of each determination, we found *A. incana* ssp. *rugosa* nitrogenase activity to be unstable at virtually all Po_2 levels so that one is unable to draw a Po_2 optimum curve. Our work suggests either that *A. incana* ssp. *rugosa* possesses a variable gas-diffusion control mechanism similar to legume

nodules (Hunt *et al.* 1987) (although not as responsive as legumes) or that it takes a very long time to establish an equilibrium acetylene reduction after a change in Po_2.

Like *Myrica gale* (Silvester *et al.* 1988), *Alnus* is able to adapt plant growth and nitrogenase activity to a wide range of root Po_2's, but unlike *M. gale*, this adaptation is not accompanied by the dramatic changes in nodule morphology and anatomy that we have reported for that plant. While the *Alnus* nodule certainly has a modified aeration pattern after exposure to varying Po_2, the changes are apparently not proportional to the eightfold change in Po_2 that was applied. In the case of *Alnus* we believe the dramatic changes in vesicle structure revealed by the dark-field microscope images are reflections of changed resistances to varying Po_2. This is in distinct contrast to the situation in *M. gale* (Silvester *et al.* 1988), where vesicles always show up under dark-field microscopy as vanishingly thin walled regardless of the Po_2 at which they are grown.

Frankia in culture shows similar adaptation to high and low Po_2 and undergoes similar changes in vesicle envelope properties when viewed under dark-field microscopy (Parsons *et al.* 1987) and these changes have been correlated with the number of lipid-like layers in the multilaminate envelope of the vesicle (Parsons *et al.* 1987). The multilaminate nature of the vesicle envelope is only clearly visualized in freeze-fracture preparations of *Frankia* and has been shown in root nodules of *Alnus* by Lalonde and Devoe (1976) and in *Elaeagnus umbellata*, a genus with very similar nodule structure to *Alnus* (Newcomb *et al.* 1987).

The oxygen balance within actinorhizal root nodules and the oxygen protection of nitrogenase are apparently not simple phenomena involving one tissue or biochemical process but are the result of a finely tuned relationship between the host nodule tissue and the bacterium.

Tjepkema (1979, 1983) showed that the oxygen protection mechanisms in legume and actinorhizal roots are vastly different and the ubiquitous presence of haemoglobin in legume nodules and its variable occurrence in actinorhizal nodules present one of the confusing aspects of attempting to compare the two systems. Actinorhizal nodules may be classified according to the form taken by *Frankia*, which is closely correlated with the probable mechanism of oxygen control and protection (Torrey 1985). At one end of the spectrum is a group, typified by *Alnus*, in which *Frankia* exists as large, round, well-developed vesicles at the periphery of large infected cells. In this case, while the nodule structure may provide some internal oxygen resistance, it appears from the present work that the major site of diffusion resistance may be the vesicle envelope. The intermediate group, typified by *Myrica gale* (Silvester *et al.* 1988), has smaller club-shaped vesicles, which generally appear to be without a substantial envelope and always show up as thin walled under dark-field microscopy. In this case, the nodule is relatively impermeable to oxygen, and gas diffusion takes place via nodule roots, which adapt in surface area according to the ambient Po_2. Thickened or modified walls of infected host cells may play a role in diffusion resistance in this case (Berg 1983), while the role of the vesicle envelope in diffusion resistance is probably minimal. At the other end of the spectrum is a group, typified by *Casuarina*, that does not form vesicles in symbiosis, despite the fact that all *Frankia* isolates that nodulate *Casuarina* produce typical spherical vesicles in aerated culture. Murry *et al.* (1985) have shown that *Frankia* strain HFP CcI3 isolated from *Casuarina* root nodules, when grown in culture at very low Po_2, does not form vesicles but shows low acetylene reduction activity. In the case of *Casuarina* root nodules it is highly likely that the lignified and suberized wall of the infected host cell provides a significant diffusion boundary to oxygen (Berg 1983).

Berg and McDowell (1987) believe they have identified the multilaminate envelope in hyphae of *Casuarina*, but only in the invasive intercellular stages. Those hyphae that are intracellular have a single lamina and this might be expected if the host cell wall provides the major diffusion resistance in these nodules. The presence of haemoglobin, which is found in high concentration both in *Myrica gale* and in *Casuarina* nodules (Tjepkema 1983; Tjepkema *et al.* 1986), remains to be understood.

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