Growth, nodule morphology, and nitrogenase activity of *Myrica gale* with roots grown at various oxygen levels

WARWICK B. SILVESTER,[1] JULIE WHITBECK, JANET K. SILVESTER,[1], AND JOHN G. TORREY[2]
Harvard University, Harvard Forest, Petersham, MA 01366, U.S.A.

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Myrica gale L. plants were inoculated with *Frankia* strain HFP M⁺gI5 and grown for 28 days with root systems exposed to 2, 5, 10, 21, and 40 kPa O_2. Plant growth was similar under all treatments, except for a small decrease in final size of plants at 2 kPa O_2. At the end of the experiment nitrogenase activity of representative plants was analyzed over a variety of Po_2 levels using an open-flow cuvette. Optimum nitrogenase activity was found at Po_2 levels close to the growth Po_2 in all cases and plants showed no short-term adaptation to oxygen. Specific activity of maximum nitrogenase was similar for all treatments, being within a factor of two. Nitrogenase activity showed rapid transient responses to step shifts in Po_2 during assay and irreversible decline in activity at Po_2 levels above optimum. Morphological responses to changing Po_2 include a dramatic increase in nodule-root growth, inversely proportional to ambient Po_2, and a variety of internal structural changes reducing nodule ventilation with Po_2 increase. No modification in vesicle envelope thickness was observed over the range of Po_2 studied. We conclude that oxygen protection in *M. gale* nodules operates through a complex suite of morphological controls, but that modification of nodule-root surface area is probably the prime mechanism.

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Des plants de *Myrica gale* L. furent inoculés avec la souche HFP M⁺gI5 de *Frankia* et cultivés durant 28 jours avec leur système racinaire exposé à 2, 5, 10, 21 et 40 kPa d'o_2. La croissance des plantes fut similaire dans tous les traitements, sauf à 2 kPa D-O_2 ou une faible diminution de la taille finale fut notée. L'activité de la nitrogénase de plantes représentatives, à la fin de l'expérience, fut analysée à plusieurs niveaux de Po_2 à l'aide de la cuvette ouverte à flot continu. L'activité optimale de la nitrogénase fut mesurée aux niveau de Po_2 voisins de celui de la croissance dans tous les cas et les plantes n'ont pas montré d'adaptation à l'oxygène à court terme. L'activité spécifique maximale de la nitrogénase fut similaire pour tous les traitements, se situant à l'intérieur d'un facteur de deux. L'activité de la nitrogénase a montré des réponses rapides passagères aux changements de Po_2 durant l'essai ainsi qu'une diminution irréversible d'activité aux niveaux de Po_2 au-dessus de l'optimum. Les réponses morphologiques aux changements de Po_2 incluent une très forte augmentation de la croissance des nodosités racinaires, inversement proportionnelle à la Po_2 ambiante, ainsi que de nombreux changements structuraux internes réduisant la ventilation des nodules suite à l'augmentation de Po_2. Aucune modification de l'épaisseur de l'enveloppe vésiculaire n'a été observée dans la gamme de Po_2 étudiée. Nous concluons que la protection contre l'oxygene dans les nodosités de *M. gale* se produit au moyen d'une suite complexe de contrôles morphologiques, mais la modification de la surface des nodosités serait le mécanisme fondamental.

[Traduit par la revue]

Introduction

Myrica gale L., a nodulated shrub of the Myricaceae, populates the wetlands of the northern United States, Canada, and northern Europe. As a widespread actinorhizal plant capable of substantial rates of dinitrogen fixation, *M. gale* has been the subject of considerable study especially concerned with its ecophysiology and the structure of the root nodules induced by the filamentous soil bacterium *Frankia* (Actinomycetales).

Myrica gale is well adapted to changing water levels and may stand fully submerged throughout the winter in many sites (Sprent and Scott 1979; Schwintzer and Lancelle 1983) or persist with root systems 10 cm or more above the water table in midsummer. Correlated with seasonal changes in the water-table level are developmental changes in roots, nodules, nodule roots, and plant biomass (Sprent and Scott 1979).

Nitrogen fixation by *Frankia* in root nodules requires available oxygen, which plays an important controlling role in symbiotic dinitrogen fixation (Bond 1961). Plants growing with submerged roots or in wet, waterlogged soils show structural modifications related to these environmental conditions and in the case of *M. gale* the most notable modification is the presence of upward growing nodule roots. Bond (1949, 1952) first described nodule roots in *M. gale* which develop from the terminal ends of nodule lobes and tend to grow vertically upward from the nodule. Such nodule roots are determinate structures (Torrey and Callaham 1978) of variable length depending on the environment in which they develop (Sprent and Scott 1979; Schwintzer and Lancelle 1983). Tjepkema (1978) provided experimental evidence that nodule roots in *M. gale* under conditions of low oxygen function to increase the availability of oxygen to the sites of nitrogenase in the nodule lobes. Structural studies of nodule roots (Torrey and Callaham 1978; Sprent and Scott 1979) indicate that the modified cortex of the nodule roots in *M. gale* is highly aerenchymatous. Rates of acetylene reduction activity are proportional to the oxygen availability (Sprent and Scott 1979; Tjepkema 1978) and Tjepkema (1978, 1983) has suggested that nodule roots serve as an important aeration function in *M. gale* nodules grown under low Po_2.

Oxygen is essential for nodule function in all nodulated plants, but free oxygen is strongly inhibitory to nitrogenase function and nitrogen fixation is sustained only under conditions of rapid oxygen flux at very low concentration. The various oxygen protection mechanisms for nitrogenase have attracted attention (Robson and Postgate 1980; Shaw 1984)

[1]Permanent address: Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.
[2]Author to whom all correspondence should be addressed.

and recent work indicates that legume nodules may posses both fixed and variable diffusion resistance boundaries that control oxygen flux and maintain a low constant oxygen concentration in the nodule (Witty *et al.* 1984). The situation in actinorhizal nodules is not as well studied, but there is good evidence that the nodules are relatively well aerated and that a major resistance to oxygen diffusion is the thickened envelope of the bacterial vesicle (Tjepkema *et al.* 1986).

Tjepkema *et al.* (1986) have suggested that the modifications of *Myrica* nodules are not sufficient to permit normal levels of nitrogenase activity when the nodules are flooded, and that flooding is generally deleterious to growth. The present study was designed to explore, using carefully controlled oxygen levels around nodulated roots of *M. gale*, the specific effects of varying oxygen on growth, anatomy, and nitrogenase activity and, by identifying changes in these parameters, attempt to define in detail the major sites of oxygen protection in these nodules.

Materials and methods

Myrica gale fruits were collected near the Harvard Forest and germinated as described previously (VandenBosch and Torrey 1983). Three weeks after germination, seedlings were transferred to water culture and inoculated with *Frankia*.

The strain of *Frankia* used was isolated by Z. Zhang in 1984 from root nodules of *M. gale* collected from the Pond Side site of the Harvard Forest (Schwintzer *et al.* 1982) and shown to be spore (+) at the time of isolation. The strain has been designated HFP M⁺gI5 (catalog no. HFP 161105), commonly called M⁺g. The strain grows well in culture on M6B medium (Baker and Torrey 1979), sporulates in culture, and when used as inoculum on seedling roots of *Myrica gale*, produces effective nodules which are consistently spore (−) in water-culture experiments in the greenhouse or in growth chambers.

Plants were maintained in a growth cabinet at 280 μE m^{-2} s^{-1} photosynthetically active radiation, 16 h light : 8 h darkness at 26:19°C (light:dark). After nodule induction plants were maintained in aerated water culture sparged with either air or an appropriate gas mixture. Water level was lowered so that nodules were in the gas phase but kept moist by the fine mist of nutrient solution created by breaking bubbles. Plants maintained in elevated or lowered oxygen levels were aerated in a closed loop with their roots in 1-L canning (preserving) jars in which the dome lid was punctured with several holes (Fig. 1). Three plants per jar were inserted through split bungs and sealed in place with a plastic adhesive compound, Holdit (Eberhard Faber Inc., Wilkes Barre, PA, U.S.A.). Gas was circulated through the jars by a sealed diaphragm pump from a 200-L polyvinyl chloride reservoir bag. Oxygen levels were measured daily. Largest daily fluctuations were <5% of the nominal value and gas additions were made when necessary to keep Po_2 within the 5% range. Carbon dioxide values were measured on occasions and these remained in the range of 0.5−1.0 kPa, depending on the root mass. The large surface area of the polyvinyl chloride bags and the differential CO_2 permeability were sufficient we believe to allow significant loss of CO_2 from the reservoir during the experiments.

Gas mixtures were prepared from commerical gas cylinders and tested by gas chromatography. For the open-cuvette assay system, mixtures were made by volumetric transfer using a 1.5-L syringe into 10-L polyvinyl chloride beach balls fitted with septum seals and pipework. Where appropriate, 10 kPa acetylene was added and during assay oxygen values were maintained within ±0.1 kPa of stated values.

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

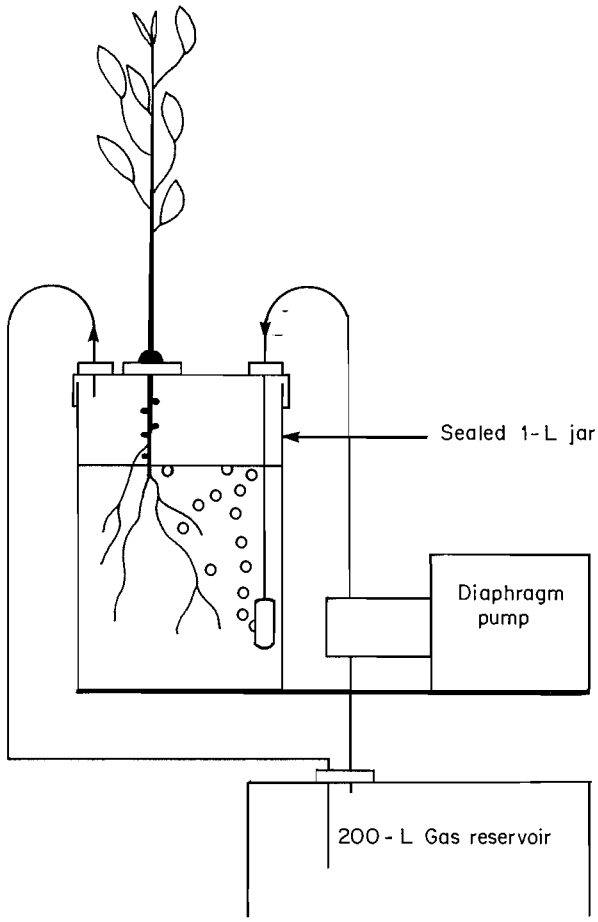


FIG. 1. Apparatus used for growing plants with root systems in defined oxygen environment. The gas mixture is made up in a 200-L polyvinyl chloride bag and pumped via a sealed diaphragm pump through the sealed jar and back to the reservoir. Up to six jars each containing up to three seedlings can be run on each apparatus.

All assays were conducted in an open-flow cuvette based on the techniques described by Minchin *et al.* (1983) (Fig. 2). 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 1−400 mL min^{-1} at constant rate. The cuvette for whole-plant studies consisted of a 60-mL plastic syringe with the head removed and a split rubber stopper inserted to hold 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 30−50 mL and flow rate controlled to give at least one gas change per minute. Normally the cuvette was installed in a water bath at 25°C.

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 them directly into the gas chromatograph. This configuration allowed samples to be taken at 40 to 60s 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 gas chromatography.

Whole-plant assays were conducted in the laboratory without added lighting, all gas mixtures were water saturated, and plants were kept in polythene bags to reduce transpirational water loss. Under these conditions rates of acetylene reduction were constant for 4−8 h.

The problem of acetylene-induced decline (Minchin *et al.* 1983), in

TABLE 1. Growth parameters of *Myrica* plants grown for 28 days at various oxygen levels

O_2, kPa	Shoot height, mm	No. of leaves	Stem diameter, mm	Total dry weight, g	Nodule dry weight, mg
2	212	26	3.90	1.72*	57.6
5	295 (91)	28	5.03	1.44 (0.69)	51.6
21	318 (67)	34	4.50	1.47 (0.45)	70.2
40	270 (59)	34	4.50	1.27 (0.81)	61.2

NOTE: Values in table are means and representative of 95% confidence limits for three plants, except at 2 kPa O_2 (one plant) (see text).
*Weight at 39 days.

which acetylene reduction declines during the first 5–10 min of acetylene exposure, was addressed in preliminary experiments. *Myrica* and *Alnus* plants showed variable evidence of decline and spontaneous return to maximum values over time. However, regardless of the decline, acetylene reduction rates stabilized after 10–15 min and remained stable for 4–8 h and the acetylene decline is not a confusion to the present results.

Microscopy

Nodule lobes were removed from plants at harvest and were treated and sectioned for light microscopy as described previously by VandenBosch and Torrey (1983). Nodules were fixed in 3% glutaraldehyde in phosphate buffer, dehydrated in acetone, and embedded in resin. Sections cut at 1 μm were mounted on glass slides and stained with toluidine blue in borate solution. Nodules from three plants per treatment were sampled. Mean cell sizes were calculated from measurements of 30 cells per treatment.

Results

Plant growth

In a preliminary experiment plants were grown for 28 days with their roots at 5, 10, 21, and 40 kPa O_2 and plants showed little, if any, difference in growth rate. It was apparent from this result that *M. gale* plants could adapt rapidly to at least an eightfold range in oxygen tension without obvious damage. A second experiment was conducted to cover a 20-fold range of Po_2 with plant roots at 2, 5, 21, and 40 kPa O_2. In this case plants were started at a smaller size and the experiment run for 28 days. Growth as measured by plant height (Fig. 3), number of leaves, stem diameter, total weight, and nodule weights (Table 1) showed very little difference except at 2 kPa O_2. Unfortunately it was possible to have only three plants per treatment and one of the plants at 2 kPa O_2 was broken partway through the experiment. Of the resulting two plants, one grew very well, while the other grew slowly throughout. The results suggest that even at 2 kPa O_2 *M. gale* plants may be healthy, adapting both nitrogenase and other root functions to the extremely wide range of oxygen levels.

Nitrogenase activity

Nitrogenase activity was tested on representative plants from all levels of Po_2 at the end of the experiment. Because nitrogenase is extremely sensitive to oxygen shock, plants for nitrogenase assay were removed inside a plastic-glove bag at the Po_2 of the circulating gas and inserted into the cuvette at that Po_2. Initial gassing in the cuvette was at the nominal Po_2 of the gas stream and then values above and below were introduced and run until equilibrium was reached. The results (Fig. 4) show that *M. gale* adapts to the ambient oxygen level, with plants at low Po_2 showing very sharp optima near to growth Po_2. Specific activity of nodules at the various Po_2 levels is remarkably similar with only the 10 kPa O_2 plant showing a noticeably greater activity than all other plants. It is very significant that over a 20-fold range in Po_2 the specific activity changes so little. This result indicates that there are similar amounts of infective tissue per unit nodule mass and that Po_2 has not induced vast changes in nodule anatomy such as production of large amounts of mechanical tissue.

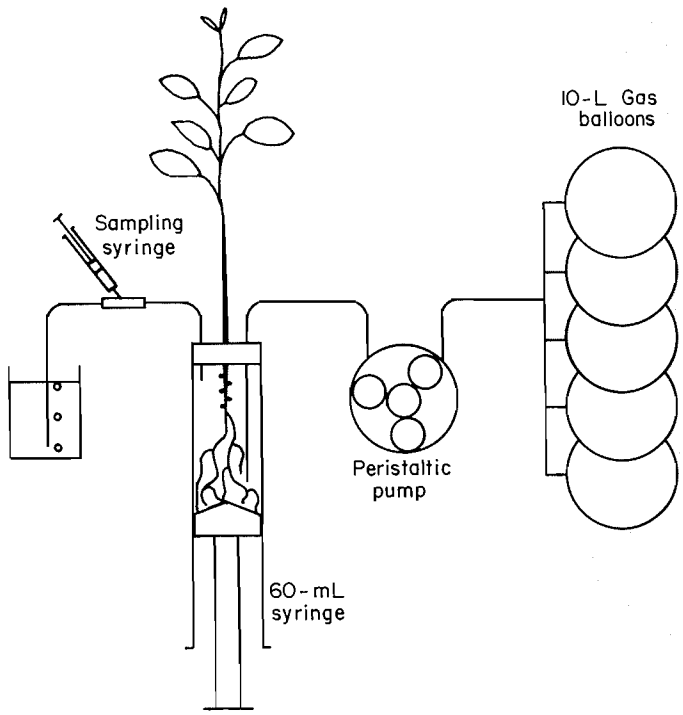


FIG. 2. Apparatus used for semicontinuous nitrogenase assay of nodulated plants. Plants are sealed into the syringe cuvette and gas is pumped at 20–100 mL min^{-1} through the cuvette from one of the gas balloons. Samples are taken at 60-s intervals from the rubber tube on the outlet.

Dynamics of nitrogenase activity

We have shown previously (W. B. Silvester and L. J. Winship, unpublished) that nitrogenase activity in actinorhizal nodules (*Myrica* in particular) responds very rapidly to changes in oxygen tension and that rapid transient changes in nitrogenase activity occur when oxygen tension is increased stepwise. Transient changes in nitrogenase activity are shown in Figs. 5–9 along with responses of representative plants grown at various oxygen tensions. All treatments show that *M. gale* responds extremely rapidly to oxygen shifts, which suggests that *M. gale* is strongly oxygen limited all the way up to the optimum. These figures and the optimum curves of

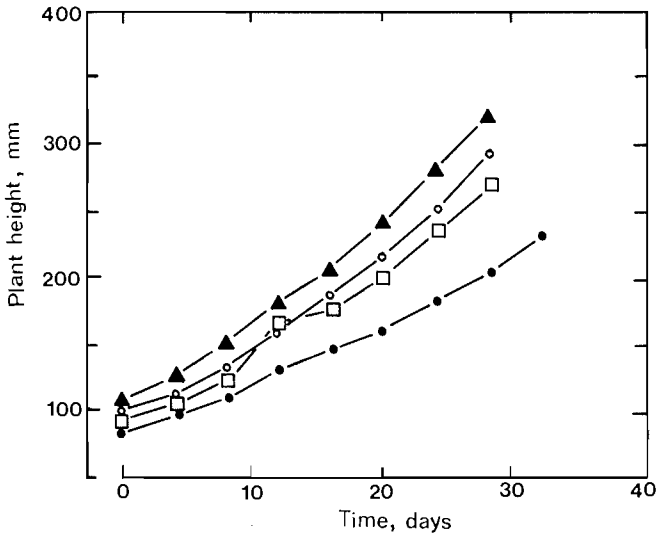


FIG. 3. Height growth of *M. gale* plants at various P_{O_2} levels. Plants were grown with root systems at 2, ●; 5, ○; 21, ▲; and 40 kPa O_2, □. Means of three plants for 5, 21, and 40 kPa O_2, one plant only for 2 kPa O_2.

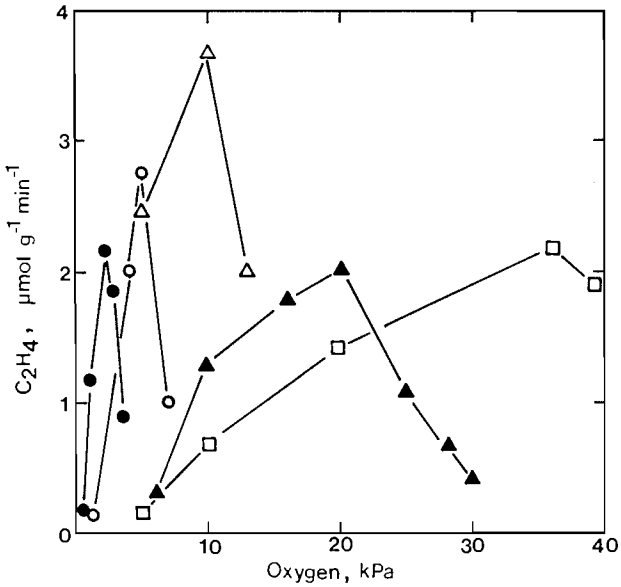


FIG. 4. Nitrogenase activity of *M. gale* plants at various P_{O_2} levels, following growth with root systems at defined P_{O_2}. Plants were grown at 2, ●; 5, ○; 10, △; 21, ▲; and 40 kPa $_2$, □.

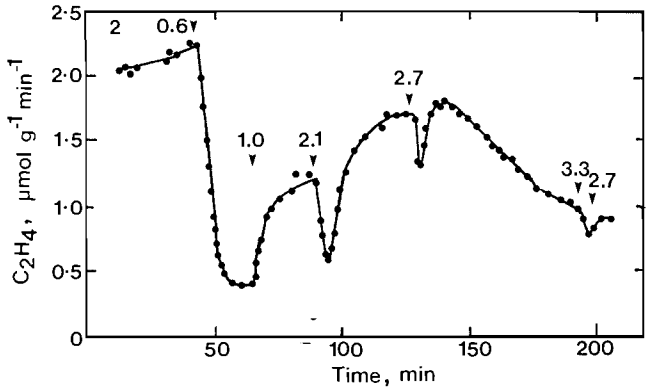


FIG. 5. Nitrogenase response curve for plant grown with root system at 2 kPa O_2. Plant equilibrated at 2 kPa O_2 then submitted to step changes in P_{O_2} as shown by the arrowheads.

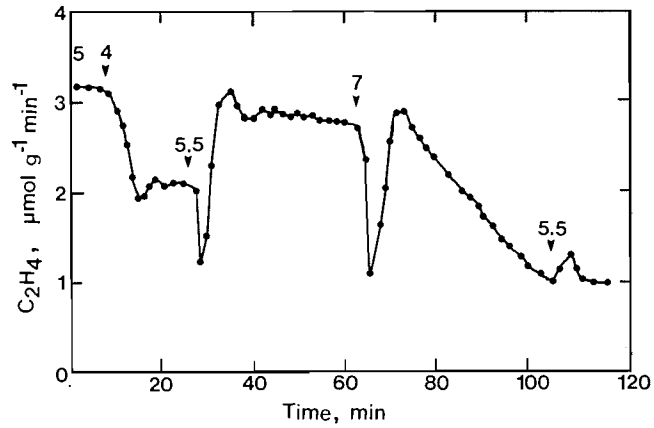


FIG. 6. Nitrogenase response curve for plant grown with root system at 5 kPa O_2. Plant equilibrated at 5 kPa O_2 then submitted to step changes in P_{O_2} as shown by the arrowheads.

TABLE 2. Effect of various oxygen tensions on the morphology of nodule roots

O_2, kPa	Length, mm	Diameter, mm	Aeration, % air space
2	62*a*	0.46*a*	32*a*
5	33*b*	0.51*a*	24*ab*
21	14*c*	0.45*a*	31*a*
40	6*d*	0.29*b*	20*b*

NOTE: Plants were grown for 28 days at the oxygen tensions shown. Numbers within columns followed by different letters are significantly different (p = 0.05).

Fig. 4 indicate a sharp P_{O_2} optimum close to the growth optimum. The curves from oxygen transients also show rapid recovery from oxygen shock; in most cases recovery is complete, but in some there are significant losses in nitrogenase especially when the transient occurs at above the optimum.

Analysis of the oxygen response curves (Figs. 5–9) reveals a number of important attributes of the physiology of nitrogenase in *M. gale* nodules. Firstly, there is no obvious adaptation to below-ambient P_{O_2}; see especially Figs. 5, 8, and 9, which show very constant nitrogenase activity at lower than optimal P_{O_2}. Secondly, nitrogenase shows steep reversible declines in response to stepwise increases in P_{O_2}. Finally, and this is most marked in plants optimized to low P_{O_2}, nitrogenase activity shows a sustained decline when assayed at above optimum P_{O_2} (Figs. 5, 6, 7). Attempts to recover activity after this slow decline by returning to optimum P_{O_2} during assay result in little recovery of nitrogenase.

We conclude from these results that *M. gale* nodules are very well ventilated and possess long-term adaptable resistance(s) to gas diffusion. There is no indication of short-term adaptation to above or below optimum P_{O_2} as seen in legume nodules.

Nodule morphology

The ability of *M. gale* to sustain high specific nitrogenase

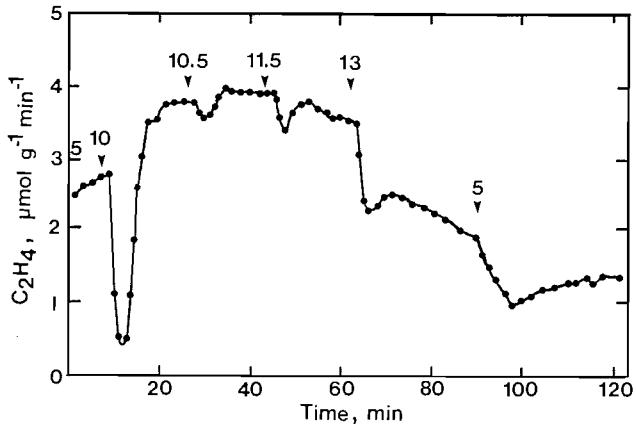


FIG. 7. Nitrogenase response curve for plant grown with root system at 10 kPa O_2. Plant equilibrated at 5 kPa O_2 then submitted to step changes in Po_2 as shown by the arrowheads.

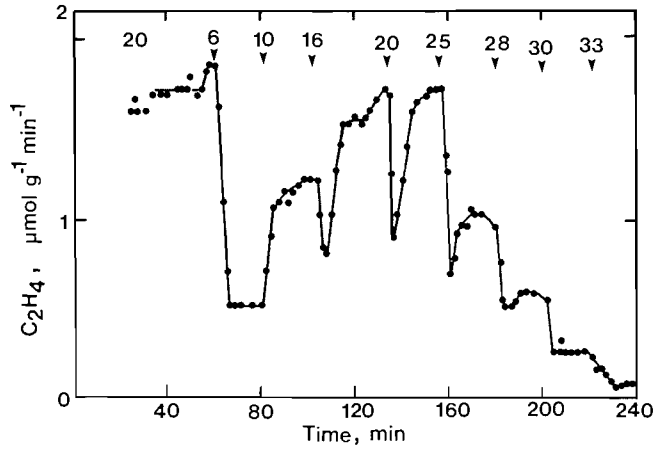


FIG. 8. Nitrogenase response curve for plant grown with root system at 21 kPa O_2. Plant equilibrated at 20 kPa O_2 then submitted to step changes in Po_2 as shown by the arrowheads.

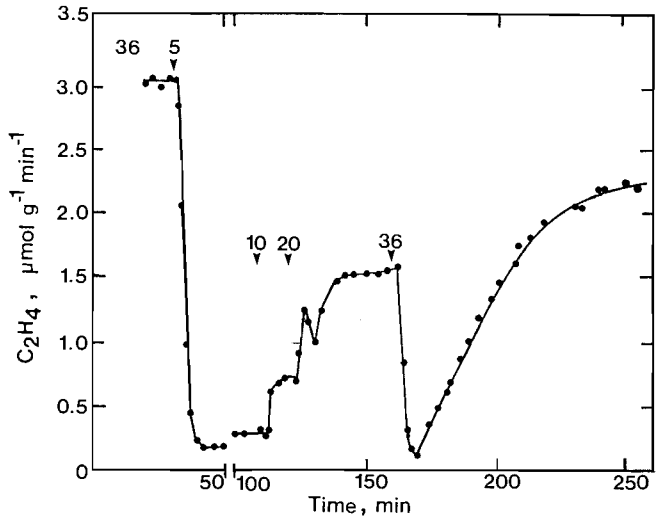


FIG. 9. Nitrogenase response curve for plant grown with root system at 40 kPa O_2. Plant equilibrated at 36 kPa O_2 then submitted to step changes in Po_2 as shown by the arrowheads.

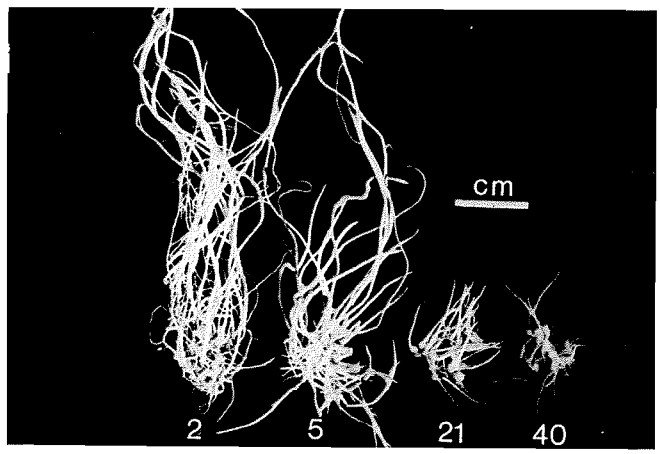


FIG. 10. Nodules with vertically oriented nodule roots from plants grown with root environments at 2, 5, 21, and 40 kPa O_2. Fine branch roots are apparent in the 2 kPa O_2 treatment. Note differences in both root length and diameter.

activity (Fig. 4) and growth (Fig. 3) over such a wide range of Po_2 implies massive changes in nodule and nodule root anatomy to maintain the high oxygen flux and low Po_2 necessary for nodule function. The fixed diffusion resistance in root nodules must be undergoing remarkable changes over the 20-fold change in Po_2 and, if there is a morphological expression of this resistance, we hypothesized that there would be evident changes in nodule anatomy.

Root nodules of *M. gale* are characterized by terminal, determinate, negatively geotropic roots (Torrey and Callaham 1978) that function as aeration systems for the nodule (Tjepkema 1978). The most obvious difference in nodule morphology is related to the effect of oxygen on the size of these roots (Fig. 10). At low oxygen the roots are extremely long, often branched, and relatively thick (Fig. 10, Table 2), while at high oxygen they are short and thin. These features are described in Table 2, which shows also that the degree of aeration is hardly, and not consistently, affected by Po_2, except for the large reduction at 40 kPa O_2.

Nodule roots cut transversely at 2 mm from the nodule apex in plants grown over the wide range of Po_2 levels (Fig. 11) all show similar structure but differ in diameter related to cell number and degree of intercellular space formation. In all cases there are two or three outer cell layers of closely packed cells and a well-aerated cortex. The cells are generally thin walled with about half of the cells being very thin walled and prone to collapse, especially in the low Po_2 roots (Figs. 11*a*, 11*b*). Estimations of percent air space are difficult as a result of this cell collapse and the values given in Table 2 must be treated with some caution. Regardless of this, there is no indication of a vast difference in degree of root aeration, although roots grown at 40 kPa O_2 do seem to be less well aerated.

The internal anatomy of nodules shows some changes over the wide range of Po_2 levels (Figs. 12 and 13), but the changes are not as dramatic as the effects on nodule-root length. In all cases there is a thin-walled periderm of one to three cells overlying a thickened hypodermis. This latter layer is of two to four cells, lacking air spaces, and there is some indication that at higher oxygen levels the layer may be wider and with thicker walls. These layers have been collectively called periderm by

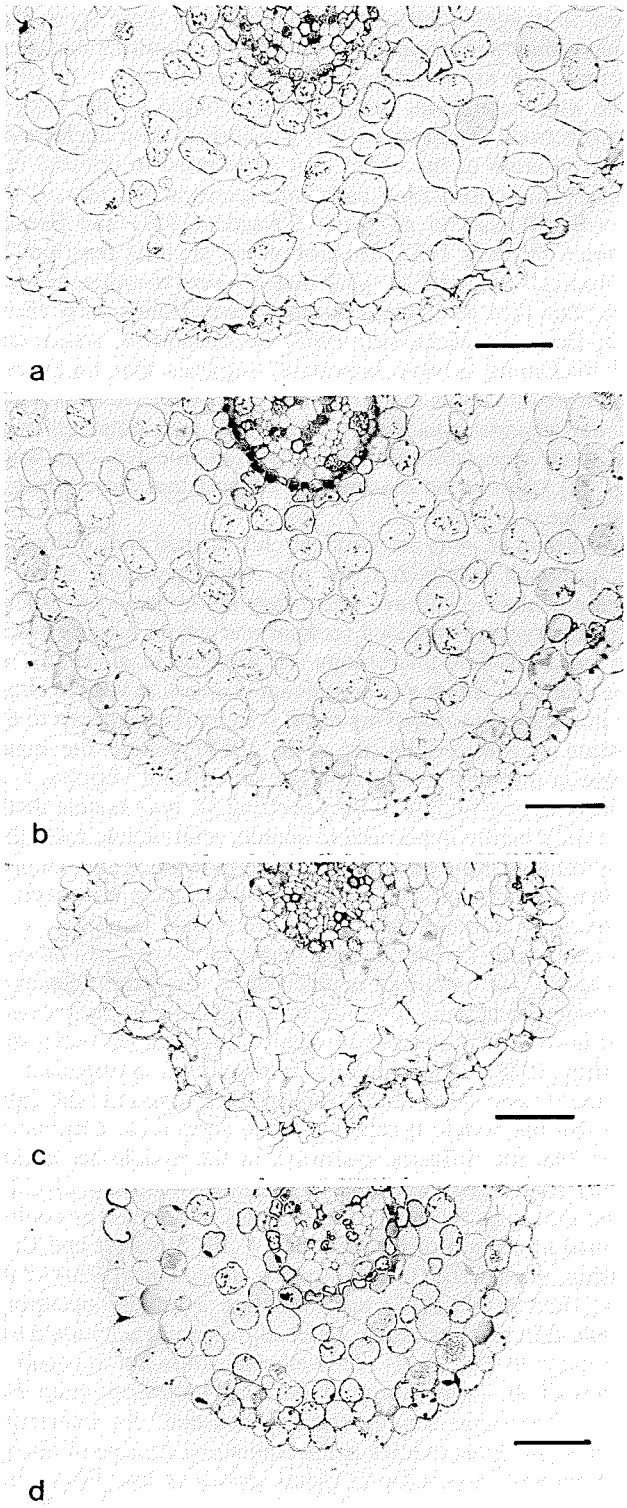


FIG. 11. Cross sections of nodule roots of plants grown with root environments at (*a*) 2, (*b*) 5, (*c*) 21, and (*d*) 40 kPa O_2. All sections were made at 2 mm from nodule apex. Scale bar is 50 μm.

Tjepkema (1983); they represent an important impermeable layer and we will continue to use this term to designate the outer layers. Underlying the periderm is an uninfected outer cortex of two to six cells, which is well aerated, possibly more so at low Po_2. At low Po_2 the aerated cortex is adjacent to the infected zone, while at high Po_2 the innermost layers of the uninfected cortex are tightly packed (Fig. 13). There is progressive widening of the uninfected layers of the cortex from 2 to 40 kPa O_2 (Fig. 12). The central cortex comprises the infected tissue in which cells occupied by *Frankia* occur singly or in groups of up to 10, with a tendency for these groups to be small (2–4 cells) at low Po_2 and large (7–10 cells) at high Po_2. In between the infected cells, channels of cortical parenchyma traverse the tissue, with large air spaces at low Po_2 and small air spaces at high Po_2 (Figs. 12 and 13). In a fully developed nodule infected cells may account for 50–70% of the tissue in the infected zone; at low Po_2 infected cells are large in diameter, while at high Po_2 they are smaller and clustered in larger groups.

The air-space distribution of the infected zone is strongly correlated with Po_2. At low Po_2, channel parenchyma has large intercellular gas spaces and infected cells have small spaces adjacent to cells. At high Po_2, spaces in channel parenchyma are small, and detectable spaces are not observed between infected cells. Walls of infected cells show some thickening, which is especially visible at 20 kPa O_2, but the effect is small and not consistently related to Po_2 (Fig. 12).

Dark-field microscopy of crushed nodules of *M. gale* was used to study vesicle envelopes of symbiotic *Frankia*. At all levels of Po_2 examined, vesicle envelopes appeared vanishingly thin and difficult to detect. This result is in contrast to the situation in *Alnus* (Silvester *et al.* 1988), where the major response to raised Po_2 is thickening of the vesicle envelope. One is led to conclude that the major resistance to oxygen diffusion in *M. gale* nodules is outside the *Frankia* vesicle.

In summary, the only major change in nodule morphology proportional to the extreme range of Po_2 is the change in nodule root surface area (i.e., length and diameter). Other significant effects are on infected cell size and aeration of the infected zone, and while these are not as dramatic as the changes in nodule roots, all the effects are cumulative and demonstrate the strong influence of the host plant in adapting and optimizing the environment of *Frankia*.

Discussion

Bond (1949, 1951) first pointed out the wide ecological range of *M. gale* and drew attention to the probable aeration function of the nodule roots (Bond 1952). More recent work (Sprent and Scott 1979; Schwintzer *et al.* 1982; Schwintzer and Lancelle 1983) has emphasized and extended our knowledge of *M. gale* ecology and it is obvious that this species is able to adapt in the field to a wide range of pH and water-table levels.

The general response of actinorhizal nodules to oxygen levels contrasts strongly with legume nodules. Actinorhizal nodules are highly aerated (Tjepkema *et al.* 1986) and show Po_2 optima for nitrogenase close to atmospheric levels, while legume nodules have distinct diffusion resistance boundaries and may show nitrogenase optima at Po_2 levels well above atmospheric (Witty *et al.* 1984; Hunt *et al.* 1987). These differences may be explained in part by the thick-walled vesicle of *Frankia*, which is pronounced both in culture (Tjepkema *et al.* 1980) and in symbiosis. The vesicle is the site of nitrogenase and the thick envelope apparently acts as a diffusion boundary, allowing *Frankia* to fix nitrogen at atmospheric levels of oxygen. In addition, when grown in culture, *Frankia* adapts to a wide range of above- and below-ambient oxygen levels by a mechanism varying the thickness of the

multilaminate vesicle envelope (Parsons *et al.* 1987). If the vesicle thickening mechanism is also operational in symbiosis, it is likely that other barriers to oxygen diffusion would not be necessary (Tjepkema *et al.* 1986).

We therefore attempted to repeat the experiments of Parsons *et al.* (1987) by varying the oxygen tension around *M. gale* roots and hypothesized that if there is significant adaptation of nitrogenase to Po_2, then variations in nodule or *Frankia* morphology would allow us to identify the site(s) of diffusion resistance or oxygen protection. It is important to emphasize that our plants were grown with most nodules exposed to the gas stream and not under water. While this does not simulate the ecological conditions under which *M. gale* grows, it does remove the major unknown variable of gas diffusion through water.

Our results show that nitrogenase adapts precisely to the ambient Po_2 and the changing root Po_2 produces a whole suite of morphological changes in the nodule, the prime one being variation in the length and surface area of nodule roots, which has also been shown in natural stands (Sprent and Scott 1979). Tjepkema (1978) demonstrated the importance of nodule roots in supplying oxygen to the nodule, especially at below ambient Po_2. The dramatic response in nodule-root growth accounts for a large proportion of the response to changing Po_2 and it is apparent that these structures act as the primary sites of gas exchange.

While the nodule roots act as variable oxygen antennae, it seems that the nodule itself which lacks lenticels is relatively impermeable to gas. Tjepkema (1983) showed that vacuum infiltration of India ink did not penetrate the periderm of *M. gale* nodules and the thickened and tightly packed nature of the outer layers suggests that it is a major barrier to gas diffusion. Despite this fact, the periderm does show significant thickening, both in cell number and wall thickness, in response to increasing Po_2, which shows that it is responsive to changes in available oxygen.

Over and above the nodule-root effects there are important quantitative differences in the degree of aeration of the uninfected and infected cortex. Chief among these is probably the compact layer just outside the infected zone, which at high Po_2 is totally lacking air spaces and must provide a significant diffusion barrier. In addition the air space distribution of the parenchyma channels and of the infected tissue is very responsive to external Po_2 and must represent a barrier to oxygen diffusion.

On the basis of India ink penetration studies of nodules and microelectrode traces of internal Po_2 of *M. gale* nodules, Tjepkema (1983) concluded that barriers to oxygen diffusion into the nodule do not exist. However, the microelectrode traces (Tjepkema 1979, 1983) indicate at least a threefold drop (10-fold in one case) in current (control electrode current in air 30–90 pA, electrode current in cortex ca. 10 pA) between air and the infected cell zone. This difference implies that the areas outside the infected zone do provide a significant outer barrier to oxygen access and the variation in these layers that we have shown in response to growth Po_2 confirms that these structures provide a variable diffusion resistance.

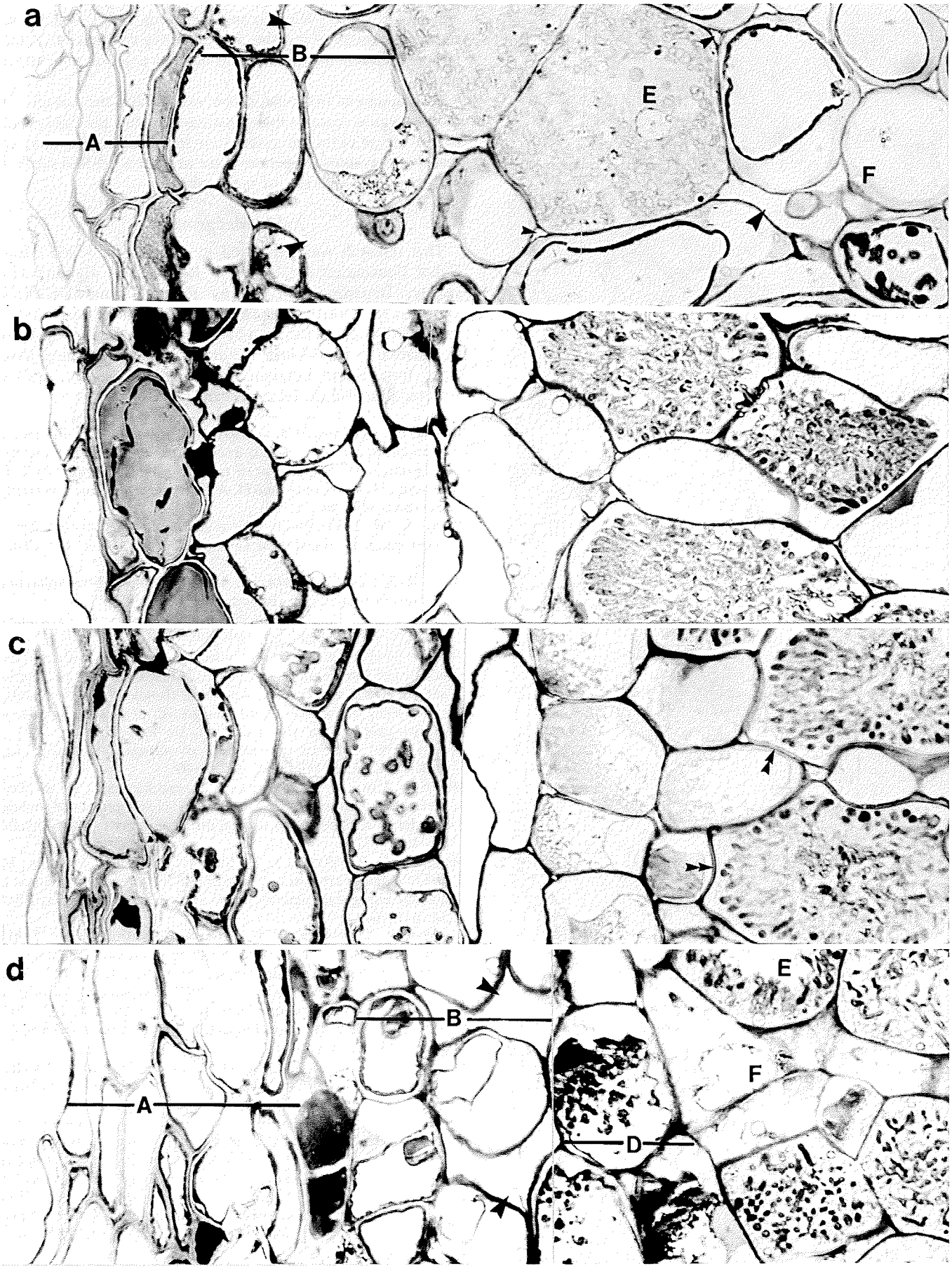
The most significant drop in Po_2 recorded by the oxygen microelectrode (Tjepkema 1983) is a 30-fold drop in electrode current in areas of the cortex that correspond to infected cell groups. This observation calls into question the role of the infected cell wall in *M. gale*. Schaede (1939) and Benson (reported by Berg 1983) have observed staining reactions in infected cell walls of *M. gale* and *M. pensylvanica*, respectively, that they attribute to lignin. More detailed work (Berg 1983; Berg and McDowell 1987) on *Casuarina*, where cell wall thickening is very important, suggests that lignin and suberin are laid down in the host cell wall. We have not studied cell wall changes specifically in *M. gale* and, although there was some detectable thickening of the infected cell walls (Fig. 12), there is no evidence to suggest that these changes were related to Po_2.

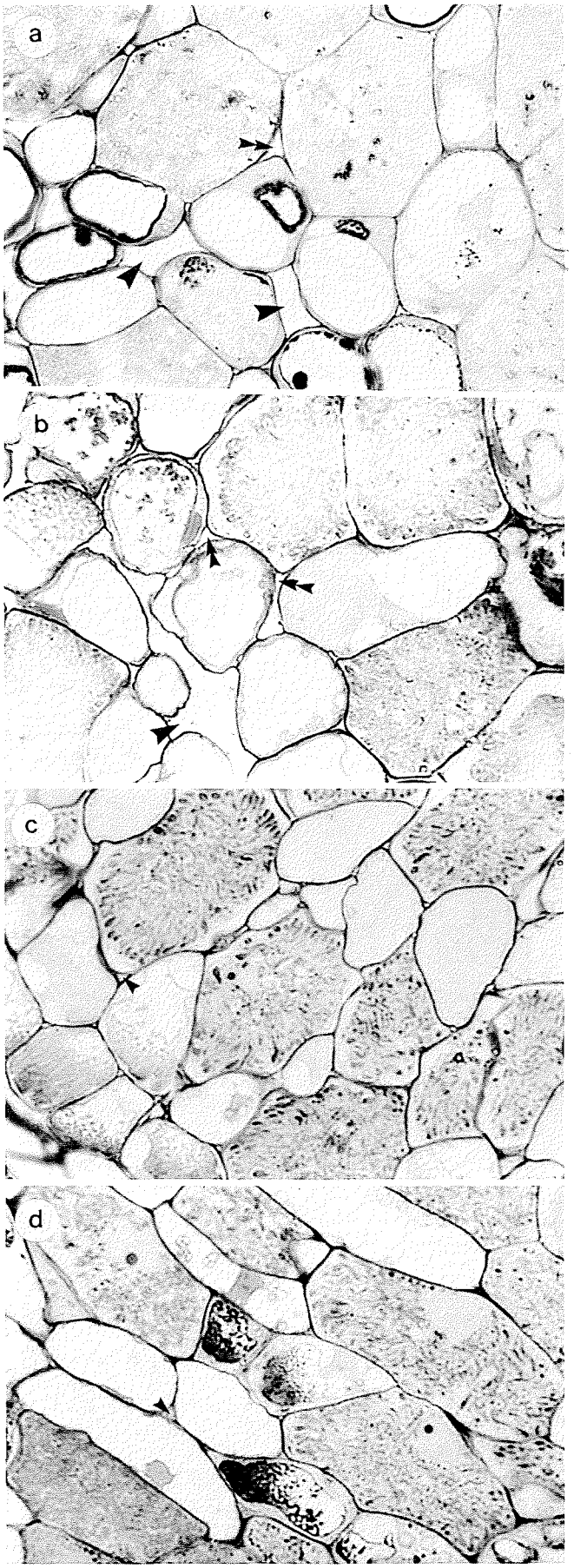
Vesicles in *M. gale* are swollen, club-shaped ends of hyphae and, although subdivided, do not reach the same stage of development as in culture, where they are large and spherical. Using both resin-embedded sections and whole mounts examined with dark-field microscopy (cf. Silvester *et al.* 1988), we were unable to detect any significant changes in vesicle structure in response to Po_2. This result is in marked contrast to the situation in *Alnus* (Silvester *et al.* 1988), where the major change in response to Po_2 is in the structure of vesicles.

The emerging picture of the operating *M. gale* nodule therefore is of a highly impermeable nodule, with nodule roots that act as variable gas-exchange antennae, in series with a number of other variable resistances, each of which acts in concert to maintain an optimum environment for nodule function.

In contrast to the above, long-term, morphological adaptations to Po_2, *M. gale* root nodules show very rapid responses in nitrogenase activity to rapid, stepwise shifts in oxygen, and these have been discussed in detail elsewhere (Silvester and Winship 1988). The rapidity with which nitrogenase is affected by step shifts in Po_2 gives further support to the argument that the nodule is relatively well aerated or, more accurately, that the diffusion pathways in the nodule are almost entirely in the gas phase. The spontaneous recovery of nitrogenase during an O_2-induced transient is interpreted as conformational protection of the enzyme (W. B. Silvester and L. J. Winship, unpublished). Recent work on legumes (Witty *et al.* 1984; Hunt *et al.* 1987) has identified a rapidly operating, variable-diffusion resistance which allows rapid adaptation to a wide range of Po_2 levels. This mechanism is not apparent in nodules of *M. gale*, where at constant below-optimum Po_2 levels, nitrogenase activity remains constant. An interesting feature of *M. gale* nodules is the continuing decline of nitrogenase activity, especially in plants grown at low Po_2, when exposed to above-optimum Po_2. This decline is interpreted as oxygen denaturation of nitrogenase since this activity was not recoverable by returning the root system to a lower Po_2. The

FIG. 12. Cross sections of root nodules from plants grown with root environments at (*a*) 2, (*b*) 5, (*c*) 21, and (*d*) 40 kPa O_2. Zones referred to in text are identified on the 2 and 40 kPa O_2 treatments only. Outer layers of periderm (A) show thinner cells to the outside and varying numbers of thick-walled cells, depending on Po_2 level. These layers are without air spaces and tightly packed. Within this is an aerated outer cortex (B), with extremely large air spaces (large arrowheads) at low Po_2. Overlying the infected cell zone, but only at higher Po_2, is a relatively tightly packed layer(s) (D), which effectively isolates the infected cells from the aerated cortex in high Po_2 grown nodules. The infected cells (E) occur in groups of 2–10 cells and are separated by interweaving channels of relatively well aerated parenchyma (F). Small arrowheads identify small air spaces associated with infected cells; large arrowheads identify large air spaces of cortex and of parenchyma channels at low Po_2. Double arrowheads identify possible wall thickening of infected cells in 20 kPa O_2 nodules. ×1000.





phenomenon of continuing decline is in strong contrast to the situation in *Alnus* (Winship and Tjepkema 1985), where some nitrogenase activity remains at well above ambient P_{O_2}, and it is hypothesized that the nodule acts as a series of compartments.

It is apparent from the above results that the complex of morphological features that constitute a nodule are finely tuned to provide an optimum gaseous environment for *Frankia*, and that this mechanism operates over at least a 20-fold range of P_{O_2} involving a variety of mechanisms.

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FIG. 13. Detail of infected zone of nodules from plants with roots grown at (*a*) 2, (*b*) 5, (*c*) 21, and (*d*) 40 kPa O_2. Large air spaces (large arrowheads) are found in the parenchyma channels only in nodules grown at 2 and 5 kPa O_2, while at high oxygen, small air spaces (small arrowheads) occur in the channels. At low oxygen, infected cells may be adjacent to quite large air spaces (double arrowheads), while at the highest P_{O_2} no visible air spaces can be seen against infected cells. ×650.

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