Chapter 5

OXYGEN RESPONSES, HEMOGLOBIN, AND THE STRUCTURE AND FUNCTION OF VESICLES

W. B. SILVESTER[1], R. H. BERG[2], C. R. SCHWINTZER[3] AND J. D. TJEPKEMA[3]

[1]*Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand*
[2]*Donald Danforth Plant Science Center, 975 N. Warson Road, St Louis, MO 63132, U.S.A.*
[3]*Department of Biological Sciences, University of Maine, Orono, ME 04469-5722, U.S.A.*

1. INTRODUCTION

Frankia nitrogenase is, like all other nitrogenases, highly sensitive to O_2 and the chemistry of its activity means it can only function in an O_2-free environment. This O_2 sensitivity is central to many aspects of the ecology and physiology of all nitrogenase-containing organisms, and many of the symbiotic states and ecological niches of nitrogenase-expressing microbes are determined by that sensitivity. For anaerobes, the problem is simply solved, but for those organisms dependent on atmospheric O_2 to generate their energy supply, the separation of nitrogenase from respiration represents a fascinating physiological dilemma. The dilemma centers on the need both to provide a high O_2 flux to drive the energy demand of nitrogenase and to protect nitrogenase from denaturation by free O_2. For all aerobic N_2-fixing organisms, this situation requires a high O_2 flux adjacent to a vanishingly low O_2 tension at the site of N_2 fixation.

A variety of strategies for solving the so-called O_2 dilemma have been described (Gallon, 1981) and they range from total avoidance, *i.e.*, the anaerobic strategy, to living with it, *i.e.*, the strategy of balancing nitrogenase synthesis with inactivation. The majority of organisms, both free-living and in symbiosis, adopt intermediate strategies that rely on external O_2 barriers coupled with high O_2 utilization at the nitrogenase site to maintain a steep O_2 gradient. Among the many physiological

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conditions that provide O_2 protection, two main families of strategies can be identified.

First, the majority of N_2-fixing bacteria rely on an external environmental source of O_2 regulation. Thus, many free-living bacteria live in microaerophilic environments in water, soil, or decaying organic matter and make use of the diffusion resistance of the medium and respiratory O_2 uptake of associated microbes to control the ambient pO_2. Similarly, the symbiotic state appears to be specifically adapted to provide the necessary pO_2 conditions for efficient nitrogenase activity. The legume nodule is the most sophisticated and physiologically intriguing example of this phenomenon and is dealt with in volume 7, *Nitrogen-fixing Leguminous Symbioses*, of this series.

Second, a few bacteria maintain their own physiological or structural O_2 diffusion barrier. These organisms can express functional nitrogenase in air and have a unique adaptability to a wide range of pO_2. This group includes only two types, the heterocystous cyanobacteria and the filamentous actinomycete *Frankia*. Both produce specialized thick-walled cells, called heterocysts and vesicles, respectively, which provide the requisite diffusion resistance to sustain nitrogenase at atmospheric pO_2. The history and verification of this phenomenon in *Frankia* vesicles forms much of the substance of this chapter.

Actinorhizal nodules then, like cyanobacterial symbioses, have two potential mechanisms of O_2 control, one at the level of the vesicle and another at the level of the nodule structure and its physiology. Because the vesicle is common to both free-living *Frankia* and symbiotic *Frankia*, knowledge of the unique structural and physiological properties of the vesicle must be central to this review. Aspects of this subject have been reviewed previously (Benson and Silvester, 1993; Huss-Danell, 1997; Silvester *et al.,* 1990). Here, we summarize and update the information on the role of O_2 in the structure and physiology of *Frankia* and its nodule systems. We also review the occurrence of hemoglobin in nodules and *Frankia* and its function in controlling O_2.

2. VESICLE STRUCTURE AND OXYGEN RESPONSES OF *FRANKIA IN VITRO*

2.1. Vesicle Structure

The *Frankia* vesicle is worthy of developmental study from a basic biology viewpoint because it is a rare example of a prokaryotic structure that is highly specialized in form and function. It holds information on how a prokaryote can accomplish formation of a sphere that contains many cells, is housed in a microaerophilic environment, and is specialized to accomplish the complex function of nitrogen fixation. An extensive review of the structural aspects of *Frankia* in plants and *in vitro* is available (Newcomb and Woods, 1987) and here we summarize the structure of the *Frankia* vesicle with respect to its development and O_2 protection of nitrogenase.

Evidence that *Frankia* vesicles are the site of nitrogen fixation comes from studies showing that: (i) *Frankia* cultures develop vesicles at tips of growing vegetative hyphae when induced to fix N_2 (Fontaine *et al.,* 1984; Torrey and Callaham, 1982); (ii) nitrogenase activity fractionates with these vesicles (Noridge and Benson, 1986); and (iii) the enzyme immunolocalizes specifically in the vesicle (Meesters, 1987).

Vesicle structure is illustrated in Figure 1A-E. The vesicle is always attached to a stalk cell (S) and, in phase-contrast images (Figure 1A), these two structures have in common a phase-bright "halo" relative to vegetative cells (V). This halo is due to birefringence from layers of lipid material that accumulate on these cells, which are stained red with the fluorescent dye Nile Red (Lamont *et al.,* 1988). These built-up layers comprise the vesicle envelope, which is the proposed O_2-diffusion barrier discussed in Section 2.2. Vesicles in cultures of *Frankia* are about 2.5μm in diameter and septate.

Because the component layers of the envelope are in the range of 35-47Å (Berg, 1994; Torrey and Callaham, 1982), the resolving power of transmission electron microscopy is required to analyze their organization in the vesicle envelope (Figure 1B-F). In thin sections of chemically fixed cells, the envelope (Figure 1B, VE) surrounds the vesicle and stalk, and component layers of the envelope can be seen as electron-translucent layers within the envelope (Figure 1D). Chemically fixed specimens show an electron-translucent zone surrounding the vesicle, the "void space" (Figure 1B, VS). This feature has been interpreted either as due to extraction of envelope lipids by dehydration solvents or as a shrinkage artifact resulting from dehydration-induced shrinkage of hydrophilic vesicle cytoplasm relative to rigid lipid-rich envelope. That the void space is due to the latter was shown by Lancelle *et al.* (1985), using thin sections of freeze-substituted *Frankia* vesicles (Figure 1B-E). In parallel samples, the void space was present in chemically fixed sections (Figure 1B) and absent in freeze-substituted *Frankia* vesicles (Figure 1C). The void space forms between the vesicle cell membrane and the envelope, evidently by shrinkage of the cytoplasm of vesicle cells. Lancelle *et al.* (1985) also show that ultra-rapid freezing preserves a number of unusual cytoplasmic inclusions (fibers and tubules) in *Frankia* vesicles that are not preserved by chemical fixation. This is the case for symbiotic vesicles as well and is discussed in Section 3.4, along with their possible role in vesicle development. Of particular interest are bundles of filaments (Figure1D-E, small arrowheads), found only in vesicles and stalks, that associate with granular bodies at the top of the stalk (Figure 1D, large arrowhead), extend longitudinally into the stalk (Figure 1D-E), and terminate at the basal septum (Figure 1E, large arrowhead). In septate vesicles, in culture or in symbiosis, the envelope is significantly thicker in the stalk cell (Figure 1C-E, electron-translucent region of the wall).

The chemical composition of the envelope has been identified as a mixture of hopanoid lipids (Berry *et al.,* 1993), which are pentacyclic triterpenoids. Vesicle envelopes have a specific class of hopanoid, bacteriohopanetetrol phenylacetate (Berry *et al.,* 1993) that reflects the difference of the vesicle envelope structure from

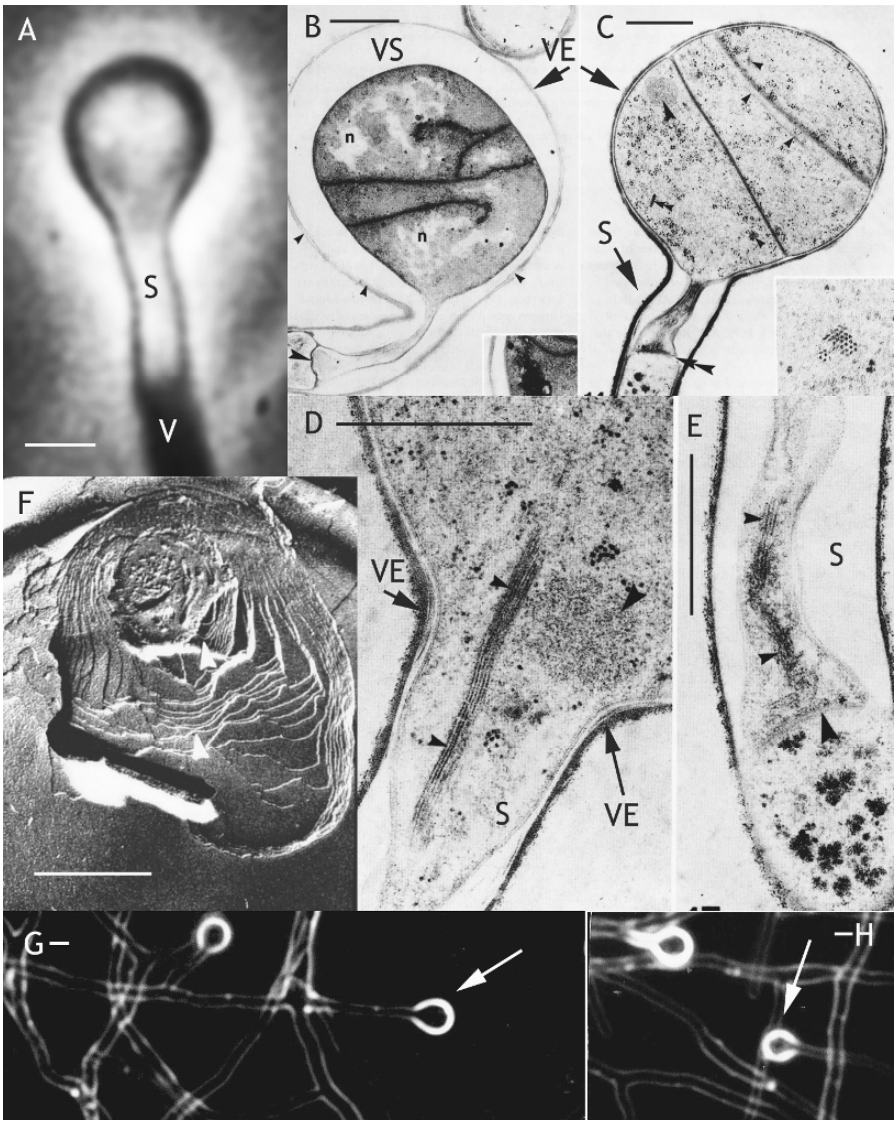

Figure 1. Frankia *vesicle structure.* Frankia *strains HFPCcI3 (A-F) and HFPArI3 (G,H). Marker = 1 µm in A,G,H and 0.5 µm in B-F.*
(A) Phase contrast of the birefringent halo of the envelope surrounding vesicle and stalk (s) (v = vegetative cell). Vesicle ultrastructure, chemically fixed (B) compared to freeze-substituted (C). (D,E) Fiber bundle in freeze-substituted stalk cell. (B-E) reproduced from Lancelle et al. (1985), with permission. (F) envelope lipid layers shown in freeze fracture. (G,H) Dark field envelope birefringence, which is much dimmer at 2kPa O_2 (G) than at 21kPa O_2 (H). (Reproduced from Harris and Silvester (1992), with permission).

the *Frankia* hyphal wall, which has relatively few, loosely organized lipid layers (Berg and McDowell, 1987). The uniform thickness of these layers suggests that they are crystals (of hopanoid), which perhaps results in a sufficient density of molecules to provide diffusive resistance to O_2 diffusion.

In their initial analysis of *Frankia* vesicle ultrastructure, Torrey and Callaham (1982) note the resistance of the envelope to conventional EM stains and report that more oxidative stains, such as provided by fixation in permanganate, improve the imaging of the envelope. Indeed, *en bloc* staining with chromic acid enables analysis of the envelope in thin sections of symbiotic vesicles (see section 3.4.2). Torrey and Callaham (1982) also show that freeze fracture provides an effective means for imaging the envelope, albeit a problematic approach for quantifying numbers of lipid layers because of the need for cross fractures.

2.2. Responses to O_2

2.2.1. Physiological Responses

The re-isolation of *Frankia* (Callaham *et al.,* 1978) made available to the scientific community an organism with many unique and fascinating properties. In total contrast to rhizobia, which are able to express nitrogenase only at extremely low (<0.1kPa) pO_2 (Tjepkema and Evans, 1975), *Frankia* shows maximum nitrogenase activity at atmospheric pO_2 (Gauthier *et al.,* 1981; Tjepkema *et al.,* 1980; 1981). Further, nitrogenase expression in cultured *Frankia* occurs coincidentally with the formation of vesicles (Tjepkema *et al.,* 1980), eliciting an interesting parallel with the heterocyst in cyanobacteria.

The normal site of nitrogenase within the vesicle has been confirmed by a number of approaches. First, when vesicles are separated from their subtending hyphae, nitrogenase activity is found only in the vesicle fraction (Noridge and Benson, 1986; Tisa and Ensign, 1987). Second, nitrogenase has been directly identified in vesicles by immunolocalization studies (Meesters, 1987; Meesters *et al.,* 1987). Third, the correlative work on vesicle induction and nitrogenase activity (Murry *et al.,* 1984a; Tjepkema *et al.,* 1980) all identify the vesicle as the site of nitrogenase and the site of O_2 protection. The above results for air-grown *Frankia* cultures contrast with those for *Frankia* grown at very low pO_2 of 0.1-0.3kPa. At these O_2-limiting conditions, vesicles do not form, but nitrogenase is expressed in the hyphae (Murry *et al.,* 1985).

The hypothesis that the vesicle, as well as being the site of nitrogenase, is also the site of O_2-diffusion limitation and control is further supported by work on the uptake kinetics of O_2 and C_2H_2 by *Frankia* in liquid culture. Isolated nitrogenase has a $K_m(C_2H_2)$ of ca. 0.6kPa, which is the same as for most intact N_2-fixing cells. However, the $K_m(C_2H_2)$ for *Frankia* is ca. 2kPa and does not change when V_{max} is altered by changing the assay pO_2 (Murry *et al.,* 1984a). This result was interpreted as a diffusion-limited system in which the apparent $K_m(C_2H_2)$ is the result of the enzyme kinetics modified by diffusion limitation. In addition, the same authors showed that in ammonia-grown cells, which lack vesicles, the uptake rate for O_2 showed a simple kinetic curve that saturated at 18μM O_2 (K_m = 1μM), whereas

nitroogenase-derepressed cultures (grown on N_2) gave two $K_m(O_2)$ values of 1μM and 170μM. This result adds further support to the conclusion that the vesicle represents a compartment with a significantly greater diffusion resistance than the hyphae filaments (Murry *et al.,* 1984a; Murry *et al.,* 1985).

2.2.2. Adaptation to varying pO_2

Perhaps the greatest insights into O_2 responses have been achieved by studying the structural and physiological responses of *Frankia* cultures to varying pO_2 in the growth media. When *Frankia* $HFPCcI_3$ vesicles were initiated in culture at pO_2 levels of 0.1, 0.3, 0.5, 5 and 20kPa, two important results were observed (Murry *et al.,* 1985; Parsons *et al.,* 1987). First, cultures at low pO_2 (< 0.3kPa) failed to produce vesicles (see above). Second, and most significantly, the pO_2 optimum for nitrogenase activity shifted with the growth pO_2. Thus, cultures grown at 20kPa O_2 had a nitrogenase-activity optimum at near 20kPa O_2, whereas those grown at 5kPa O_2 had a nitrogenase-activity optimum at near 5kPa O_2 (Figure 2). This result demonstrated for the first time that not only is *Frankia* able to express nitrogenase at atmospheric pO_2, it also is able to adapt to a wide range of pO_2 and "….that vesicle formation and the development of the O_2-protection mechanism of nitrogenase are regulated by ambient pO_2…" (Murry *et al.,* 1985).

Although *Frankia* is able to adapt to a wide variety of pO_2, nitrogenase is still sensitive to O_2 outside the ambient ranges and pulses of O_2 have been used to destroy nitrogenase in order to determine recovery rates (Baker and Huss-Danell, 1986; Harris and Silvester, 1992).

The thick walls of the vesicles were first postulated as the site of O_2-diffusion resistance by Torrey and Callaham (1982) and they also showed that the envelope

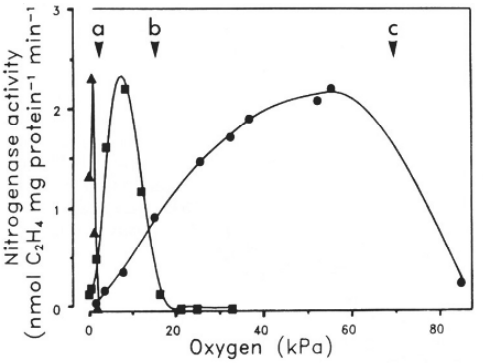

Figure 2. Effect of pO_2 on nitrogenase activity in Frankia *grown at three O_2 levels.* Frankia *was grown at 2, 16 and 70kPa O_2 and assayed for nitrogenase at various pO_2 starting at 0.4kPa. Response curves are for cultures grown at 2kPa, 16kPa and 70kPa. The arrows a, b and c indicate the level of O_2 at which the culture was grown (reproduced from Parsons* et al. *(1987), with permission).*

outside the wall is a multi-laminated structure made up of many layers of lipid. The vesicle envelope shows up as a crystalline array, which responds to polarized light and, under TEM, is shown to be made up of many lipid monolayers.

Assuming that the vesicle envelope is the site of diffusion resistance, Parsons *et al.* (1987) postulated that any adaptation to varying pO_2 must be evident in the properties of that envelope. They showed that the vesicle envelope displayed remarkable bright properties when viewed under dark-field microscopy, where the apparent envelope thickness could be readily viewed and measured (Figure 1G-H). Cultures of HFPCcI$_3$ grown at 2, 16, or 70kPa O_2 grew well and adapted their nitrogenase activity to those levels of O_2 (Figure 2). The vesicle envelopes at 2kPa O_2 were almost transparent under bright field, whereas those at 70kPa O_2 were bright glowing spheres. Apparent thickness varied from 1.38 to 3.17 units over the O_2 range. Most significantly, however, was the change in lipid layers, which ranged from an average of 17 at 4kPa O_2 to an average of 40 at 70kPa O_2.

The adaptive nature of the vesicle envelope has been confirmed by the use of Nile Red fluorescent stain, which is specific for lipid (Lamont *et al.,* 1988) and which showed the fluorescent staining to be coincident with changes in dark-field images of pO_2-adapted cultures.

O_2 protection within a thick-walled cell, such as a vesicle, can only be effective if it is accompanied by an O_2 sink. Support for the dual role of diffusion resistance and respiratory O_2 consumption is provided by the interaction between C substrate and optimum pO_2 for nitrogenase activity. Early work on requirements for heterotrophic growth of *Frankia* showed an absolute requirement for an organic acid, such as malate, fumarate or succinate (Tjepkema *et al.,* 1981). However, Lopez *et al.* (1986) showed that other substrates were useable and that the type of substrate affected the pO_2 optimum of nitrogenase activity. Cells using organic acids, *e.g.*, propionate, had maximum nitrogenase activity at 15-20kPa O_2, whereas cells able to assimilate glucose or trehalose showed lower nitrogenase activity at a pO_2 optimum of 10kPa. The conclusion from these results is that the higher respiration rate associated with organic-acid consumption provides a lower pO_2 and better O_2 control.

Growing *Frankia* in continuous culture has allowed the dynamics of adaptation to be followed (Harris and Silvester, 1992). They grew HFPCcI3 at various pO_2 at dilution rates of 0.100-0.125 day^{-1} and used an O_2 shock to destroy nitrogenase. Although the induction of vesicle formation was shown to be independent of pO_2, both the thickness of the vesicle envelope and the timing of nitrogenase recovery were specifically controlled by the growth pO_2. Thus, a culture, which was grown at 2kPa O_2, shocked with air at 21kPa O_2, and then maintained at 21kPa O_2, took 36h for nitrogenase activity to reappear, whereas a culture returned to 2kPa O_2 after the O_2 shock regained activity within a few hours. The lag in activity was measured as the time required for both existing and new vesicles to achieve sufficient thickness in the envelope.

Prior to the discovery of the vesicle and its properties, the heterocystous cyanobacteria were considered the only genuinely aerobic N_2 fixers capable of

growth in the dark under N_2-fixing conditions (Jensen and Cox, 1983). Now, the full range of *Frankia* species has been added to this class of organisms.

3. RESPONSES IN SYMBIOSIS

3.1. Nodule Structure

Root nodules of legumes and actinorhizal plants show some external similarities but they differ enormously in many of the details both of structure and function. The legume nodule is remarkably similar across many species studied (Bergersen, 1982), having a central zone of infected tissue that is bounded by a tightly packed diffusion-limiting inner cortex (Figure 3). A similar structure occurs in *Parasponia*, the only non-legume genus to have a rhizobial endophyte. In both cases, the infected cells contain high concentrations of hemoglobin and the vascular bundles are outside the infected zone.

In keeping with the diverse taxonomic status of actinorhizal species, the actinorhizal nodule is diverse in both morphology and anatomy and is quite distinct in many respects from the legume nodule (Figure 3). Nodules are bounded by a periderm, which may have either lenticels or, if not, nodule roots attached to the nodule apex as in the genera *Casuarina*, *Datisca*, *Myrica*, *Comptonia* and *Gymnostoma*. These roots have large air spaces and likely function in O_2 transport to the nodules (Bond, 1952; Tjepkema, 1978). The infected cells are variously disposed in actinorhizal nodules (Figure 3) and the disposition of lenticels and nodule roots and the situation of vascular bundles either amongst or within the infected cortex provides preliminary evidence of well ventilated nodules.

3.2. Early Physiological Studies

Early physiological studies on actinorhizal nodules were pioneered by George Bond and colleagues at Glasgow University, mostly using detached nodules and a ^{15}N tracer as an assay for nitrogenase. Using detached *Alnus glutinosa* nodules, which were exposed to 1, 5, 12 or 21kPa O_2 in the presence of $^{15}N_2$, Bond and MacConnell (1955) showed a proportionate reduction in $^{15}N_2$ uptake at the lower pO_2 and, with parallel respiration studies, suggested that respiration and N_2 fixation were strongly linked. Similar experiments with *Myrica gale* nodules (Bond, 1957) showed little reduction in $^{15}N_2$ uptake down to 5kPa O_2, but complete loss of N_2-fixation activity at 1kPa O_2.

This work was extended to four actinorhizal species (Bond, 1961) and showed that optimal N_2 fixation occurs at 12-25kPa O_2, depending on species, and that there is a steep decline in activity at higher pO_2. Bond concluded that the relationship between pO_2 and nitrogenase activity at the lower values of the pO_2 curve was due to the requirement for respiration to drive nitrogenase. On the other hand, with considerable prescience, he concluded that the inhibition of nitrogenase activity at higher pO_2 was a direct effect of O_2 on nitrogenase. By the 1970s, a number of other experiments, with many actinorhizal species, had confirmed the optimal range

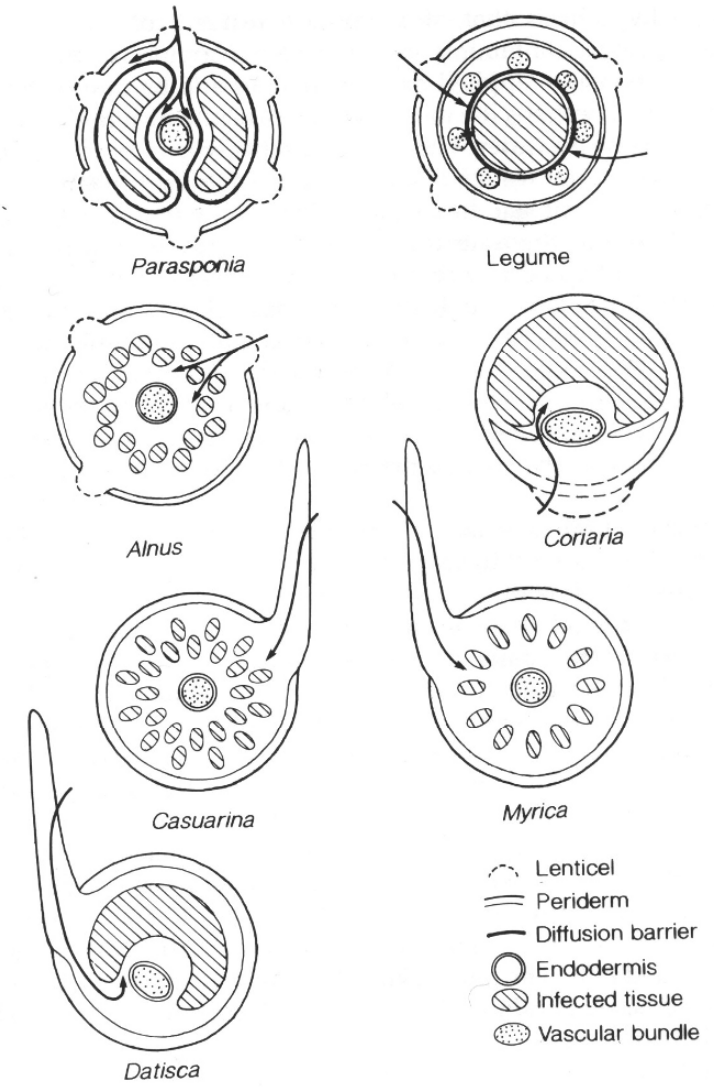

Figure 3. Diagrammatic representation of nodule structure demonstrating the main types. Arrows represent putative major O_2-diffusion pathways (reproduced from Silvester et al. *(1990), with permission).*

for nitrogenase activity as 12-25kPa O_2 (Fessenden *et al.,* 1973; Waughman, 1972; Wheeler *et al.,* 1979) and also concluded, despite the limitations of the available techniques, that the sensitivity to O_2 could not be entirely explained by O_2 needed

for respiration and that there was an apparent nitrogenase-specific O_2 inhibition in actinorhizal nodules.

Growth of whole plants with root systems exposed to a range of pO_2 (MacConnell, 1959) showed that growth of *Alnus glutinosa* is reduced by 50% at 5kPa O_2, whereas the growth of unnodulated plants was reduced by only 15%. This species has been shown to have a thermo-osmotically mediated gas-transport system from shoots to roots (Schröder, 1989) and thus, the result emphasizes the importance of O_2 to fuel the energy needs of nitrogenase.

An unusual method of supplying O_2 to the nodule is seen in *Myrica gale*, which like *Alnus glutinosa*, is adapted to wet soils with very low pO_2. *Myrica gale* produces upward–growing roots at the tips of its nodule lobes. These nodule roots enhance nitrogenase activity by enhancing O_2 uptake under flooded conditions (Tjepkema, 1978). *Myrica gale* nodules are also able to resume nitrogenase activity in spring under flooded conditions but at reduced rates (Schwintzer, 1985).

Two major advances allowed rapid progress in the understanding of many aspects of *Frankia* physiology. First, the advent of the C_2H_2-reduction assay, initially used on actinorhizal nodules by Stewart *et al.* (1967), and second, the re-isolation of *Frankia* as a pure culture (Callaham *et al.,* 1978).

3.3. Comparisons with Legume Nodules and the Internal O_2 Environment

Comparisons with legume nodules emphasize that, although the structure and biochemistry of the nitrogenase enzyme system is identical, the physiology of nitrogenase and nodule structure are very different for *Frankia*. These essential differences center on the ventilation and, thus, the gas exchange of the two nodule types. An O_2 microelectrode inserted into a legume nodule indicates a dramatic drop in pO_2 in the inner cortex and shows the center bacteroid zone to be at very low pO_2 (Tjepkema, 1979; Tjepkema and Yocum, 1974). Similarly, vacuum infiltration of India ink into nodules confirms that there is a distinct barrier to diffusion in the inner cortex of legume nodules, which has been identified as a layer of compact cells lacking intercellular spaces (Tjepkema and Yocum, 1974). Although all legume nodules studied show the above structure and physiology, the actinorhizal nodule shows a wide variety of structures and responses (Figure 3).

O_2-microelectrode analysis and India-ink infiltration show that the actinorhizal nodules of *Alnus*, *Datisca* and *Coriaria* (Silvester and Harris, 1989; Tjepkema, 1979; Tjepkema *et al.,* 1988a) are well aerated with a continuous air spaces leading from lenticels in the surrounding periderm to the surfaces of infected cells. *Myrica* nodules are somewhat intermediate in having pockets of infected cells, which show low pO_2 are not penetrated by India ink, and are embedded in a well-aerated cortex (Tjepkema, 1979; Tjepkema, 1983a). In strong contrast, the nodules of *Casuarina* possess a strong discontinuity and the groups of infected cells are in a low O_2 environment. Dye infiltration (Zeng *et al.,* 1989) and electron micrograph studies (Berg and McDowell, 1987) confirm that the infected cells of *Casuarina* are surrounded by a dense layer of cortical cells and are not in contact with air.

Further weight to the distinction between legume and actinorhizal nodules is provided by their contrasting temperature/O_2 interactions. Legume nodules show a low Q_{10} for respiration and for nitrogenase activity (Tjepkema and Yocum, 1973; Waughman, 1977), which has been interpreted as the center of the nodule being essentially diffusion limited due to the inner cortical diffusion barrier. In contrast, nitrogenase activity in *Alnus* nodules is strongly temperature limited (Waughman, 1972) and O_2 consumption at 5 and 20kPa O_2 shows a Q_{10} of approximately 2.0 (Tjepkema, 1979).

So, in contrast to legume nodules, actinorhizal nodules, with the exception of *Casuarina,* are relatively well ventilated, apparently lack significant diffusion boundaries, show a high Q_{10} for respiration and nitrogenase activity, and in keeping with a well-ventilated system, show an inhibition of nitrogenase activity at pO_2 values above ambient.

3.4. The Vesicle in Symbiosis

In symbiosis with plants, *Frankia* in most cases fix N_2 in vesicle structures equivalent to those formed *in vitro*. The story becomes even more interesting because vesicle structure is different in different hosts, the apparent result of the environment unique to each host's symbiotic cell. For example, the same *Frankia* strain forms typical alder-type vesicles in alder and typical *Myrica*-type club-shaped vesicles in the *Myrica* relative, *Comptonia* (Lalonde, 1979). Here, we summarize vesicle structure in different plant hosts as represented by five different classes of N_2-fixing *Frankia* structures (Torrey, 1985). In all cases, these are in symbiotic host cells, which are cortical cells of modified lateral roots. For more details, the reader is referred to an excellent review (Newcomb and Wood, 1987). We adopt their terminology in that vesicles formed during symbiosis are termed *symbiotic vesicles*, whereas those formed *in vitro* are termed *Frankia vesicles*.

3.4.1. Symbiotic Vesicle Structure and Development - Alder Symbiotic Vesicles

Considered the classic example of the symbiotic vesicle, the alder-type has a form that is the closest to that of the *Frankia* vesicle, which is a septate (multi-cellular) sphere with subtending stalk (Figure 4). This vesicle type is found in a variety of actinorhizal plants (Newcomb and Wood, 1987), including members of *Alnus*, the Elaeagnaceae, and some members of the Rhamnaceae. This vesicle is about 4-5µm in diameter compared to a diameter of about 2.5 µm for vesicles in culture. As is universal with *Frankia*, the vesicle forms at the tips of hyphae. The *Frankia* hyphae enter a plant cell *via* infection threads (Berg *et al.,* 1999) and, in alder, these invasive hyphae branch and proliferate in the cell center in the form of vegetative hyphae (Figure 4A; v). Vesicles differentiate in the cell cortex (Figure 4A; sv) to produce a large number in the periphery of a cortical cell of the nodule. Interspersed with the infected cells are uninfected cells (Figure 4A; uc) that contain amyloplasts.

The alder-type vesicle is highly septate (Figure 4B-C), with some septa forming complete cross walls (Figure 4C; double asterisk) and others terminating in the

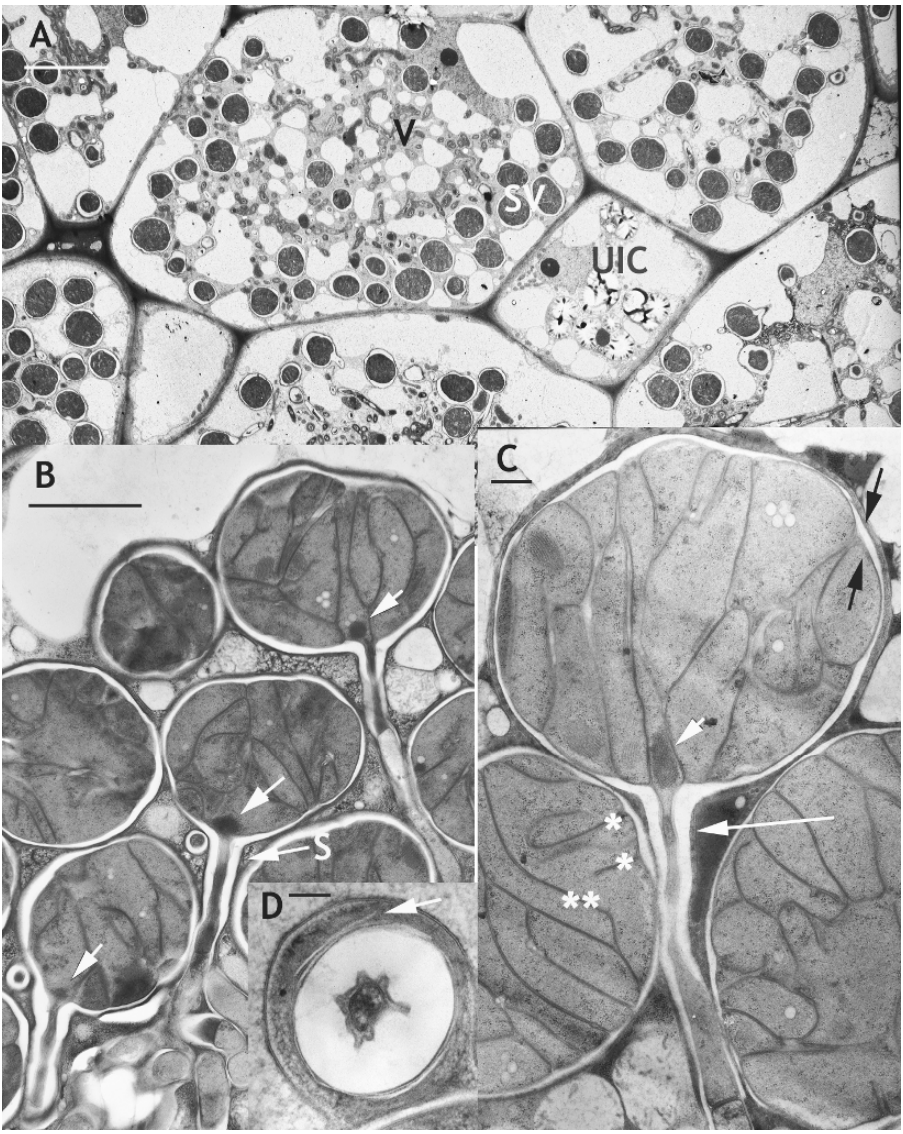

Figure 4. Ultrastructure of the alder-type vesicle, in Alnus serrulata
(A, chemically fixed, B-D freeze substituted;
marker = 10 µm in A, 2 µm in B, 0.5 µm in C, and 0.1 µm in D).
(A) Vegetative hyphae (V) proliferate at cell center, symbiotic vesicles (SV) differentiate at cell periphery, UIC = uninfected cell. (B) Stalk cell (S) joins vesicle near granular bodies (arrows), envelope is electron-translucent and thickest on stalk. (C) Complete (double-) and incomplete (single-asterisks) septa; envelope is uneven at septa-surface junctions (opposing arrows). (D) Stalk envelope in cross section.
(From Berg et al. *(1999) [A]; 1994 [B,D]; and Berry (1994) [C], with permission).*

cytoplasm (Figure 4C; single asterisk). The former make the vesicle multi-cellular and the latter, in effect, increase the surface area of vesicle cell membranes in perhaps an adaptation that increases available sites for membrane electron transport and energy production for nitrogen fixation. Immunolabeling shows that nitrogenase appears in alder-type vesicles only after septa are formed (Huss-Danell and Bergman, 1990), with the highest immunolabeling density only after full development of vesicle septation. By this stage in vesicle development, the vesicle envelope is fully developed, providing a diffusion barrier for protecting nitrogenase from O_2 inactivation.

The structure of the envelope is discussed below, where it is explained that the envelope can be imaged as an electron-translucent halo when tissue is prepared by freeze substitution (see also Section 2.1.2 for the structure of the vesicle *in vitro*). In Figure 4B-C, high pressure-frozen and freeze-substituted tissue alder vesicles are medially sectioned to show the envelope structure on both vesicle and subtending stalk (S). The envelope halo sometimes varies in thickness around the vesicle (Figure 4C), with the greatest number of lipid layers at septa junctions with the vesicle surface (Figure 4C, opposing arrows), which may result from accumulation of envelope lipid diffusing from septal membrane sites of synthesis (Berg, 1994).

Stalk cells are remarkable for the amount of lipid layers in their envelope (Figure 4B-D), especially at junctions with the vesicle (Figure 4C, arrow). This accumulation constricts stalk-cell cytoplasm to a small channel (Figure 4D, arrow indicates loosely associated surface lipid layer). This accumulation might act to reduce O_2 diffus ion from vegetative cells, which have very few lipid layers and therefore likely a higher pO_2.

Another interesting feature of stalk cells is the inclusions seen in freeze-substituted cells. Bundles of fibers, terminating at the basal septum of the stalk cell (Figure 5A, s and arrow; Figure 5B, asterisk) extend longitudinally along the stalk, terminating where stalk cytoplasm joins vesicle cell cytoplasm. Always adjacent to this terminus in the vesicle is a granular body (Figure 5A, asterisk; see also Figure 1D-E). Because the fibers always associate with the granular body (Figure 4B-C, arrowheads), it is likely that together they have an important purpose in either vesicle development or function. As is the case for vesicles *in vitro* (Lancelle *et al.,* 1985), fibers in symbiosis are only found in stalks or vesicles and the cytoplasm of the stalk is continuous with vesicle-cell cytoplasm. In the early stage of vesicle differentiation before septation, a fibrous body appears in the center of the vesicle (Figure 5C; Berg, 1994) and is oriented longitudinally to the stalk axis. In some cells of nearly mature vesicles, bundles of fibers traverse the vesicle-cell cytoplasm (Figure 6A) and some of these clearly bind the vesicle-cell septal membrane (Figure 6B, arrow). The possibility exists, then, that these fibers are involved in vesicle morphogenesis and bind developing septal walls and stalk septum or other anchors. Perhaps related to this situation, it is not unusual to find vesicle septa terminating at the neck of the stalk, associated with the stalk fiber bundle (Figure 4B-C; Figure 5A, arrowhead). These fibers might function as contractile proteins in vesicle-cell division and morphogenesis, and perhaps hold the key to understanding how *Frankia* accomplishes the feat of building a spherical, multi-cellular prokaryotic structure.

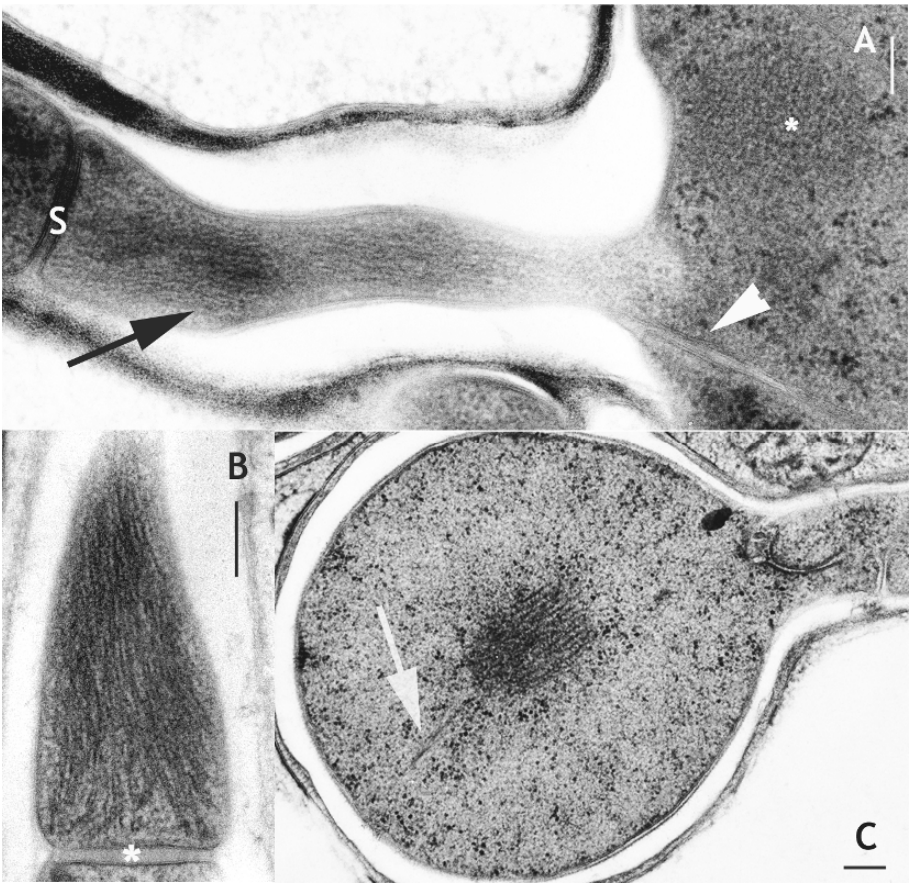

Figure 5. Stalk ultrastructure in high pressure-frozen/freeze-substituted Alnus serrulata. *Markers = 0.2 µm. (A) Stalk cell longitudinal fiber bundle (arrow) terminates at the basal end near the stalk cell septum (S) and at the apical end where stalk cell cytoplasm merges with that of vesicle cells, always associating with a granular body (asterisk, also see arrows in Figure 5B-C). Vesicle septa commonly terminate near apex of stalk cell (arrowhead). Stalk cell envelope (electron-translucent surface) is thickest at vesicle juncture, tapering towards stalk basal septum. (B) Fibers terminate at septum and at lateral walls of stalk cell (reproduced from Berg, 1994, with permission). (C) Early in vesicle differentiation a fibrous body in vesicle center orients longitudinally with the stalk axis, in this case with fibers extending toward the vesicle cell wall (arrow).*

When molecular approaches to understanding *Frankia* biology become available, it will be worthwhile to investigate whether these fibers are homologous to the bacterial protein FtsZ, a tubulin homolog that forms fiber arrays that contract to carry out bacterial cell division (Bramhill, 1997).

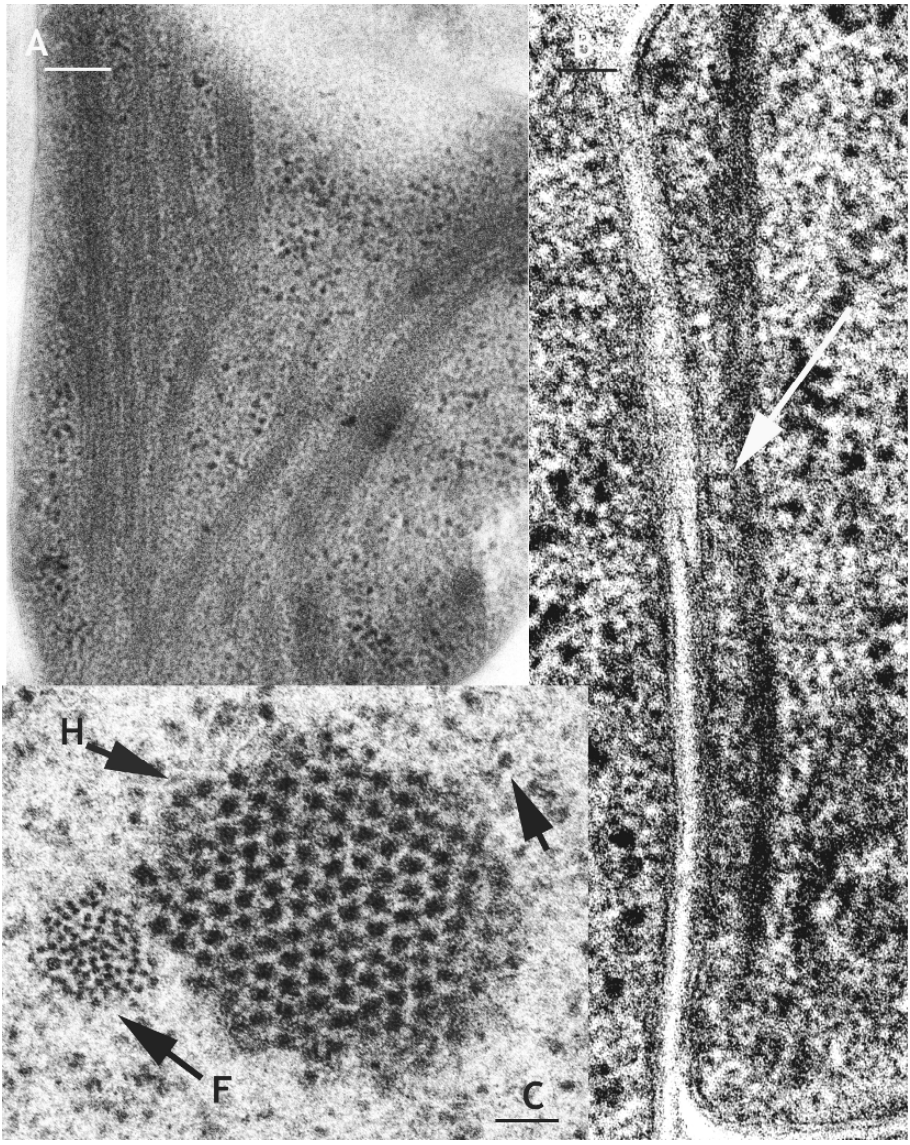

Figure 6. Cytoplasmic inclusions in high pressure-frozen/freeze-substituted alder-type vesicles (Alnus serrulata)*. Markers = 100 nm.*
(A), 50 nm (B,C). A number of fiber bundles traverse this vesicle cell cytoplasm. (Reproduced from Berg (1994), with permission).
(B) A single fiber bundle with several cross-links (e.g., arrow) to a septum membrane.
(C) Hexagonal inclusion (H) of particles which also are present free in vesicle cytoplasm (arrow). Fiber bundle cross section (F).

An abundant inclusion within vesicles is a hexagonal crystal (Figure 6C, h) that is formed from particles, which are also found free in the vesicle-cell cytoplasm (Figure 6C, unlabeled arrow). These associate with, and are sometimes bound to, septa (Berg, 1994). Their function is unknown, but they may be an excess of the free particles, which could be some sort of macromolecular complex. The smaller inclusion in Figure 6C is a cross section of a fiber bundle (also present in vesicles *in vitro*; Lancelle *et al.,* 1985).

3.4.2. Envelope Ultrastructure - Alder Symbiotic Vesicles

The symbiotic vesicle envelope, like the envelope of vesicles *in vitro*, does not stain with conventional stains for electron microscopy and instead produces an electron-translucent halo, the "void space", that is both shrinkage artifact and unstained envelope. Using chromic-acid fixation, it is possible to contrast lipid layers of the envelope in symbiotic vesicles *in vivo* (Berg, 1994), so providing a means both for quantitative analysis of envelope ultrastructure and for evaluating the void space artifact. The vesicle in Figure 7A tells the story. The envelope is stained with chromic acid (arrows in Figure 7A-B point to the same region), showing it is retained after processing in dehydration solvents. A prominent void space (Figure 7A, vs) is an artifact due to shrinkage of vesicle cytoplasm away from the envelope during solvent dehydration, which occurs in relatively big increments in chemical-fixation protocols. As is the case for vesicles *in vitro* (Lancelle *et al.,* 1985, see Section 2.1.2), freeze substitution eliminates the shrinkage artifact and allows ultrastructural analysis of the envelope as an electron-translucent halo. This effect is clearly shown by a comparison of the alder symbiotic-vesicle envelope stained by chromic-acid fixation (Figure 7C) with that imaged by high pressure-freezing/freeze-substitution (Figure 7D). Occasionally, individual lipid layers of the envelope can be discerned in freeze-substituted material, as shown for the envelope of *Myrica cerifera* in Figure 7E.

Quantitative analysis by either counting the number of chromic acid-stained layers or by measuring envelope thickness in freeze-substituted samples gave similar results (Berg, 1994). For *Alnus serrulata*, the vesicle envelope averages about 65nm thick or about 14 lipid layers. For *Elaeagnus umbellata* (alder-type vesicle), the envelope is about 39nm thick or about nine layers. These are statistically significant differences in thickness and likely reflect differences in nodule aeration. The morphology of the envelope is similar in these two hosts, with the stalk envelope having the greatest number of layers (about 40 in *A. serrulata* and 30 in *E. umbellata*).

3.4.3. Symbiotic Vesicle Structure and Development - Ceanothus *Symbiotic Vesicles*

The symbiotic vesicle in *Ceanothus* and in members of the Rosaceae (the *Ceanothus*-type) is pear-shaped, non-septate, and lacks a stalk cell (Figure 8B). In *Ceanothus*, the vesicle diameter is about 2μm and, in the other symbioses, this diameter varies up to 4μm (Newcomb and Wood, 1987). In *Ceanothus*, as in the alders, vesicles form at the periphery of the host cell (Figure 8A, sv, v = vegetative

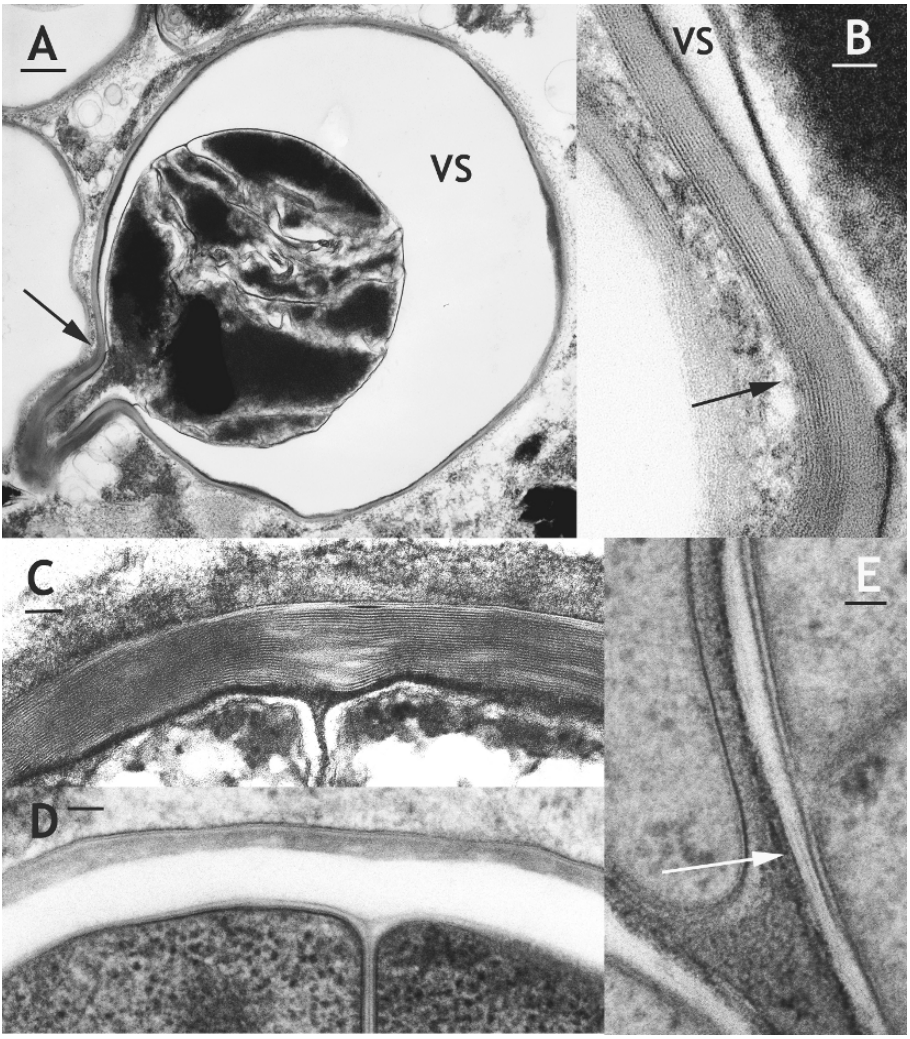

Figure 7. Cytochemistry of the vesicle envelope.
Markers = 0.5 µm (A), 50 nm (B-E).
(A,B) Chromic acid-fixed symbiotic vesicle in Elaeagnus umbellata*, showing the void space artifact (VS) relative to the envelope, which is stained (e.g., arrow, pointing to the same in both micrographs).*
(C,D) comparison of envelope structure stained with chromic acid (C) or imaged as an electron-translucent region free of the void space artifact, in freeze-substituted material (D); Alnus serrulata*, reproduced from Berg, 1994, with permission.*
(E) Vesicle envelope in freeze-substituted Myrica cerifera*—verification that individual lipid layers survive dehydration solvents (arrow).*

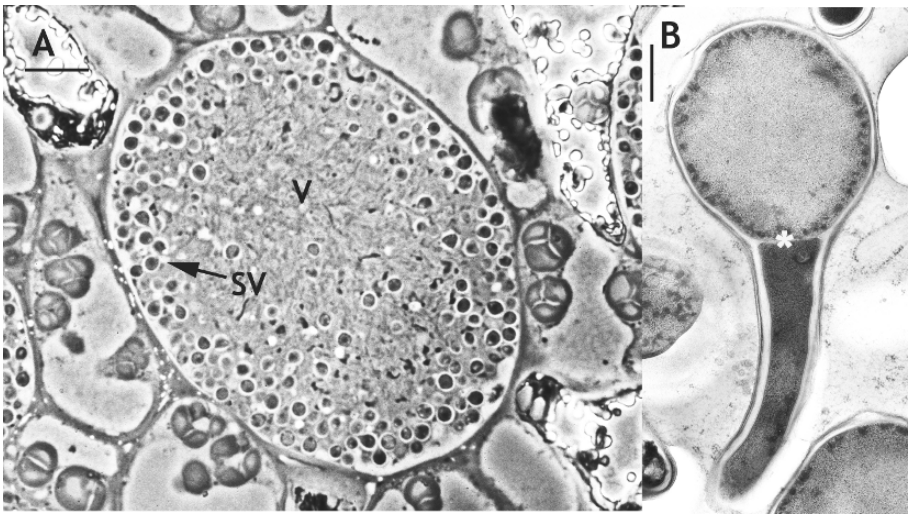

Figure 8. The Ceanothus*-type vesicle, in* Ceanothus americanus*.*
Markers = 10 µm (A), 0.5 µm (B).
(A) In the large infected cells of Ceanothus*, vegetative hyphae (V) proliferate in cell center and symbiotic vesicles (SV) differentiate at cell periphery.*
(B) The Ceanothus*-type vesicle is pear-shaped and unicellular, being separated from vegetative hypha by a septum (asterisk) (reproduced from Berg (1994) with permission).*

hyphae), which is much larger than symbiotic cells of alder. The vesicle is separated from the vegetative hypha by a septum (Figure 8B, asterisk). This vesicle type differs from the alder-type in being smaller and single-celled. Correlated with the lack of septum formation and stalk cell, there are neither fiber inclusions nor hexagonal bodies, and the envelope is uniform in thickness (Figure 8B) because there are no septa junctions with the vesicle surface. Envelope thickness in *C. americanus* (Berg, 1994) measures 43nm (about 8 lipid layers) with no significant increase in thickness in the adjacent hyphal cell, unlike a stalk cell. This value is not statistically different from the vesicle envelope thickness in *Elaeagnus umbellata* measured in that study.

3.4.4. Symbiotic Vesicle Structure and Development - Myrica *Symbiotic Vesicles*
The *Myrica*-type vesicle (in *Comptonia* and *Myrica*) departs from the spherical motif by being club-shaped and elongate (Figure 9). They can extend over a third of the length of the host cell (Figure 9A, arrows), differentiating peripherally (towards one side of the cell) from vegetative hyphae at cell center (Figure 9A, v). They gradually increase in diameter towards their tip, which is about 2µm in diameter (Figure 9D). There are disorganized septa (Figure 9D, arrowhead), some that join with the vesicle surface and, as in the alder-type vesicle, in these regions the envelope is usually thicker (Figure 9D, arrow). There are cross-wall septa (Figure 9D, asterisk), but there is no stalk cell. There is an inclusion, the “striated

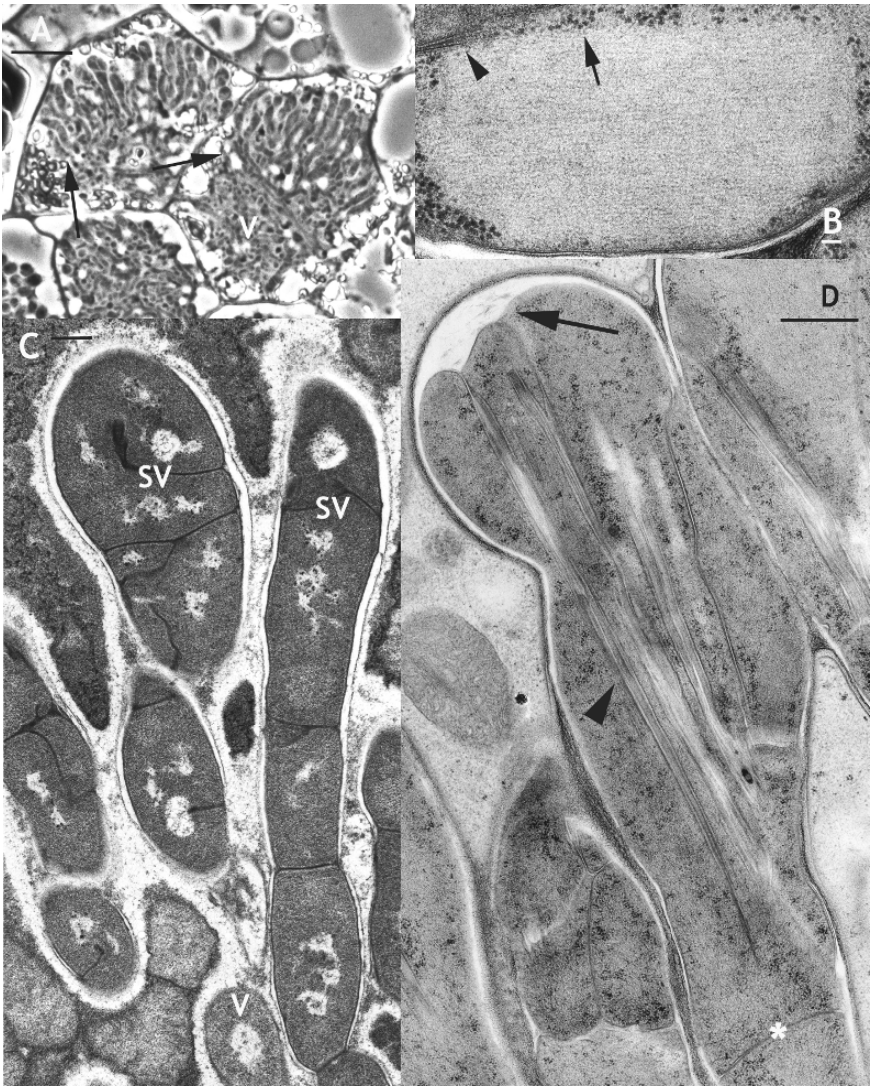

Figure 9. The Myrica*-type vesicle, in* Myrica cerifera*.*
Markers = 10 µm (A), 100 nm (B), and 0.5 µm (C,D).
(B,D) freeze-substituted, (C) permanganate-fixed.
(A) Club-shaped vesicles (arrows) differentiate on one side of the cell relative to vegetative hyphae (V). (B) A striated body inclusion in the vesicle associates with septum (arrowhead) and particles (arrow) in vesicle cytoplasm. (C) Vesicle envelope thickness increases from very little on vegetative hyphae (V) to its greatest thickness at vesicle apex (SV).
(D) Complexes of septa align axially in the vesicle (arrowhead), which also contains a cross septum (asterisk). Envelope is thickest where septa junction its surface (arrow).
(D) Reproduced from Berg (1994) with permission.

body" (Figure 9B), that may be homologous to the fibers or hexagonal crystals of the alder-type vesicle. The striated body sometimes binds septa (Figure 9B, arrowhead) and particles, which are similar in structure to those that bind hexagonal bodies in alder (Figure 7H), bind its surface (Figure 9B, arrow). Based on the likely involvement of alder-type inclusions in septum formation, one would expect similar inclusions in this septa-forming vesicle. There might be a causal relationship between formation of longitudinal arrays of septa and the club shape. The envelope is about 64nm thick in non-thickened (*i.e.*, not at septa) regions, corresponding to about 6 lipid layers (Berg, 1994); both values are not statistically different from the envelope in alder-type vesicles but significantly higher than the envelope thickness in *Elaeagnus*. When nodules are fixed in permanganate, the envelope is an electron-translucent halo in contrast to the heavily oxidized tissue components and shows a gradual envelope thickening from little on the vegetative hyphae (Figure 9C, v) to maximum thickness in the tip region of the vesicles (Figure 9C, sv).

3.4.5. Symbiotic Vesicle Structure and Development – Casuarina, a Special Case

Symbiotic vesicles do not form in *Casuarina* and *Frankia*, in this case, fixes N_2 while in the hyphal form. Figure 10B is an image of a 1µm-thick section (using a high voltage EM) clearly showing that only hyphal filaments exist in mature infected cells of *Casuarina* and are distributed evenly throughout the cell (Berg and McDowell 1987; 1988). A second remarkable feature is that these N_2-fixing hyphae are non-septate (Berg and McDowell, 1987) and are separated from invasive hyphae by a septum. A third unusual feature is that walls of infected cells in *Casuarina* become lignified in response to infection (Berg and McDowell, 1988) and can be stained with the lignin stain phloroglucinol (Figure 10A, longitudinal section, files of infected cells marked with asterisks, x = xylem). As discussed below (Section 3.5.), by modifying the physical characteristics of the wall, this lignification may have a role in suppressing vesicle formation during nitrogen fixation.

3.4.5. Symbiotic Vesicle Structure and Development – Datisca/Coriaria Vesicle

Two features distinguish this vesicle type; its location and structure. In contrast to other actinorhizal symbioses, in *Datisca* and *Coriaria*, the vegetative hyphae proliferate at the cell periphery (Figure 10C, v) and symbiotic vesicles differentiate near the cell center (Figure 10C, sv) and "point" at the intact vacuole in the cell center (C, vac) to form a hollow sphere of tightly packed vesicles (Berg *et al.,* 1999; Newcomb and Pankhurst, 1982). The vesicle is essentially a single-cell rod-shaped hypha with the diameter of vegetative hyphae (about 1µm) and 3-4µm in length. The envelope is no different in thickness than that of vegetative hyphae (Silvester *et al.,* 1999). An unusual feature of the sphere of symbiotic vesicles is that a layer of mitochondria covers them (Hafeez *et al.,* 1984a; Mirza *et al.,* 1994; Silvester *et al.,* 1999). The implications of this arrangement in O_2 protection are discussed below. The confocal micrograph in Figure 10D is of living *Coriaria* nodule cells stained with rhodamine 123, which loads into mitochondria, and illustrates the ring of mitochondria (arrow) surrounding the bases of the sphere of symbiotic vesicles.

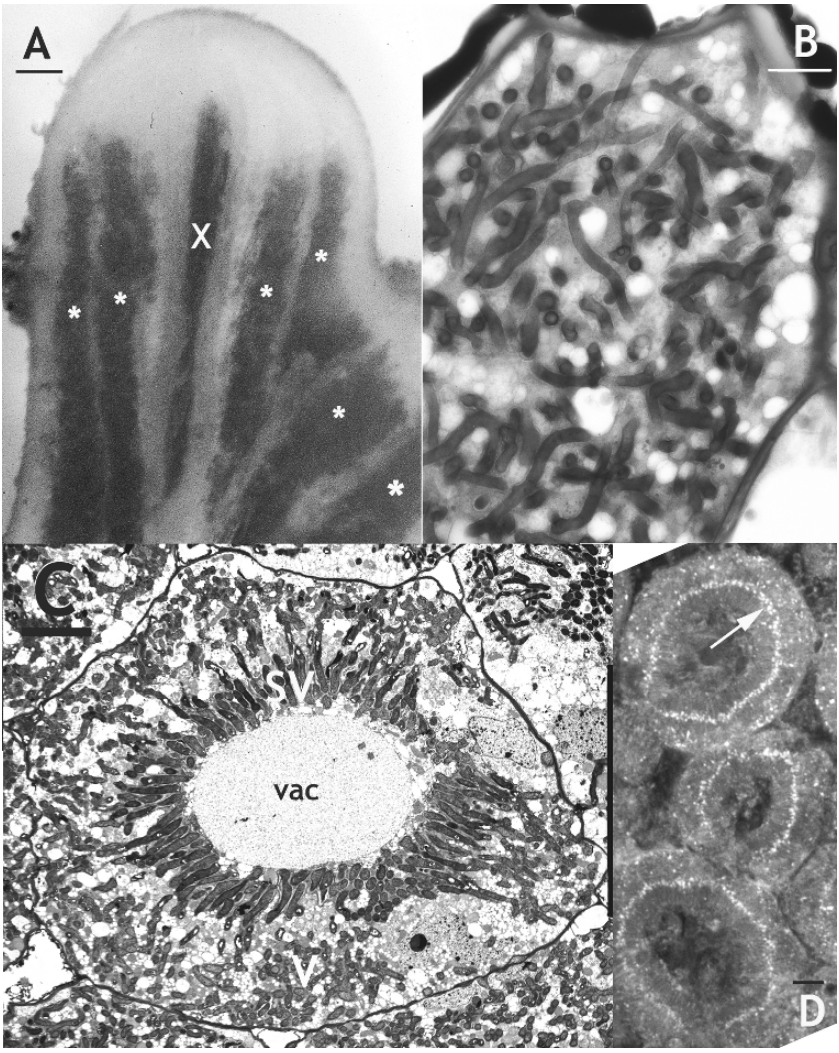

Figure 10. Special adaptations in Casuarina *(A,B) and* Datisca/Coriaria *nodule types (C,D). Marker = 100 µm (A), 5 µm (B-D).*
(A) Walls of infected cells in Casuarina *become lignified, shown here by staining with phloroglucinol (asterisks mark longitudinal files of infected cells, x = xylem). (B) Hyphae fix nitrogen in symbiotic cells of* Casuarina *(high voltage EM micrograph of 1 µm thick section). (C) Symbiotic cell in* Datisca *showing "reversed" anatomy: vegetative hyphae (V) at cell cortex and tightly-packed sphere of rod-shaped vesicles (SV) pointing to the central vacuole (vac). (D) Blanket of mitochondria (arrow) covers the sphere of vesicles; confocal micrograph of live tissue stained with rhodamine 123* (Coriaria).
Reproduced (C) from Berg et al. *(1999), and (D) from Silvester* et al. *(1999), with permission.*

3.5. Responses to O_2

It is tempting to seek one simple model for O_2 protection in actinorhizal nodules as found in legume nodules. However, unlike the legume nodule, in which O_2 protection is provided solely by the nodule, we would anticipate that at least some part of the O_2-protection system in actinorhizal nodules is in the *Frankia* vesicles themselves as well as in the nodule. Added to this complication is the variation in nodule structure as determined by the wide variety of host families and genera of actinorhizas. Thus, at one end of a spectrum, in the classical *Alnus* nodule, normal *Frankia* vesicles occur and are organized within infected cells as clusters that face outwards. In this nodule type, we might predict that much of the O_2 protection resides in the vesicle. At the other extreme is the *Casuarina* nodule in which *Frankia*, though forming good vesicles in culture, lacks vesicles in symbioses. By inference from work on *Frankia in vitro*, the lack of vesicles indicates a very low pO_2 environment within the nodule. Intermediate conditions are seen in nodules of *Myrica*, *Coriaria* and *Datisca* in which club-shaped vesicles are produced, either centrifugally in *Myrica* or centripetally in *Coriaria* and *Datisca*.

With such a wide range of nodule anatomy and *Frankia* differentiation, it is difficult to generalize about O_2 responses and associated physiologies and so the different types of nodules are considered separately. Given the range of physiology, it is surprising that the energy cost of nitrogenase, measured as CO_2 evolved per unit of C_2H_4 production, neither varies significantly across five actinorhizal genera nor between these genera and eight legume genera (Tjepkema and Winship, 1980).

The O_2 responses are of three distinct varieties. First, they may be the immediate effect of different pO_2 levels applied to unadapted (or air-grown) nodules. Second, they may involve short-term adaptation (minutes-hours) to a changed pO_2 in which case physiological adaptation is implied. Finally, they may involve longer-term application of altered pO_2, which brings about both structural and physiological adaptation to the altered environment. The short-term effects are considered prior to describing the major types of response by the plants.

3.5.1. Short-term Effects of Acetylene and O_2 on Nitrogenase Activity

Root nodules of actinorhizal plants, whether attached or excised, show two important short-term effects (transients) that are fundamental to understanding O_2 effects. First is the so-called C_2H_2-induced decline, which was first observed in legume nodules (Minchin *et al.,* 1983) and is also apparent to varying degrees in actinorhizal nodules (Rosendahl and Huss-Danell, 1988; Silvester and Harris, 1989; Tjepkema *et al.,* 1988b; see section *3.5.2.*). This effect arises with some plants when C_2H_2 reduction is used to measure nitrogenase activity. The second effect is the transient decrease in nitrogenase activity in response to rapid small shifts in pO_2 during nitrogenase assays (Silvester and Winship, 1990; see section *3.5.3.*).

3.5.2. Acetylene-induced Decline in Nitrogenase Activity

The effect of adding C_2H_2 or removing N_2 (replaced by Ar) on legume nodules is to set in motion a chain of activities that causes a rapid decline in electron transfer

through nitrogenase. This phenomenon, termed the C_2H_2-induced decline, is well documented for many, but not all, legumes (Minchin *et al.,* 1983; Witty *et al.,* 1984). In some cases, there may be a partial recovery of activity (Schuller *et al.,* 1988), but full recovery has not been recorded in legumes. Both C_2H_2- and Ar-induced declines occur only in those legume nodules that show a tolerance to O_2 levels above ambient; nodules that are not well protected against high pO_2 show no decline (Witty *et al.,* 1984). The decline phenomenon, which is also initiated by root disturbance, moisture stress, and defoliation (Durand *et al.,* 1987; Hartwig *et al.,* 1987; Minchin *et al.,* 1986) has been explained as a sequence of events that starts with the cessation of ammonia production and triggers a rapid increase in the diffusion resistance of the nodule (Witty *et al.,* 1984). Those nodules, which are already highly limited for O_2, then show a rapid decline in nitrogenase activity, which can be reversed by increasing the pO_2 of the assay.

Actinorhizal nodules show a similar decline but, in keeping with the wide range of nodule structures and *Frankia* morphologies, there is a wide variety of resulting effects. The decline does not occur with *Frankia in vitro* as shown by fast-flow C_2H_2-reduction assays in liquid culture on two species of *Frankia*, which were suspended on either nylon mesh or filter paper, where no decrease in C_2H_4 production occurred over a 12-minute assay period (Silvester and Winship, 1990). Thus, the conclusion must be that the decline is a nodule effect and the mechanism sought, as in the legume nodule, is in structures outside the symbiotic vesicle.

A variety of actinorhizal species have been investigated for C_2H_2-induced decline but, in contrast to legumes, the decline is often followed by either a partial or full recovery (Silvester and Winship, 1990; Tjepkema *et al.,* 1988b). The magnitude of the decline and its subsequent recovery is dependent on the host species, growth conditions, and plant age.

The extent of the problem is illustrated by the work on C_2H_2 decline in several species of *Alnus*. *Alnus incana* spp. *rugosa*, when grown aeroponically in the laboratory, showed a marked decline and normally a complete recovery within 20 minutes (Silvester and Winship, 1990). The same species taken from the field showed little or no recovery (Schwintzer and Tjepkema, 1997), however when grown hydroponically, the plants recovered slowly but only partially. The related European species *Alnus incana* spp. *incana* showed no recovery (Rosendahl and Huss-Danell, 1988), whereas *Alnus rubra* showed full recovery (Tjepkema *et al.,* 1988b). *Elaeagnus*, a genus with similar nodule anatomy to *Alnus*, exhibited a decline with partial recovery (Johnson *et al.,* 1997). Furthermore, in *Casuarina cunninghamiana*, both a large decline and full recovery (Tjepkema and Murry, 1989) and a very small decline with minimum recovery (Silvester and Winship, 1990; Figure 11) have been reported.

In contrast to the above observations, the performance of *Coriaria* and *Datisca* is unequivocal. These genera, from separate but related families, have very similar nodule anatomy and display large C_2H_2- and Ar-induced declines with no recovery (Harris and Silvester, 1994; Tjepkema, 1997; Tjepkema *et al.,* 1988b).

Without doubt, the most intensively studied genus is *Myrica*. In unstressed laboratory-grown plants, nitrogenase in *Myrica* typically shows a marked C_2H_2-induced decline with a recovery to 80% or more of the original rate (Schwintzer and

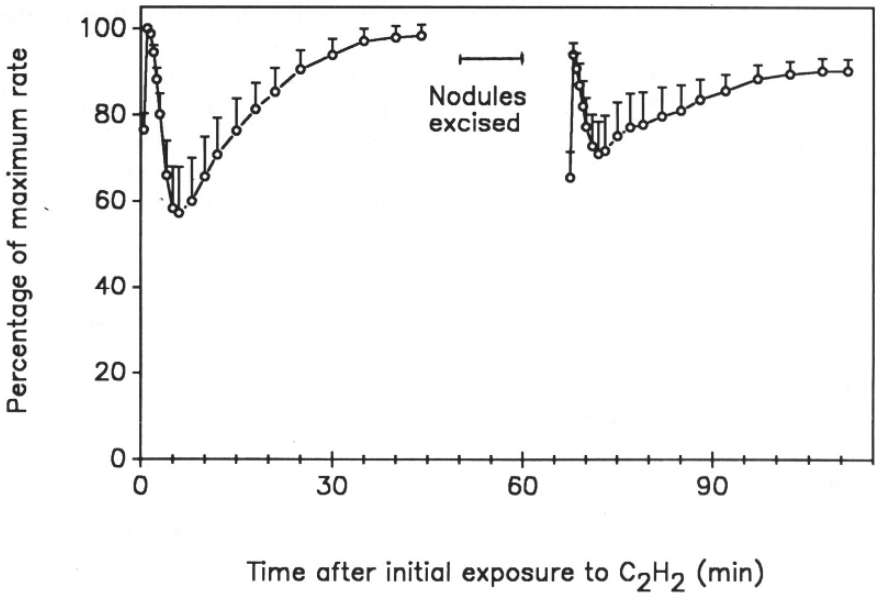

Figure 11. Acetylene-induced decline in nodules of Casuarina cunninghamiana *before and after excision.*
(Reproduced from Tjepkema and Murry, 1989, with permission).

Tjepkema, 1994; Silvester and Winship, 1990; Tjepkema and Schwintzer, 1992; Tjepkema *et al.,* 1988a). In contrast, laboratory-grown plants, which are stressed by extended darkness or water deficit prior to the acetylene-reduction assay (Schwintzer and Tjepkema, 1994), and field-collected nodules (Schwintzer and Tjepkema, 1997) have substantially deeper declines and much less recovery. As with legumes, the decline is initiated by cessation of ammonia production, which in turn may lead to depletion of either an amino acid or other substance involved in the generation of reductant for nitrogenase (Tjepkema and Schwintzer, 1992).

Although it may appear that the presence or absence of an C_2H_2-induced decline is fundamental to our understanding of gas exchange in nodules, other explanations are possible. For example, plant (nodule) age is known to be important in defining the presence of an C_2H_2-induced decline. In *Coriaria* nodules, the equilibrium rate changes from *ca*. 60% of the maximum rate in three-month-old nodules to 83% in six-month-old plants, with only a negligible decline in both nine- and 12-month-old plants (Harris and Silvester, 1994). This result was attributed to increases in the time required for gas diffusion in older nodules rather than a lack of an C_2H_2-induced decline, which was simply being masked by the extra tissue in the gas pathway of older nodules (Harris and Silvester, 1994). Thus, the presence or absence of a measured C_2H_2-induced decline may be due mainly to the age or

response time of the nodule, but the spontaneous recovery from a decline is of fundamental significance in understanding the O_2-protection mechanism.

3.5.3. O_2-induced Transients

When root nodules are exposed to higher pO_2 in a flow-through continuous assay system, they often show a transient decrease in nitrogenase activity. These transients were first observed in legume nodules (Hunt *et al.,* 1987) and were later shown, by using spectroscopic monitoring of leghemoglobin during H_2 evolution by nitrogenase, to be accompanied by a sharp rise in internal pO_2 of the nodule.

The same effect has been observed in *Alnus* (Rosendahl and Huss-Danell, 1988; Silvester *et al.,* 1988b), *Casuarina* (Silvester and Winship, 1990), *Myrica* (Silvester *et al.,* 1988a) and *Coriaria* (Silvester and Harris, 1989). The phenomenon manifests itself as a massive drop in nitrogenase activity following a small increase in pO_2 in the flow-through cuvette; nitrogenase activity then spontaneously recovers often to a rate higher than the initial nitrogenase-activity rate. The event can only be witnessed when continuous steady-state monitoring of nitrogenase activity is conducted and, in many cases, nitrogenase activity may decrease to zero for a short period of time. The event is very short lived (1-5 minutes) and is not associated with a short-term change in diffusion resistance (Silvester and Winship, 1990).

O_2-induced transients in legumes were considered to be an effect either close to or within the bacterium (King *et al.,* 1988) and related to O_2 concentrations close to nitrogenase. Although this suggestion cannot be tested for rhizobia because of their intense sensitivity to O_2, it has been tested for *Frankia in vitro* (Silvester and Winship, 1990). In order to overcome the problem of gas diffusion through liquid media, *Frankia* were suspended on nylon mesh in a cuvette and exposed to changes in pO_2. Transient decreases in nitrogenase activity were observed that are identical to those seen with nodules, in that the transient response is rapid, is proportional to the magnitude of the applied O_2 pulse, and is spontaneously recoverable (Silvester and Winship, 1990).

It now appears likely that the step-up in pO_2, which initiates the transient decrease in nitrogenase activity, produces a transient increase in pO_2 at the nitrogenase site, which directly or indirectly affects either nitrogenase itself or its electron-transport chain. The spontaneous recovery of nitrogenase activity is explained as an increase in O_2-limited respiration (Winship and Tjepkema, 1985) within or close to the bacterium, which then restores the low internal equilibrium pO_2 level. The rapid switch-off of nitrogenase activity that is required to support this mechanism was first described in *Azotobacter* by Dalton and Postgate (1969) and given the name of conformational protection. This mechanism involves an FeS protein, which binds to nitrogenase making it inactive, but tolerant to O_2, for short periods (Robson and Postgate, 1980).

O_2-induced transients therefore appear to be a feature common to N_2-fixing bacteria, whether in culture or in symbioses, and they have features in common across many different species and systems. In many ways, the recoverable C_2H_2-induced declines that are seen in *Myrica, Alnus*, and *Casuarina* resemble these

transients, although the fact that an C_2H_2-induced decline is not observed with *Frankia in vitro* argues for a somewhat different mechanism.

3.5.4. Adaptation to Varying pO_2 and O_2 Protection

Four major nodule anatomies are displayed by actinorhizas and these give rise to differences in *Frankia* anatomy and the physiology of nitrogenase. Each type will be described separately prior to an attempt to distill an overall general hypothesis of O_2 protection. As both *Frankia* and the nodule display possible gas-diffusion properties and responses to O_2, it is the balance between these two responses that is liable to change with different nodule types. Any consideration of nodule physiology will need to explain such phenomena as C_2H_2-induced decline, O_2-induced transients, and both long- and short-term adaptation to pO_2 as well as nodule structure.

3.5.5. O_2 Adaptation and O_2 Protection in Alnus

Alnus nodules are exceptionally well-ventilated, they have many lenticels (Bond, 1974), and they contain an interlacing network of air spaces (Wheeler *et al.,* 1979). When infiltrated under vacuum with India ink, the spaces are shown to be connected to the outside (Tjepkema, 1979). Nitrogenase activity in *Alnus rubra* shows a broad optimum response to O_2 being largely independent of pO_2 over the range from 15-30kPa O_2, but is severely limited by pO_2 both above and below that range (Wheeler *et al.,* 1979; Winship and Tjepkema, 1985). The infected cells form a broad band of clumps of cells in the mid cortex surrounding the central stele and *Frankia* shows well formed vesicles towards the outside of the infected cells (Figure 3).

Protection of nitrogenase from the effects of O_2 in *Alnus* nodules is the result of a combination of diffusion limitation coupled with a high rate of O_2-sensitive respiration (Winship and Tjepkema, 1983). The permeability of the O_2 pathway in nodules is extremely temperature sensitive and the combined effects of temperature and pO_2 on nodules are consistent with a nodule in which nitrogenase is located within a series of compartments. Some compartments of high permeability are active at low pO_2, whereas others of low permeability are only active at high pO_2 (Winship and Tjepkema, 1985). This model helps explain the physiological responses but does not identify the actual sites of diffusion resistance.

Alnus plants grown at different pO_2 levels showed very little difference in growth between 10 and 30kPa O_2 (MacConnell, 1959), thus implying that plants can adapt to varying pO_2. Such adaptation must be accompanied by physiological and/or anatomical changes that can be used to identify the sites of O_2 protection and diffusion limitation. Using this hypothesis, plants were grown at 5, 21 and 40kPa O_2 and shown to adapt nitrogenase activity to each of the growth pO_2 levels (Silvester *et al.,* 1988b). Thus, plants grown at 5kPa O_2 had a broad nitrogenase optimum at 5-9kPa O_2, whereas those grown at 40kPa O_2 optimized nitrogenase activity at 15-30kPa O_2. Significant changes in nodule anatomy were also observed, including lenticel size, intercellular spaces, and most significantly the dark-field appearance of vesicles. Thus, at high pO_2, nodules had restricted lenticel development, smaller

intercellular spaces (with very small ones between infected cells), and relatively thick-walled vesicles (Silvester *et al.,* 1988b).

Furthermore, the vesicles from this experiment were examined by freeze-fracture electron microscopy (Abeysekera *et al.,* 1990). The distal parts of vesicles showed significant variation in the number of lipid laminae; a two-fold increase in the number of laminae was observed with the eight-fold increase in pO_2 from 5-40kPa. These results indicate that, although vesicle-envelope thickening is certainly a component of the O_2-protection mechanism, it is not the only part.

The emerging picture of O_2 protection and adaptation in *Alnus* nodules is a combination of changes in vesicle envelope and nodule structure, backed up by respiratory O_2 uptake. The results of physiological studies (Winship and Tjepkema, 1983, 1985), structural adaptation (Silvester *et al.,* 1988b), anatomical study (Abeysekera *et al.,* 1990), and theoretical network simulation (Winship and Silvester, 1989) all support the model in which nitrogenase exists in several compartments in a nodule. The O_2-diffusion pathway is to some extent controlled by diffusion through the nodule, but this is a minor part of the resistance. A second source of resistance is through the packing of cells together in clusters, with some cells or parts of cells exposed to intercellular spaces and some not. Finally, the major control component of the O_2-diffusion pathway is the vesicle, which in all cases contains many lipid layers and adapts significantly to changing ambient pO_2. The situation in the *Alnus* nodule is probably the easiest to explain because it implicates the symbiotic-vesicle envelope as a major diffusion barrier.

3.5.6. O_2 Adaptation and O_2 Protection in Casuarina

Frankia in *Casuarina* nodules do not form vesicles (Berg, 1983; Berg and McDowell, 1987) despite the fact that these frankiae are capable of forming vesicles when grown in culture. Work on vesicle formation and function (Murry *et al.,* 1985) concluded that infected cells are at a very low pO_2 and operate in an environment similar to legume nodules. This situation is consistent with the high level of hemoglobin found in *Casuarina* nodules (see section *4.1.2.*). *Casuarina* nodules are surrounded by a solid periderm that lacks lenticels; the connection to the outside is *via* the nodule root (Figure 3). Dye infiltration (Zeng *et al.,* 1989) and EM studies (Berg and McDowell, 1987) show that the internal air spaces, although connected to air *via* the nodule root, become attenuated near the infected cell and are absent amongst infected cells.

Casuarina nodules show responses in both nitrogenase activity and respiration to changes in pO_2 that are remarkably similar to those of legume nodules (Tjepkema and Murry, 1989). Both nodule types show a linear increase in both respiration and nitrogenase activity over the full range of sub-optimal pO_2 that is consistent with a diffusion barrier within the nodule. *Casuarina* nodules also show a respiration component that is saturated at *ca*. 5kPa O_2, suggesting the presence of a compartment that is presumably outside the diffusion barrier (Tjepkema and Murry, 1989).

These results led to the conclusion that *Casuarina* is unique among actinorhizal nodules in that the nodule provides a major component of the diffusion barrier and

O_2 protection to *Frankia*. This barrier could be represented either by the close packing of cells in the infected area so presenting a cytoplasmic diffusion requirement for O_2 or by the lignified/suberized cell walls of the infected cells (Berg, 1983; Berg and McDowell, 1988). Moreover, both these components could act in concert to determine an inner low pO_2 area in which *Frankia* can express nitrogenase in hyphae rather than in vesicles.

3.5.7. O_2 Adaptation and O_2 Protection in Myrica

Isolates from nodules containing *Myrica*-type vesicles form typical *Frankia* vesicles *in vitro* and alder-type vesicles in alder (Lalonde, 1979; Torrey and Callaham, 1982). This illustrates the potential for host-plant influence on vesicle structure. As shown in a study of *Myrica gale* (Tjepkema, 1983a), the O_2 concentration is low in the region of infected cells due, at least in part, to smaller intercellular air spaces near infected cells and the lack of these spaces in groupings of infected cells. The walls of infected cells in *Myrica* become lignified during infection (Tjepkema and Asa, 1987) and this wall modification might be an important component in slowing the diffusion of O_2 into infected cells (Zeng and Tjepkema, 1994).

The *Myrica*-type vesicle could be considered as an intermediate between the alder-type and the lack of vesicles in *Casuarina*. Like *Casuarina,* hemoglobin concentration is high in *Myrica* nodules (Tjepkema and Asa, 1987; see section *4.1.2.*). However, in contrast to *Casuarina*, there is an envelope on the *Myrica*-type vesicle (Figure 9) that is comparable in thickness to that in the alder-type vesicle (Berg, 1994), indicating a microsymbiont response to O_2.

Myrica gale nodules, when subjected to various O_2 levels, show a response in nodule anatomy but not in vesicle envelope (Silvester *et al.,* 1988a). At 40kPa O_2, the nodules formed substantially smaller nodule roots (a source for nodule aeration) and decreased intercellular air spaces in both the nodule cortex and the region of infected cells. Changes in the walls of infected cells were not evident by light microscopy. Overall, these nodules adapt structurally to increased pO_2 by altering the entry of O_2 into nodule tissue through nodule roots.

The sites of O_2 resistance within nodules have also been studied by using silicone grease to eliminate gas entry into nodules. These experiments indicate that the nodule root is a major entry point for O_2 into nodules (Zeng and Tjepkema, 1994) and they have been likened to O_2-gathering antennae (Silvester *et al.,* 1988a). However, when nodules are sliced (0.1-mm thick), nitrogenase activity is hardly affected, being still O_2 limited up to 18kPa O_2 (Zeng and Tjepkema, 1994). Further proof that the resistance to O_2 diffusion is at the host-cell surface is provided by the observation that 2kPa CO added to the gas phase had little effect on nodule respiration (Zeng and Tjepkema, 1994).

All of these results strongly indicate that the site of O_2-diffusion resistance lies within the nodule and most likely at the host-cell walls. Because the diffusion resistance of *Myrica* nodules does not respond to pO_2 changes (Tjepkema and Schwintzer, 1992; Silvester *et al.,* 1988a) but is very sensitive to temperature (Zeng and Tjepkema, 1995), it may involve the lignified wall. The lignified wall would have a fixed resistance at constant temperature but may decrease in permeability at

lower temperature, analogous to polyethylene, which has a Q_{10} of 1.8 over the temperature range 0-30°C (Zeng and Tjepkema, 1995).

O_2 protection in *Myrica* appears to be a combination of cell-wall resistance coupled with a contribution from the vesicle envelope that exists in *Myrica* symbiotic vesicles.

3.5.8. O_2 Adaptation and O_2 Protection in Ceanothus

There are no experimental data on the response to varying pO_2 in *Ceanothus* nodules. The nodules appear well aerated because very little nodule hemoglobin is present (Silvester *et al.,* 1990, see also section *4.1.2*.). The thickness of the vesicle envelope in the *Ceanothus*-type vesicle is not as thick as in the alder-type nodule (Berg, 1994, and see discussion above), but this does not mean it is inadequate to function as an O_2-diffusion barrier. The difference in vesicle morphology relative to the alder-type may be genetically determined. Based on 16S-rRNA gene sequences, the *Ceanothus* microsymbiont is placed with the microsymbiont of *Dryas* (Ritchie and Myrold, 1999), another *Frankia* that forms *Ceanothus*-type vesicles. It would be interesting to see if nitrogen-fixing cultures of these microsymbionts form *Ceanothus*-type vesicles *in vitro*.

3.5.9. O_2 Adaptation and O_2 Protection in Coriaria *and* Datisca

These two genera, belonging to two distinct angiosperm families, show remarkably similar nodule anatomy and physiology that is quite distinct from all other actinorhizal plants. The nodules develop asymmetrically with a mass of infected cells displacing the stele to one side (Calvert, 1979; Mirza *et al.,* 1994). Unlike other actinorhizal nodules, all cells in the infected cortex contain *Frankia* (Figure 3). *Frankia* proliferates in the perimeter of the cell and develops tightly packed symbiotic vesicles facing towards a central vacuole (Berg *et al.,* 1999). Anatomical studies and India-ink infiltration shows that all the infected cells of both genera are surrounded by a network of air spaces that are connected to the outside (Silvester and Harris, 1989; Tjepkema *et al.,* 1988a) *via* a nodule root in *Datisca* and *via* a single lateral lenticel in *Coriaria*. The relatively unrestricted gas-diffusion path in nodules of both species is further illustrated by the short time required to reach peak ethylene concentration in fast-flow C_2H_2-reduction assays. When either *Coriaria* (Silvester and Harris, 1989) or *Datisca* (Tjepkema, 1997) are exposed to C_2H_2 in a fast-flow system, which is capable of resolving reactions at 5-s intervals, maximum ethylene output is achieved in approximately 30s. The speed of these responses is greatly in excess of any other actinorhizal species and confirms ready access of gas to the interior of the nodule.

Both genera show significant C_2H_2-induced declines with either no recovery in *Coriaria* (Silvester and Harris, 1989) or a small recovery in *Datisca* (Tjepkema, 1997). This decline in nitrogenase activity is accompanied by a parallel decline in respiration, results that are similar in many ways to those of legume nodules.

When *Coriaria* plants are grown with root systems at pO_2 levels from 5-40kPa, there is no significant change in either plant performance or level of nodulation,

which indicates that these plants, like *Alnus*, can adapt their root and nodule activities to a wide pO_2 range (Silvester and Harris, 1989). The effects on root structure include a minor increase in periderm thickness at high pO_2 and an increase in lenticel size at low pO_2. Most notably, however, was the increase in suberization of the periderm around the whole circumference and the increased thickening of the internal periderm that wraps around the infected cell mass. Long-term morphological responses to a wide range of pO_2 are minor in *Coriaria* compared to those seen in *Alnus* and *Myrica*. What are important however, are the short-term adaptations.

When *Coriaria* nodules of an air-grown plant are exposed to quite rapid changes in pO_2 from 10-60kPa over a period of 4.5h, the plant quickly adapts nitrogenase activity to the new pO_2. Similarly, a plant grown with roots at 5kPa O_2, when exposed to pO_2 levels from 1.7-30kPa, adjusted the nitrogenase rate to within 20% of the initial control rate (Silvester and Harris, 1989). Each change in pO_2 is accompanied by an O_2-induced transient decrease in nitrogenase activity, which is then followed by a return to the initial control rate. These results indicate probable changes in diffusion resistance in the tissue in response to changing pO_2. Lag-phase measurements of C_2H_4 production at various pO_2 levels also indicate significant changes in nodule-diffusion resistance. The time constant (half the time required to return to the equilibrium C_2H_4 concentration) changed from *ca*. 13s at 21kPa O_2 to *ca*. 28s at 40kPa O_2 (Silvester and Harris, 1989). The most likely site of these changes is the 2-3 cell layers between the internal periderm and the endodermis. Although this layer allows India ink to penetrate into the nodule interior, it does represent a significant choke, which could osmotically change its configuration.

The situation in *Datisca* has been interpreted quite differently (Tjepkema *et al.,* 1999). Like *Coriaria* nodules, *Datisca* nodules respond very rapidly to C_2H_2 exposure and are extremely well ventilated, but in contrast to legume nodules, *Datisca* nodules show only very small changes in both respiration and nitrogenase activity when exposed to 16-30kPa pO_2 during C_2H_2 exposure. There is also little difference in nitrogenase activity when measurements are made in He/O_2 mixtures compared with those made in Ar/O_2 mixtures. These results are consistent with a system lacking significant gas-phase limitation on diffusion.

The interpretation by Tjepkema *et al.* (1999) of O_2 protection in *Datisca* is similar to that proposed for *Alnus*; nitrogenase exists in a number of compartments, maybe a continuum, of differing accessibility to O_2. They conclude that the major resistance to O_2 lies in the infected cells. The vesicles, which lie tightly packed towards the center of the cell, present only a small proportion of their surface area to the surrounding cell cytoplasm and this orientation is considered sufficient to offer significant resistance to O_2 and to explain the effects.

The inward facing vesicles of *Coriaria* and *Datisca* are highly reducing sites, as shown by tetrazolium reduction (Hafeez *et al.,* 1984a, 1984b; Silvester *et al.,* 1999), and nitrogenase has been located in these sites by *in situ* hybridization of *nif*H transcripts (Mirza *et al.,* 1994). These structures, although rod-shaped, are comparable to symbiotic vesicles, but they do not have a thickened envelope (Silvester *et al.,* 1999) and, therefore, do not function in the same way as *Alnus* vesicles. There is no doubt that the vesicles are operating at a very low pO_2 as

defined by intense cyanotetrazolium-chloride (CTC) fluorescence in vesicles of normal *Coriaria* nodules.

An unusual concentration of mitochondria at the base of symbiotic vesicles occurs both in *Datisca* (Hafeez *et al.,* 1984b) and in *Coriaria* (Mirza *et al.,* 1994), indicative of a possible role in O_2 regulation. A detailed study of the location and possible role of these mitochondria (Silvester *et al.,* 1999) showed that the dense blanket of mitochondria at the base of the vesicle area was absolutely defined by the reduced-CTC volume of the symbiotic vesicles. This mitochondrial blanket also forms a discontinuous blanket around the intercellular spaces. This mitochondrial blanket was assigned a major role in mopping up O_2 (Silvester *et al.,* 1999), but this proposal has been questioned (Tjepkema *et al.*, 1999) because the respiration rate required for these mitochondria to protect the symbiotic vesicles is many times greater than the actual observed respiration rate of the nodules. Despite this uncertainty of any role in the O_2-protection catena, their very specific location, concentrated as a blanket, is indicative of a significant role for these mitochondria in nodule physiology.

O_2 protection and control in the *Coriaria/Datisca* nodule is enigmatic and unresolved. The evidence suggests that the two nodule types behave differently. In *Datisca*, it is proposed that there is no variable diffusion barrier and the very small response to external pO_2 is explained by the presence of a series of compartments with varying degrees of O_2 protection. These compartments are sequentially activated and deactivated during changes in pO_2, thus keeping the mean activity relatively unchanged. The major resistance to O_2 is proposed to be the tightly packed mass of symbiotic vesicles (Tjepkema *et al.,* 1999).

In *Coriaria*, although the evidence is interpreted as a variable O_2 barrier being present, it is also possible that the lag-phase results represent various compartments along a continuum of O_2 accessibility in the same manner as *Datisca*. However, additional O_2 protection is provided in the infected cell. Between the gas-filled intercellular space and the frankiae, there are (in order) a plant-cell wall, cell membrane, vegetative hyphae, mitochondria, and the vesicle wall. Each of these components likely contributes to O_2 reduction by either its permeability or uptake capacity. These resistances in series, a catena, act in concert to provide the reducing environment in which nitrogenase can operate (Silvester *et al.,* 1999).

4. HEMOGLOBINS IN ACTINORHIZAL NODULES AND *FRANKIA*

4.1. Hemoglobins in Legume Nodules

Legume nodules contain a hemoglobin that is produced by the host plant. This hemoglobin, termed leghemoglobin, is localized in the infected (*Rhizobium*-containing) cells that are the site of nitrogen fixation. These cells occur at the center of the nodule and are surrounded by a layer of tightly packed cortical cells that forms a diffusion barrier (Tjepkema and Yocum, 1973). Leghemoglobin is thought to function by facilitating the transport of O_2 within the infected cells, such that the O_2 concentration is

sufficient in the interior of the cell to support respiration while also being low enough at the cell periphery to avoid damage to nitrogenase (Appleby, 1992).

4.2. Hemoglobins in Actinorhizal Nodules

4.2.1. Presence and Amounts of Hemoglobin

There has long been evidence for the presence of hemoglobin in actinorhizal nodules (Davenport, 1960) and now it has been established that actinorhizal nodules contain one or more hemoglobins (Christensen *et al.,* 1991; Fleming *et al.,* 1987; Tjepkema and Asa, 1987). The amounts of hemoglobin present vary widely among actinorhizal genera (Silvester *et al.,* 1990) with the highest concentrations being found in the nodules of *Casuarina* and *Myrica*, both of which contain hemoglobin concentrations approaching those found in legume nodules (Fleming *et al.,* 1987; Tjepkema and Asa, 1987). In contrast, *Alnus* nodules contain only *ca*. 25% of the hemoglobin in *Casuarina* and *Myrica* and other genera, including *Ceanothus*, *Elaeagnus*, *Coriaria*, and *Datisca*, contain even less (Silvester *et al.,* 1990).

4.2.2. Plant-produced Hemoglobins

Casuarina glauca nodules contain a soluble hemoglobin that has a typical optical absorption spectrum. Moreover, the spectrum shows the expected shifts in the optical absorption peaks between the ferrous (Hb), carboxyferrous (HbCO), and oxyferrous (HbO_2) forms of hemoglobin (Fleming *et al.,* 1987). This hemoglobin is a monomer with a molecular mass estimated by gel filtration of 17.5kDa. The kinetics of its reactions with O_2 and CO are similar to those of soybean leghemoglobin and *Parasponia* hemoglobin. The O_2-association rate is very rapid ($41\mu M^{-1}s^{-1}$) and the dissociation rate is moderate ($5.5s^{-1}$) (Fleming *et al.,* 1987). This hemoglobin has three components, termed Hb I, Hb II, and Hb III, identified by isoelectric focusing. Of these, Hb I is the most abundant (Fleming *et al*., 1987) and has a 44% amino-acid sequence identity with the leghemoglobin of soybean nodules (Kortt *et al.,* 1988).

C. glauca has two types of hemoglobin genes, symbiotic and non-symbiotic. Three closely related symbiotic genes have been identified and designated as *symA*, *symB*, and *symC* (Jacobsen-Lyon *et al.,* 1995). These genes are only expressed in the nodule. The deduced amino-acid sequence of *symA* is almost identical with the protein sequence for Hb I. The sequences for *symB* and *symC* are at least 97% identical with *symA* (Jacobsen-Lyon *et al.,* 1995). A non-symbiotic gene, which encodes Hb II (Christensen *et al.,* 1991), has also been identified. This gene is expressed in roots and shoots and is only barely detectable in nodules (Jacobsen-Lyon *et al.,* 1995). The deduced amino-acid sequence for Hb II is 80% homologous with the hemoglobins of *Trema* (a non-symbiotic genus closely related to *Parasponia*) and *Parasponia*, but only 53% homologous with *Casuarina* Hb I (Christensen *et al.,* 1991). Thus, this gene is very different from the family of symbiotic genes.

Within *C. glauca* nodules, hemoglobin has been localized using immuno-cytochemistry and is found in the N_2-fixing cells that contain *Frankia* (the

infected cells), but not in the uninfected cells. Within infected cells, hemoglobin is found in the host cytoplasm and nucleus, but not in the *Frankia* hyphae (Goodchild and Miller, 1997). Localization of hemoglobin-gene (*hb*) transcripts in *C. glauca* also shows that hemoglobin is restricted to the infected cells (Gherbi *et al.,* 1997). Developmentally, *hb* transcripts are first detected in the youngest infected cells near the nodule lobe apex and reach maximum concentration in the mature infected cells just below this region. Comparable to the developmental sequence observed in legumes, transcripts of *nif*H mRNA, a *Frankia* nitrogenase structural gene, are not detectable in the youngest infected cells but are found in the mature infected cells (Gherbi *et al.,* 1997). Thus, production of hemoglobin precedes production of nitrogenase in the development of *Casuarina* nodules as it does in legume nodules.

Hemoglobin has also been extracted from root nodules of *Casuarina cunninghamiana*, *Casuarina equisetifolia*, and *C. glauca* that were inoculated with four different *Frankia* sources each (Sellstedt *et al.,* 1991). For each plant species, there is a strong linear correlation between concentration of hemoglobin in the nodules and dry mass of the whole plants. There is also a moderate linear correlation with nitrogen content of the whole plant, but no correlation with nitrogen concentration within the nodules (Sellstedt *et al.,* 1991). Varying amounts of hemoglobin also occur in root nodules of these same *Casuarina* species, when inoculated with two different *Frankia* sources. Moreover, the nodules contain lignin-like compounds in the cell walls which may serve as a diffusion barrier (Sellstedt *et al.,* 1991). These results suggest that hemoglobin plays an important role in nitrogen fixation by these plants.

Hemoglobin from *Myrica gale* nodules has been less extensively studied. *M. gale* produces a typical plant hemoglobin with optical absorption peaks for Hb, HbCO, and HbO_2 similar to those of hemoglobins from *Casuarina*, *Parasponia*, and various legume nodules. This hemoglobin has a molecular mass estimated by gel filtration of 38.5kDa and is probably a dimer (Pathirana and Tjepkema, 1995). In addition, *M. gale* produces a second hemoglobin-like compound with a molecular mass of 16.7kDa that is consistent with a monomeric hemoglobin. The absorption spectrum of the CO form of this compound is also consistent with it being a hemoglobin. This compound may be a separate hemoglobin and not just a monomeric form of the dimer because its absorption maximum is at 419nm in the Soret region instead of 416.2nm characteristic of the dimer (Pathirana and Tjepkema, 1995).

The function of the plant-produced *Casuarina* and *Myrica* hemoglobins is probably facilitated O_2 diffusion similar to that of hemoglobins in legume nodules. Evidence for this function is suggested both by the presence of relatively high concentrations of hemoglobin (Pathirana and Tjepkema, 1995) and by the likely diffusion barrier in the walls of the infected cells that probably restricts O_2 diffusion into the cells (Berg and McDowell, 1988; Zeng, Tjepkema, and Berg, 1989).

4.2.3. Alder Hemoglobin: A Possible Frankia *Hemoglobin*

A hemoglobin has also been purified from the nodules of *Alnus glutinosa* (Suharjo and Tjepkema, 1995). This hemoglobin is a monomer, has typical optical absorption spectra for the Hb, HbCO, and HbO_2 forms, and a molecular mass estimated by gel

filtration of 15.3kDa. There is no evidence for a second hemoglobin in these nodules. In nodule homogenates, much of this hemoglobin is associated with *Frankia* vesicle clusters. The relatively low concentrations of hemoglobin (Suharjo and Tjepkema, 1995; Tjepkema and Asa, 1987) found in these nodules and its association with *Frankia* vesicle clusters suggests that at least part of this hemoglobin may be produced by *Frankia* rather than the host plant. This suggestion is further supported by the recent discovery that several strains of *Frankia,* including a strain isolated from *Alnus,* produce a hemoglobin when grown in culture (Beckwith *et al.,* 2002; Tjepkema *et al.,* 2002).

4.3. Hemoglobins Produced by Frankia in vitro

4.3.1. Characteristics of Frankia *Hemoglobins*

Cultures of *Frankia* strain CcI3, originally isolated from root nodules of *C. cunninghamiana*, produce a hemoglobin with optical absorption spectra for Hb, HbCO, and HbO_2 typical of a hemoglobin, and a molecular mass determined by gel filtration of 14.1kDa. This hemoglobin has extremely rapid O_2-binding kinetics with an association rate constant of $206\mu M^{-1}\ s^{-1}$ and an O_2-dissociation rate constant of $56s^{-1}$; the equilibrium O_2-binding constant is 274nM (Tjepkema *et al.,* 2002). A similar hemoglobin is produced by cultures of *Frankia* strain EAN1pec, originally isolated from *Elaeagnus angustifolia*. This hemoglobin has typical optical absorption spectra, a molecular mass of 13.4kDa, and an O_2-dissociation rate constant of $131s^{-1}$ (Beckwith *et al.,* 2002).

4.3.2. Production of Hemoglobin is Widespread among Frankia *Strains*

All five strains of *Frankia* examined produce hemoglobin (Beckwith *et al.,* 2002). These are, in addition to strains CcI3 and EAN1pec (previously discussed), strain ArI3 that was originally isolated from *Alnus rubra*, strain EUN1f that was originally isolated from *Elaeagnus umbellata*, and strain Cc.1.17 originally isolated from *Colletia cruciata*. These five strains represent a wide range of the genetic diversity that was previously identified in the genus *Frankia* and include four genomic species (Fernandez *et al.,* 1989) and three genospecies (Akimov and Dobritsa, 1992). The presence of hemoglobin in all of these genetically diverse strains suggests that hemoglobin may be produced throughout the genus *Frankia*. It is also probable that the hemoglobin, which is produced by *Frankia* in culture, is also produced in the nodules of actinorhizal plants. As discussed earlier, the hemoglobin extracted from *Alnus glutinosa* nodules is likely to be produced at least in part by *Frankia*. Moreover, *M. gale* produces low concentrations of a hemoglobin-like monomer in addition to the plant-produced dimmer, which is found in relatively high concentrations (Pathirana and Tjepkema, 1995). This monomer may be a *Frankia* hemoglobin.

4.3.3. Conditions that Affect Production of Frankia *Hemoglobins*

All five *Frankia* strains (see above) produce hemoglobin in N_2-fixing cultures, *i.e.*, without supplied fixed-N, as well as in cultures supplied with ammonium, *i.e.*,

where nitrogen fixation is absent or minimal (Beckwith *et al.,* 2002). Hemoglobin production in 14-day-old cultures and 28-day-old cultures of these five strains varies with strain, culture age, and nitrogen nutrition. In the N_2-fixing cultures, the hemoglobin concentration in four of the five strains increases with age, but remains unchanged in one strain. In contrast, in cultures supplied with ammonium, the hemoglobin concentration decreases with age in two strains and remains unchanged in three strains. The effect of the amount of O_2 present in the culture has only been examined with strain EAN1pec. In this strain, O_2 strongly influences hemoglobin concentration in N_2-fixing cultures, but has little effect in cultures supplied with ammonium (Beckwith *et al.,* 2002).

4.3.4. Relationship of Frankia *Hemoglobin to Other Hemoglobins*

Hemoglobins exist in a wide variety of non-vertebrate organisms, including microbes, plants, and invertebrate animals. Their molecular adaptations and functions have been reviewed by Weber and Vinogradov (2001). Many bacteria produce a flavohemoglobin that has two domains; one domain contains heme that binds O_2 and is homologous to classical hemoglobins, whereas the second domain has reductase activity. These flavohemoglobins have several possible functions, including removal of excess NO (Poole and Hughes, 2000). A single domain hemoglobin that is related to the flavohemoglobins is produced by *Vitreoscilla*. Various lines of evidence suggest that this hemoglobin enhances O_2 uptake under conditions of low O_2 concentration (Ramandeep *et al.,* 2001).

Many bacteria also produce truncated hemoglobins (trHb). These hemoglobins share little amino-acid sequence similarity with classical hemoglobins and may be of very ancient origin (Wittenberg *et al.,* 2002). Besides having a shorter amino-acid sequence than the classical hemoglobins, their tertiary structure has a novel two-over-two α-helical sandwich fold rather than the three-over-three fold of the classical hemoglobins (Pesce *et al.,* 2000). Three major groups, Groups I, II, and III, of the truncated hemoglobins have been proposed and some bacterial species have genes for two or even all three of these groups (Wittenberg *et al.,* 2002). *Mycobacterium bovis* produces two hemoglobins, trHbN and trHbO, and these have different functions; trHbN provides protection from NO (Ouellet *et al.,* 2002), whereas trHbO enhances O_2 uptake under hypoxic conditions (Pathania *et al.,* 2002). At present, generalizations cannot be made about the extent of occurrence of truncated hemoglobins in bacteria and uncertainties about their functions remain.

Nostoc commune, a N_2-fixing cyanobacterium, produces a truncated hemoglobin that is expressed under conditions of low O_2 and fixed-N starvation. Its gene is located between two genes needed for nitrogen fixation, suggesting a function in this process (Potts *et al.,* 1992). However, in a survey of sixteen genera representing all five sections of the cyanobacteria, the gene was found only in five strains of *Nostoc* (Hill *et al.,* 1996). The *Nostoc* hemoglobin may function to scavenge O_2 to support respiration under conditions when O_2 is limiting (Hill *et al.,* 1996), and thus may not play a direct role in nitrogen fixation.

Evidence has accumulated to conclude that *Frankia* hemoglobin is a truncated hemoglobin. This evidence includes both a molecular mass and O_2-binding

properties that are similar to those of the truncated hemoglobin from *Nostoc commune*. Moreover, a 202-bp gene sequence from four *Frankia* strains shares about 80% homology on average with the sequence of the truncated hemoglobin of *Streptomyces coelicolor*. Genetic analysis, which included the two strains, EAN1pec and EUN1f, from which hemoglobin was previously extracted (Beckwith *et al.*, 2002), indicates that all four sequences fit into the trHbO subgroup of Group II of the truncated hemoglobins (J. Niemann and L. S. Tisa, unpublished data).

4.3.5. Possible Functions of Frankia *Hemoglobins*

The function of *Frankia* hemoglobin is probably not directly related to nitrogen fixation based on the following two lines of evidence. First, hemoglobin is produced in *Frankia* cultures in the absence of nitrogen fixation (where fixed-N is present) and in concentrations that are roughly comparable to those produced under N_2-fixing conditions. Second, O_2 concentration in the medium does not affect the amount of hemoglobin produced in the absence of nitrogen fixation (Beckwith *et al.,* 2002).

One possible function is facilitated O_2 diffusion over short distances within *Frankia* cells because this hemoglobin has extremely rapid O_2-binding kinetics that would be well suited for this function. However, hemoglobin concentrations within *Frankia* cells may not be high enough for this function unless the hemoglobin is concentrated in a specific region of the cell, such as the area near the plasma membrane (Tjepkema *et al.,* 2002). Another possible function is in protection against excess NO as found in *Mycobacterium bovis* (Ouellet *et al.,* 2002). Clearly, much uncertainty remains about the function(s) of *Frankia* hemoglobin.

5. CONCLUDING DISCUSSION

The *Frankia* vesicle represents one of the most fascinating models of physiological response in the prokaryote world. Its role appears to be solely related to nitrogenase because cultures grown on combined-N fail to produce vesicles. Further, its response to varying pO_2 indicates its adaptive significance to the functioning of *Frankia*, both in the free-living and the symbiotic state. Thus, in the free-living state, *Frankia*, like the cyanobacteria, is able to function at high efficiency in air and by altering the vesicle envelope is able to adapt nitrogenase to a wide variety of ambient pO_2. Whether it actually does this in nature can only be conjecture because these responses have only been observed *in vitro*. The role of *Frankia* hemoglobin remains a mystery, it does not respond as if it were a fundamental part of the *in vitro Frankia* O_2-protection mechanism and appears to be a constitutive property.

The legume nodule has always been the model by which other nitrogenase-containing nodules are compared but, because a primary function of the legume nodule is that of both short- and long-term O_2 control and regulation, the comparison of legume nodules with those of actinorhizal and cyanobacterial nodules is tenuous. There is no doubt that no single physiological mechanism operates across the wide spectrum of actinorhizal nodules, where both the vesicle and the nodule contribute to O_2 protection. The variety of mechanisms constitutes very elegant combinations of structure and physiology which, acting in concert,

bring about equal energetic efficiency across a wide diversity of structures. These structures, which range from the *Alnus* nodule where the vesicle represents the major O_2 barrier through to *Casuarina* where significant low-pO_2 sites exist within the nodule, represent a continuum of physiology and structure. The role of host hemoglobin, in contrast to that of *Frankia* hemoglobin, may be significant in *Myrica* and *Casuarina.* Both these symbioses possess high levels of hemoglobin and both have infected cells and cell masses with significant wall thickenings that support the notion of cellular O_2 transport by hemoglobin in these nodules.

It is apparent from this review and from work on energetics (Tjepkema and Winship, 1980) that no nodule system, whether legume or actinorhizal, is more efficient than any other. As respiratory O_2 uptake is an essential part of the O_2-protection mechanism in all systems, it appears that all the mechanisms, which have evolved, have done so with equal efficiency or perhaps that equally efficient systems have been selected for.

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