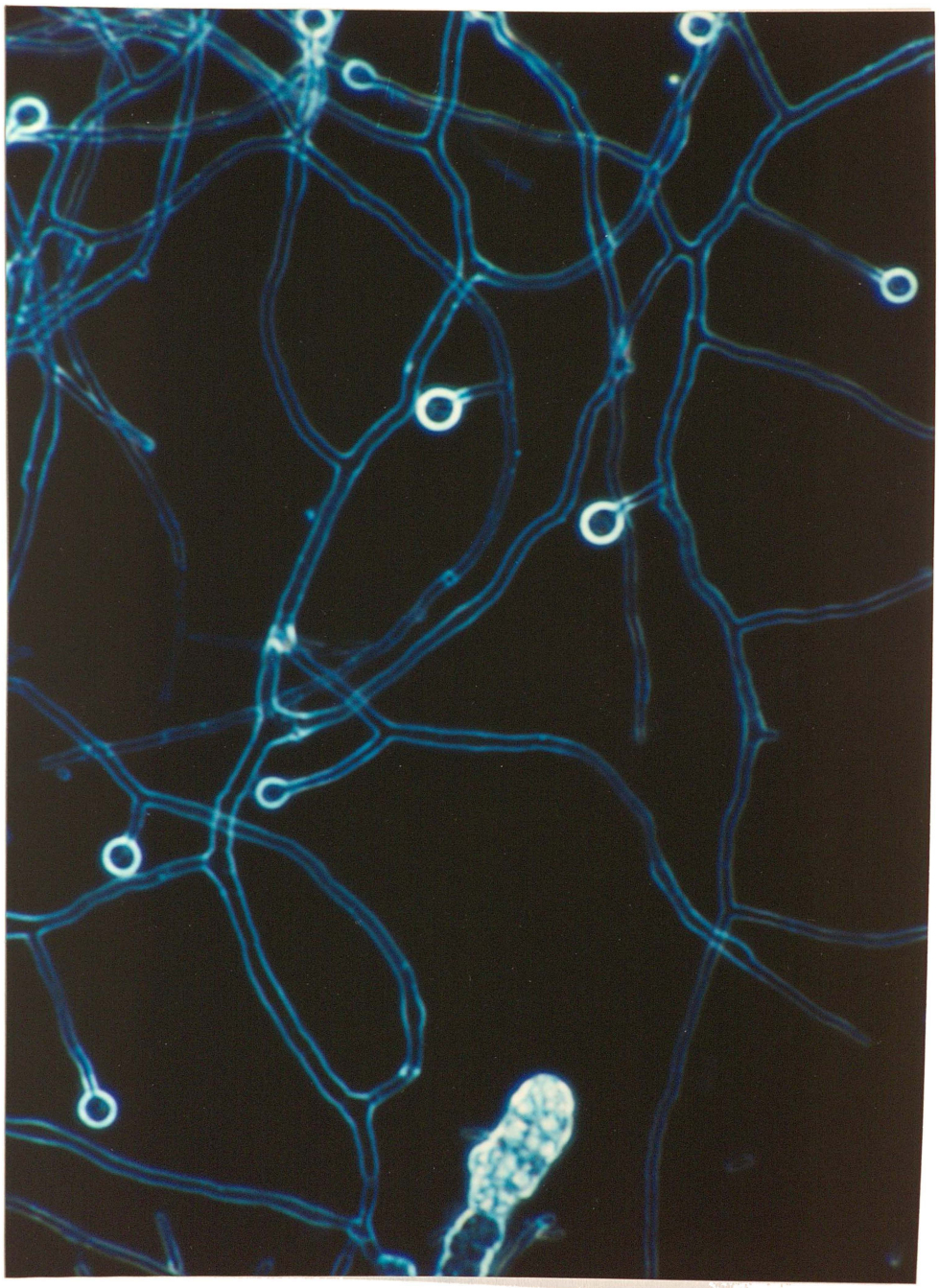

Frontispiece

Dark field photograph of *Frankia* sp. strain HFPCcI3

showing hyphae, vesicles and sporangia.

OXYGEN CONTROL OF NITROGEN FIXATION IN *FRANKIA* AND *CORIARIA ARBOREA*

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ABSTRACT

Oxygen has a central role in regulation of nitrogen fixation activity in both asymbiotic *Frankia* and *Frankia* - actinorhizal plant symbioses. Daily addition of fresh medium at a low dilution rate ($D=0.125\ day^{-1}$) allowed *Frankia* to be maintained in continuous culture with a stable rate of growth and nitrogenase activity for periods of more than 30 days. Use of continuous cultures permitted the effects of oxygen on vesicle morphology and nitrogenase activity to be investigated independent of normal developmental changes occurring in batch culture. *Frankia* grown at higher oxygen concentrations formed thicker-walled vesicles which allowed adaptation of nitrogenase activity over a pO_2 range of 5 - 40 kPa O_2. Recovery of nitrogenase activity after oxygen shock (2 to 21 kPa O_2) followed formation of thicker-walled vesicles. Vesicle walls also showed a thickening response to pO_2 level under conditions where nitrogenase activity was inhibited (in Ar : O_2 atmospheres) or not detected (in cultures grown on nitrate-containing medium). These results indicated the presence of vesicles and the induction of nitrogenase activity in culture is not as synonymous as was originally believed. GC/MS analysis of lipid extracts from *Frankia* cultures and actinorhizal nodules confirmed the high hopanoid content of *Frankia*. Differences in the relative proportions of hopanoids present were also detected between samples and the total amount of hopanoids measured in *Frankia* cultures was slightly higher at higher oxygen concentrations.

Most work on *Frankia* symbioses focussed on the *Coriaria arborea* association and highlighted startling similarities between this actinorhizal symbiosis and *Rhizobium* - legume associations. *Coriaria* showed a legume-like, non-recoverable decline in nitrogenase activity and nodule respiration following exposure to 10 kPa acetylene or argon. The extent of the acetylene-induced decline was diminished with increasing plant age and adaptation to above atmospheric oxygen concentrations. Lag-phase measurements indicated acteylene-induced declines in *Coriaria* are due to an apparent increase in nodule diffusion resistance which has also been observed in legumes.

Defoliation of *Coriaria* also caused a decline in nitrogenase and respiratory activity associated with an apparent increase in nodule diffusion resistance although the effect was delayed for approximately two hours. Continuous flow assay of nitrogenase activity in several actinorhizal species and one legume, exposed to alternate gas streams of nitrogen and helium at varying oxygen tensions, indicated a small component of the diffusion pathway in *Coriaria arborea* and *Myrica gale* nodules is an air-filled pathway. In *Alnus glutinosa*, *Casuarina cunninghamiana* and *Medicago sativa* nodules the diffusion pathway has some "water-filled" or "solid" diffusion barrier. Speculation is offered on the location and functioning of fixed and variable diffusion resistance in Coriaria nodules based on results from this study and previous work.

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Oxygen Control of Nitrogen Fixation in *Frankia* and *Coriaria arborea*

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CHAPTER ONE

INTRODUCTION

1.1 Nitrogen fixation

Nitrogen is, after hydrogen, carbon and oxygen, the most abundant elemental constituent of living organisms and it has been calculated that plants and animals together contain approximately 1.5 x 10^{10} tonnes of nitrogen. Nitrogen is essential to all organisms because it is a component of major biological molecules such as nucleic acids, proteins and amino acids and quantitatively less significant compounds such as coenzymes, vitamins and pigments (Postgate 1987). In many situations availability of nitrogen in a form suitable for assimilation is the limiting factor for plant growth. By far the most abundant source of nitrogen is the atmosphere, where nitrogen (dinitrogen) contributes approximately 78 % of atmospheric gases. However, ability to exploit this nitrogen source i.e. nitrogen fixation, is restricted to some prokaryotes, therefore plants and animals which obtain nitrogen from the atmosphere must do so indirectly either through symbiotic associations with nitrogen-fixing bacteria or through other processes in the nitrogen cycle. Nitrogen fixation is an important process in the nitrogen cycle and probably over 60 % of the input of soil and water nitrogen is supplied by nitrogen-fixing bacteria in one form or another (Gallon and Chaplin 1987; Postgate 1987).

1.2 Nitrogen fixation and nitrogenase

The ability of microorganisms to fix nitrogen is dependent on the presence of the nitrogenase enzyme complex. Nitrogen fixation by cell free extracts was initially demonstrated by Carnahan *et al.* (1960) who obtained the first cell-free suspension of nitrogenase from *Clostridium pasteurianum*. Nitrogenase has since been isolated, and in

many cases purified, from representatives of all known types of nitrogen-fixing organism. The properties of nitrogenase are remarkably constant regardless of the source of the enzyme and in all cases requires a low redox potential reductant, Mg^{2+} cation and large amounts of ATP for its operation.

The active enzyme complex consists of two Fe-S protein components with different prosthetic groups and properties. The larger MoFe protein (nitrogenase) binds nitrogen, as well as other substrates containing a triple bond such as acetylene, cyanide and nitrous oxide, and catalyses their reduction. The smaller Fe protein (nitrogenase reductase) supplies equivalents for the reduction. A low redox potential electron donor supplies an electron to nitrogenase reductase enabling it to react with Mg^{2+} - ATP. Meanwhile a nitrogen molecule combines with the Mo on the nitrogenase subunit. The two protein components then combine to form the active enzyme complex and electrons flow singly from nitrogenase reductase to nitrogenase with simultaneous hydrolysis of two ATP molecules. After transfer of each electron the enzyme complex dissociates. Once a sufficient level of electrons has been built up electron flow proceeds to the bound nitrogen molecule which is reduced in three two-electron steps thus requiring six nitrogenase reductase - Mg^{2+} - ATP complexes. Formation of the resultant ammonia also requires protons which are derived from the cellular pool (Burris *et al.* 1981; Burgess 1985; Thornley and Lowe 1985).

It should be noted that two alternative nitrogenase enzyme complexes have been discovered in *Azotobacter vinelandii* (Bishop *et al.* 1980). One of these alternative nitrogenases (nitrogenase-2) contains vanadium instead of molybdenum while the other (nitrogenase-3) contains neither prosthetic group. Nitrogenase-2 has also been found in *Azotobacter chroococcum* (Robson *et al.* 1986). The alternative nitrogenase complexes differ from standard nitrogenase by their high rates of hydrogen evolution in the presence of nitrogen, low rates of acetylene reduction and conversion of acetylene to ethane in addition to ethylene. Expression of alternative nitrogenases is regulated by the presence or absence of the respective prosthetic groups (Bishop *et al.* 1980).

Biological reduction of nitrogen to ammonia is only about 80 % efficient largely due to concurrent evolution of hydrogen. Electrons bypass the bound nitrogen and instead combine directly with protons to form hydrogen gas. Thus nitrogen fixation may be summarised as:

$$N_2 + 8H^+ + 8e^- \text{ -----> } 2NH_3 + H_2$$

Associated hydrogen evolution not only wastes reducing power but also energy since ATP is utilised before reduction of protons can occur. However some aerobic nitrogen-fixing organisms possess uptake hydrogenase enzyme (Hup) which may oxidise some or all of the hydrogen produced by the nitrogenase complex. The resulting electrons are passed down the oxidative phosphorylation pathway so reclaiming some ATP lost during nitrogen fixation. Two other potential advantages of having Hup are that consumption of hydrogen helps to maintain a low level of hydrogen as well as consuming oxygen, both of which may inhibit nitrogen fixation (Dixon 1972).

Reduction of nitrogen to ammonia requires 28 moles of ATP for every mole of dinitrogen fixed (Dixon and Wheeler 1986) which includes energy required for activation of the enzyme complex and for supply of reductant. Thus large amounts of ATP must be generated close to the site of nitrogenase and the ATP-generating system must have either a high turnover rate and/or be present in high concentrations. In aerobic organisms ATP is supplied by phosphorylation so sufficient oxygen must be present to support respiration (Orme-Johnson *et al.* 1971). However the nitrogenase enzyme complex, and in particular nitrogenase reductase, is irreversibly inactivated by exposure to oxygen. Nitrogenase reductase for example has a $T_{1/2}$ in air of 30 to 45 seconds while the nitrogenase protein has a $T_{1/2}$ in air of approximately ten minutes. Therefore the requirement for oxygen coupled with the extreme oxygen sensitivity of nitrogenase creates a physiological dilemma for nitrogen-fixing aerobes. In response to this problem, free-living and symbiotic nitrogen-fixing organisms have developed a variety of so-called "oxygen protection mechanisms" (Gallon 1981).

1.3 Range of nitrogen-fixing bacteria

Prokaryotes may be separated into three divisions, archaebacteria, bacteria and cyanobacteria, encompassing approximately 370 genera, 72 of which contain species capable of nitrogen fixation (Bond 1983; Silvester and Musgrave 1991). Nitrogen-fixing organisms have little in common and range from free-living species to symbionts. Free-living species comprise three main groups:

(1) nitrogen-fixing heterotrophic bacteria which are mostly gram negative rods and may be anaerobic, e.g. several *Clostridium* genera, or aerobic, e.g. *Azotobacter, Rhizobium and Frankia*

(2) phototrophic bacteria including some strains of *Rhodospirill um, Rhodopseudomonas* and *Chlorobium*

(3) autotrophic cyanobacteria including non-heterocystous unicells, e.g. *Gloeocapsa*, filamentous species, e.g. *Oscillatoria*, and heterocystous, filamentous organisms such as *Anabaena* and *Nostoc* (Sprent 1979).

Nitrogen-fixing bacteria are generally carbon-limited so those which utilise carbon compounds symbiotically from plants are at a great advantage. Nitrogen-fixing bacteria living in symbiosis with higher plants belong to three taxa, Cyanobacteria (mainly *Anabaena* and *Nostoc*), *Rhizobium* and *Frankia.*

The associations between cyanobacteria and plants are not rigid since the bacteria can usually grow and fix nitrogen independent of the host. In symbiosis however the ability to reduce carbon is lowered, so the main role of cyanobacteria is to fix nitrogen for both partners. Cyanobacteria symbioses range from extracellular associations e.g. with bryophytes and Azolla, to intracellular infection which occurs in the angiosperm genus *Gunnera.* Here *Nostoc* invades excretory glands near the *Gunnera* apex and forms dense nodules within the stems (Fay 1980).

The most specialised combination of diazotroph and plant is the root nodule symbiosis. Formation of root nodules is induced by the invading bacterium which uses carbon compounds supplied by the plant as a source of carbon and energy. In return the endosymbiont excretes fixed nitrogen into the cytoplasm of the plant host. Therefore the root nodule endosymbiont has the advantage over free-living nitrogen-fixers of a protected environment where it does not compete with other microbes for nutrients (Akkermans and Houwers 1983).

Perhaps the best known and economically most important root nodule symbiosis is between *Rhizobium* and members of the Leguminosae. *Rhizobium* also forms a nitrogen-fixing symbiosis with one non-legume, *Parasponia*. However, it is the root nodule symbiotic relationship between the actinomycete *Frankia* and a range of non-leguminous or actinorhizal plants that is the focus of this study.

1.4 Acinorhizal Symbioses

1.4.1 Research history

The presence of root nodules on an actinorhizal plant, *Alnus glutinosa*, was first reported in 1829 but it was not until 1896 that Hiltner provided evidence of nitrogen fixation activity associated with *Alnus* nodules. However, substantial research of the nitrogen-fixing ability of non-legumes did not begin until the 1950's when Bond listed eight actinorhizal, nitrogen-fixing genera: *Alnus*, *Casuarina*, *Ceanothus*, *Coriaria*, *Elaeagnus*, *Hippophae*, *Myrica* and *Shepherdia* (Bond 1955). Early work with these species showed nodulated plants grew more vigorously than unnodulated plants in rooting medium free of combined nitrogen (Bond *et al.* 1954; Gardner and Bond 1957; Bond 1959) and, based on ^{15}N isotope evidence, Bond (1959) stated that the better growth of nodulated plants was due to nitrogen fixation activity in the root nodules.

In contrast to study of the actinorhizal plant hosts, research on the endosymbiont is much more recent. The identity of the endosymbiont was a subject of speculation for some time and it has been variously described as a fungus (Woronin 1866), a species of Plasmodiophora (Moller 1885) and a bacterium (Hiltner 1896). Electron microscope studies of *Alnus* and *Myrica* nodules (Becking *et al.* 1964) revealed the endosymbiont to be a prokaryote closely related to the Actinomycetes and in 1970 Becking introduced a new genus, *Frankia*, to include all endosymbionts of actinorhizal plants. The successful isolation and cultivation of the endosymbiont from *Comptonia peregrina* (Callaham *et al.* 1978), and subsequently a number of other *Frankia* strains, has permitted characterisation of *Frankia* and study of the morphology, physiology and biochemistry of the bacterium in the free-living state and in symbiosis with actinorhizal plants .

1.4.2 Actinorhizal plants

The present list of actinorhizal plants encompasses around 200 species distributed among 23 genera in eight phylogenetically unrelated families (Moiroud and Gianinazzi-Pearson 1986) (Table 1.1). All actinorhizal plants are dicotyledonous angiosperms and, except for one genus, *Datisca*, all are perennial woody trees or shrubs (Torrey 1978). Most actinorhizal plants occur in temperate climates, unlike the warm temperate-to-tropical distribution of legumes, while some are circum-polar e.g. *Dryas*. Those genera which do extend into the tropics are usually restricted to higher altitudes although *Casuarina* is an exception to this trend (Silvester 1977).

Actinorhizal species are important as pioneer plants in nutrient-poor environments due to their ability to fix nitrogen and therefore survive under nitrogen-limited conditions. Consequently they have been used for replanting on mine wastes and areas affected by deforestation, forest fires and volcanic eruption. Some genera e.g. *Casuarina* and *Allocasuarina*, are also being introduced into arid and saline habitats (Resch 1979).

Table 1.1 The taxonomy and distribution of actinorhizal plants (Torrey 1978; Bond 1983).

Order	Family	Genus	Distribution
Casuarinales	Casuarinaceae	*Allocasuarina*	Australia
		Casuarina	Australia, S.E. Asia
		Gymnostoma	Sumatra to Fiji
Coriariales	Coriariaceae	*Coriaria*	NZ, Chile to Mexico, Japan to Spain
Cucurbitales	Datiscaceae	*Datisca*	Central Asia, N. America
Fagales	Betulaceae	*Alnus*	temperate N. hemisphere
Myricales	Myricaceae	*Comptonia*	N. America
		Myrica	tropical to teperate to subarctic
Rhamnales	Elaeagnaceae	*Elaeagnus*	Asia, Europe, N. America
		Hippophae	Asia, Europe
		Shepherdia	N. America
	Rhamnaceae	*Ceanothus*	N. America
		Colletia	S. America
		Discaria	Andes, Brazil, Aust., NZ
		Kentrothamnus	Bolivia, Argentina
		Retanilla	S. America
		Talguenea	Chile
		Trevoa	Andes
Rosales	Rosaceae	*Cercocarpus*	W. USA, Mexico
		Chamaebatia	California
		Cowania	N. America
		Dryas	Alaska, Canada
		Purshia	W. USA

However, unlike legumes, actinorhizal plants have a limited use as agricultural species although *Ceanothus* and *Purshia* are valued as stock fodder while timber of some *Alnus* and *Casuarina* species is merchantable (Silvester 1977).

This study includes experiments using several actinorhizal species, *Alnus glutinosa*, *Casuarina cunninghamiana* and *Myrica gale*, although most plant work focusses on a single species, *Coriaria arborea.*

1.4.3 Coriaria

Coriaria is the only genus in the family Coriariaceae and has no close relatives or close morphological affiliation with any other family. *Coriaria* is widely but disjunctly distributed and inhabits warm temperature regions of southern South America and, in tropical areas, high altitudes in Central America and Mexico. It is also found in southern Europe, north Africa, islands off the Pacific coast of Asia and the western Pacific Ocean including New Zealand (Skog 1972).

Coriaria grows vigorously on poor sandy, gravelly or clay soils in both lowland and subalpine habitats (Burke 1963). *Coriaria* has an important role in the succession to forest in New Zealand and species of *Coriaria* occur as pioneer plants where they readily invade alluvium, moraines, alpine scree and disturbed areas (Silvester 1977). *C. arborea*, for example was the first woody species to grow on Mt Tarawera, New Zealand, following the 1886 volcanic eruption (Cockayne 1967). *C. arborea* also exists as understorey in exotic Pinus radiata forest (Silvester 1977) but has no commercial use in either forestry or agriculture due partly to its poisonous nature (Aston 1935).

Surveys show all eight New Zealand species of *Coriaria* (*C. angustissima, C. arborea, C. kingiana, C. lurida, C. plumosa, C. pottsiana, C. pterioides* and *C. sarmentosa*), as well as five hybrids, are always nodulated (Burke 1963). Evidence for the involvement of

Coriaria root nodules in nitrogen fixation was provided by ^{15}N isotope studies (Morrison 1961) and growth experiments using *C. myrtifolia* (Bond 1967).

1.4.4 Host - endosymbiont specificity

Research in the area of host - endosymbiont specificity has been complicated by the unavailability of *Frankia* isolates from approximately half the actinorhizal species and the limited number of inoculation trials on actinorhizal genera using existing isolates. Further problems arise from the long time taken by some isolates to nodulate plants or the low number of resulting nodules and the fact that effectivity of nodules may vary considerably (Tjepkema *et al.* 1986).

Initial studies using crushed nodule inocula (Rodriguez-Barrueco and Miguel 1979; Rodriguez-Barrueco *et al.* 1981) indicated frequent cross-infectivity between nodule endosymbionts of different actinorhizal genera. Since then isolation and cultivation of *Frankia* strains has allowed cross infectivity to be investigated using pure cultured inocula. Studies using a small number of isolates (Lalonde 1979; Dillon and Baker 1982) demonstrated single *Frankia* strains could infect more than one genus of actinorhizal plants and, using a larger number of strains, Baker (1987) defined four host-specificity groups:

(1) strains which nodulate *Alnus* and *Myrica*

(2) strains which nodulate *Casuarina* and *Myrica*

(3) strains which nodulate *Myrica* and Elaeagnaceae

(4) strains which nodulate only the Elaeagnaceae.

Baker pointed out additional groupings may be expected following isolation and testing of strains from root nodules of other families not included in his summary i.e. Coriariaceae, Datiscaceae, Rhamnaceae and Rosaceae. Baker's summary is also complicated by reports of strains which fail to nodulate plants of the host genus from which they were originally isolated (Normand and Lalonde 1986). This has been proposed as evidence for the

presence of more than one *Frankia* strain in nodules but direct observation of dual occupancy among actinorhizal nodules has yet to be reported.

1.4.5 Infection and nodule development

Infection of potential actinorhizal host plants and consequent nodule development relies on a series of recognition steps requiring cooperation of the host and endosymbiont. Current theories suggest the recognition mechanism is linked to a series of gene regulated biochemical interactions involving both symbionts. Key substances possibly involved in these processes include root exudates, mucilages or the host-derived encapsulation layer. Potential symbioses may fail at several steps if the correct biochemical interactions do not occur (Newcomb and Wood 1987; Berry and Sunell 1990).

In the presence of the host plant, *Frankia* proliferates in the rhizosphere surrounding the elongating region of the root (Lalonde 1977) and, as in most legumes, the endosymbiont may enter the host via deformed root hairs. Root hair infections have been described for *Alnus*, *Casuarina*, *Comptonia* and *Myrica* (Callaham *et al.* 1979; Berry and Torrey 1983; Berry *et al.* 1986). Infection of only one or a few root hairs is sufficient for nodule development in contrast to the multiple infections observed in legumes although multiple root hair infections have been reported for some species of *Comptonia* and *Myrica* (Berry and Sunell 1990). Infection may also occur through the root epidermis as reported for *Elaeagnus* and *Hippophae* and it was recently shown the route of infection is at least partly under host control (Miller and Baker 1985*b*; 1986).

Following root hair deformation and penetration by the *Frankia*, cortical cells divide and are infected by hyphae which form clusters within the host cells (Akkermans 1978), but outside the host plasma (Newcomb and Wood 1987). In contrast, *Frankia* invading through the root epidermis colonise the intercellular spaces prior to entering the cortical cells (Miller and Baker 1985*a*).

Cellular activity within the cortex results in the formation of a prenodule observable as a small protuberance on the root surface (Callaham and Torrey 1977). In the region of the prenodule a root primordium is then initiated and meristematic cells become infected as they grow through the cortex. Continued growth of the infected root primordium results in a visible, active root nodule (Akkermans 1978).

1.4.6 Nodule structure

Actinorhizal root nodules consist of numerous lobes, each of which arises from a modified lateral root with successive nodule lobes developing from the pericycle of previous lobes (Newcomb and Wood 1987). Thus nodules are perennial and increase in size each season (Berry and Sunell 1990).

Two groups of actinorhizal nodules with different external morphologies are recognised. In the *Myrica*-type grouping, nodules usually consist of discrete lobes and are characterised by the presence of determinate nodule roots which arise from the distal region of each mature nodule lobe. Members of this group include the Myricaceae, Casuarinaceae and Datiscaceae. *Alnus*-type nodules have a knobbly, coralloid appearance and consist of densely packed lobes which lack nodule roots. Representatives of this second group are found among the Betulaceae, Coriariaceae, Elaeagnaceae, Rhamnaceae and Rosaceae (Newcomb and Wood 1987, Berry and Sunell 1990).

The internal arrangement of tissues in actinorhizal nodules is similar to that of lateral roots. All actinorhizal nodules possess a stele which is usually centrally located and surrounded by an endodermis, several layers of cortical cells and an epidermis or periderm of variable thickness (Newcomb and Wood 1987).

Frankia occupies only certain cells, usually those in the middle cortex, although the distribution of infected tissue does vary among actinorhizal species. In *Alnus* for example

infected cells are randomly located through the middle cortex while in *Myrica* infected cells occur in clumps interspersed with uninfected cortical cells(Bond 1957; 1967; Lalonde and Knowles 1975; Newcomb *et al.* 1978, Wheeler *et al.* 1979).

Coriaria and *Datisca* show a very different arrangement of nodule tissues (Calvert *et al.* 1979; Newcomb and Pankhurst 1982*a,b*; Silvester and Harris 1989). In these nodules the stele is displaced to one side of the nodule and infected cells occupy a semi-lunar zone in which every cell is infected. *Coriaria* is also distinct from other actinorhizal genera by the absence of a cortex in nodules.

In most actinorhizal plants *Frankia* forms terminal hyphal swellings or vesicles which vary in shape and distribution depending on the host plant. In *Alnus* and *Elaeagnus* nodules the vesicles are spherical and arranged around the periphery of infected cells (Lalonde and Knowles 1975). In *Ceanothus,* vesicles are also peripherally oriented but are pear-shaped (Strand and Laetsch 1977) while in *Myrica* and *Comptonia,* vesicles are club-shaped with the swollen ends toward the periphery (Newcomb *et al.* 1978; Benson and Eveleigh 1979). In contrast, *Coriaria* and *Datisca* vesicles are elongate and oriented toward the central vacuole of infected cells (Calvert *et al.* 1979; Newcomb and Pankhurst 1982a). Perhaps the most unusual situation occurs in *Casuarina* and *Allocasuarina* nodules where *Frankia* bears no vesicles at all (Zhang and Torrey 1985).

1.4.7 Carbon metabolism

Frankia in symbiosis is dependent on the host plant for a source of carbon compounds which are essential for maintenance and growth, delivery of energy and reductant to nitrogenase and for supply of acceptor molecules in the assimilation of ammonia produced by nitrogen fixation. Root nodules obtain carbon compounds produced in photosynthesis which are transported in the phloem (Huss-Danell 1990). However the close relationship between *Frankia* and the host plant means it is difficult to separate the carbon metabolism of the symbionts and identify the carbon transfer compounds. *Frankia* metabolism in the

host plant has been studied using vesicle cluster suspensions derived from crushed nodules (Vikman and Huss-Danell 1987*a*,*b*). From these studies it appears the carbon compounds used by *Frankia* in symbiosis are similar to those used *in vitro* and include short chain fatty acids, fatty acid derivatives, TCA cycle intermediates and a small number of other organic acids (Benson *et al.* 1985).

1.4.8 Nitrogen metabolism

Nitrogen fixation reactions of *Frankia* in symbiosis are identical to those occurring in culture and in other nitrogen-fixing organisms. This was confirmed by characterisation of purified nitrogenase from *Alnus* glutinosa nodules (Benson *et al.* 1979). However, following reduction to ammonia, the fate of nitrogen in symbiosis appears to differ from the asymbiotic state.

In symbiosis *Frankia* glutamine synthetase (GS) and glutamate dehydrogenase (GDH) activity is suppressed and the fixed nitrogen is excreted from the endosymbiont to the host plant cell probably as ammonia (Wheeler 1984). But high concentrations of ammonia are toxic in plants and therefore ammonia is assimilated into amino acids and amides (Huss-Danell 1990). Once in the plant tissue ammonia is most likely assimilated via plant GS combined with glutamate synthase (glutamine oxo-glutarate aminotransferase; GOGAT). High levels os GS are present in *Alnus* root nodules (Hirel *et al.* 1982) but GOGAT could not be detected, possibly due to inactivation of the enzyme by some compound released during nodule homogenisation (Blom 1982). GDH has also been shown to be present in nodules but its role in ammonia assimilation is unresolved (Akkermans and Roelofsen 1980).

The products of ammonia assimilation in actinorhizal plants are extremely varied. Examples include citrulline in *Alnus* species (Blom 1981; Schubert 1986) and *Casuarina equisetifolia* (Walsh *et al* 1984), arginine and glutamine in *Myrica* species, *Elaeagnus*

species and *Ceanothus* species (Wheeler and Bond 1970; Schubert 1986) and arginine, glutamate, glutamine and asparagine in *Datisca* species and *Coriaria* species (Wheeler and Bond 1970; Schubert 1986).

1.4.9 Hydrogen metabolism

Actinorhizal nodules, like other nitrogen-fixing systems, form hydrogen during reduction of nitrogen to ammonia. However little or no hydrogen is detected when actinorhizal nodules are placed in a cuvette with air (Schubert and Evans 1976; Winship *et al.* 1987) in contrast to most legumes which evolve large amounts of hydrogen (Evans *et al* 1987). The low level of hydrogen evolution in actinorhizal symbioses is due to the action of uptake hydrogenase (Hup) which oxidises hydrogen. All actinorhizal nodules tested to date, with the exception of one *Alnus incana* - *Frankia* symbiosis (Sellstedt and Huss-Danell 1984), have hydrogen-oxidising activity . An interesting property of Hup in actinorhizal nodules is that some, but not all, are irreversibly inhibited when nodules are exposed to 10 kPa acetylene (Winship and Martin 1985).

1.5 Frankia

1.5.1 Frankia taxonomy

Frankia is a filamentous streptomycete and the sole genus in the Frankiaceae family, order Actinomycetales. Actinomycetes are gram-positive bacteria most of which are free-living saprophytes inhabiting such substrates as soil, compost and plant litter as well as smaller numbers in air and water. A few are associated with plants or animals, both as a component of their natural flora or as pathogens (Lechevalier 1981; Tjepkema *et al.* 1986). Based on the homology of *Frankia* DNA with DNA of other actinomycetes

having the same cell wall type, the closest relative to *Frankia* may be Geodermatophilus (An *et al.* 1987).

All *Frankia* are characterised as slow-growing sporactinomycetes producing three cell types under certain conditions: vegetative hyphae, spores and vesicles. Baker (1981) listed ten criteria for the recognition of *Frankia*:

(1) slow-growing

(2) aerobic actinomycete

(3) gram positive

(4) contain meso-diaminopimelic acid

(5) filamentous with septa

(6) bear vegetative sporangia with non-motile spores

(7) may be pigmented

(8) fix nitrogen via specialised vesicles

(9) have a multilamellar envelope exterior to the cell wall

(10) are cross-reactive with anti-*Frankia* sera.

Although these criteria allow ready identification of *Frankia*, considerable morphological and physiological variation occurs within the genus making species characterisation difficult. Consequently it has been agreed that no species names should be used until a more complete understanding of what constitutes a *Frankia* species is reached. Instead strains are denoted by a code derived from the host plant generic name and the isolate number e.g. the *Frankia* strain isolated from *Comptonia peregrina* is denoted CpI1.

Frankia are presently divided into two broad groups based on their physiology, infectivity and genomic characteristics. Group A strains are physiologically heterogenous, relatively aerobic, grow relatively rapidly, can be maintained in slant culture, can utilise carbohydrates and are usually not infective on their host plants. DNA - DNA hybridisation studies have also shown Group A isolates have low genomic homology levels. In contrast, Group B strains are serologically, genetically and chemicall y more homogenous, tend to be less oxygen tolerant, grow relatively slowly, cannot be

maintained on slants, do not use carbohydrates and are generally both infective and effective on the host plant (Baker 1981; An *et al.* 1985; Lechevalier and Lechevalier 1989).

1.5.2 Ecology

For some time *Frankia* was regarded as an obligate symbiont but growth of *Frankia* in culture (Callaham *et al.* 1978), together with isolation from soil (Baker and O'Keefe 1984), has shown *Frankia* to be capable of extranodular survival. However comparatively little is known about the survival of *Frankia* in soil outside the host plant. While distribution of *Frankia* can be partly related to the distribution of host plants, actinorhizal plants are known to nodulate in regions where they have not been growing for many years. For example, Wollum *et al.* (1968) found *Frankia* from *Ceanothus velutinus* in soil under timber stands devoid of *Ceanothus* for almost 100 years while strains from *Alnus glutinosa* and *Myrica gale* were detected in soils that had been free of these plants for many years (Rodriguez-Barrueco 1968). Recently an *Alnus* nodulating *Frankia* strain was found to be surprisingly abundant in soil under stands of a non-host species, *Betula pendula* (Smolander and Sundman 1987).

Van Dijk (1979) suggests existence of populations of extranodular *Frankia* in soil is dependent on regular influx of viable endosymbiont from nodule decay, immigration, and possibly extranodular growth; to compensate for death, emmigration and immobilisation. While studies have shown the contribution of nodule decay to soil populations of *Frankia* is significant (Akkermans and Van Dijk 1976), experimental evidence of endosymbiont growth and migration in soil is limited. Reports of *Frankia* at sites free of host plants for a number of years cannot be explained by migration of the endosymbiont alone and the possibility of extranodular growth must therefore be considered. Proliferation of *Frankia* in the rhizosphere surrounding *Alnus* roots at the time of nodulation was obtained from electron microscopy observations (Lalonde 1977). While growth of *Frankia* in soil

therefore appears likely there is not yet any evidence to indicate whether it is also capable of extranodular nitrogen fixation.

1.5.3 Frankia morphology (in vitro)

Frankia in liquid culture grows as a dense hyphal mat or spherical colonies composed of branched, septate filaments, or hyphae, 0.5 to 1.5 μm in diameter. The vegetative hyphae often appear white although, under certain culture conditions, some strains produce soluble pigments giving a light brown colour (Newcomb *et al.* 1979; Fontaine *et al.* 1984; Zhang and Torrey 1985).

When cultured on complex medium or in the presence of combined nitrogen, terminal or intercalary sporangia develop from enlarged portions of the hyphae. Sporangia vary in both shape and size but are usually club-shaped measuring 10 μm by 30 to 50 μm (Newcomb and Wood 1987) and contain a few to several hundred non-motile, spherical or angular spores (Newcomb *et al.* 1979). *Frankia* spores also occur in symbiosis in nodules of some actinorhizal genera where they probably serve as resistant propagules which, when released into the soil, persist and infect further generations of host plants (Tjepkema *et al.* 1986).

A feature of all *Frankia* strains in culture is their ability to form spherical structures termed vesicles. Vesicle development is usually initiated in nitrogen-limited cultures since the presence of a source of combined nitrogen in the growth medium suppresses vesicle formation in most *Frankia* strains. The effect of various sources of combined nitrogen on vesicle development is investigated in more detail in chapter four. While vesicle form in symbiosis varies between plants, all cultured strains possess vesicles which are light-bulb shaped with a spherical body and a stalk attaching the vesicle to the subtending hyphae. Young vesicles, termed provesicles, are 1.5 to 2.0 μm in diameter and usually appear phase dark under phase contrast microscopy. As vesicles mature they increase in size,

reaching a mean diameter of 2.5 μm, develop internal septa continuous with the multi-layered wall structure and become phase bright. Late in development some vesicles become damaged due to breakdown of the cytoplasm and internal septa leading to collapse of the vesicle wall. At this point the vesicles ("ghost" vesicles) lose their brightness and appear empty (Fontaine *et al.* 1984).

1.5.4 Carbon metabolism

In culture *Frankia* is capable of utilising a wide range of carbon sources for growth although the range of carbon sources varies greatly between strains, with some isolates able to use only a few compounds. The most effective include short chain fatty acids like propionate and acetate, fatty acid derivatives such as Tween 80, TCA cycle intermediates like malate or succinate, and other organic acids such as pyruvate (Benson *et al.* 1985). Most strains have glycogen or trehalose as storage compounds implying an ability to catabolise carbohydrates (Benson and Eveleigh 1979; Lechevalier *et al.* 1983). However only a small number of strains can grow on carbohydrates such as glucose, fructose, mannitol, trehalose, maltose or sucrose (Burggraaf and Shipton 1983; Lechevalier *et al.* 1983; Tisa *et al.* 1983). This inability of most *Frankia* to utilise carbohydrates has been attributed to lack of sugar transport systems (Lechevalier and Ruan 1984).

Little information is available concerning the pathways of carbon metabolism in *Frankia* although assumptions may be based on the compounds used. TCA cycle, glyoxylate cycle and gluconeogenic enzyme activities have been demonstrated in *Frankia* sp. strain AvcI1 (Blom and Harkink 1981; Akkermans *et al.* 1983) and studies on ArI3 indicate glucose is catabolised through the Embden - Myerhof - Parnas pathway (Lopez and Torrey 1985). Propionate is utilised via the propionyl - CoA pathway (Stowers *et al* 1986).

1.5.5 Nitrogen metabolism

Frankia can use a wide range of nitrogen compounds including amino acids, urea, adenine, nitrate, ammonia, dinitrogen and uracil, with ammonia the most preferred source of most strains (Blom 1982; Shipton and Burggraaf 1983). The effect of different nitrogen sources on nitrogenase activity and vesicle development in *Frankia* sp. strain CcI3 is examined in chapter four.

Studies of ammonia transport and assimilation in *Frankia* sp. strain CpI1 indicate ammonia is assimilated via one of two glutamine synthetases (GSI or GSII) depending on the nitrogen status of the cells (Edmands *et al.* 1987). Interestingly, the only other organisms shown to possess a dual ammonia assimilating system are genera of the Rhizobiaceae, also capable of symbiosis (Benson 1988). In *Frankia*, like most nitrogen-fixing bacteria, assimilation of fixed nitrogen follows the nitrogenase - glutamine synthetase (GS) - glutamate synthase (GOGAT) pathway (Benson and Schultz 1990).

1.6 Nitrogenase and oxygen

1.6.1 Oxygen protection mechanisms

Although nitrogenase is inactivated by oxygen therefore requiring an anaerobic environment, only some nitrogen-fixing organisms are anaerobes which maintain active nitrogenase by avoiding oxygen e.g. *Clostridium pasteurianum*. As outlined above, oxygen protection of nitrogenase is complicated in aerobic bacteria by the need for oxygen to support respiration which in turn provides ATP for nitrogenase activity. Gallon (1981) categorised a number of strategies for protection of nitrogenase which are employed by a range of nitrogen-fixing organisms.

Some aerobic nitrogen-fixing organisms provide a physical barrier around nitrogenase so limiting oxygen diffusion. Cyanobacteria such as *Nostoc* and *Anabaena* locate their nitrogenase in specialised heterocyst cells which are surrounded by a thick lipid envelope. Walsby (1985) showed the heterocyst envelope presents a considerable barrier to oxygen diffusion compared with the vegetative cell wall. Oxygen protection of nitrogenase in legumes and actinorhizal nodules and in *Frankia* itself also involves physical barriers to oxygen diffusion and this is discussed in more detail below.

Alternatively, nitrogen-fixing organisms may spatially or temporally separate nitrogenase activity from other metabolic processes which involve or produce oxygen. Heterocystous bacteria for example separate nitrogenase activity in the heterocyst from oxygen producing photosynthesis reactions (photosystem II) which is restricted to vegetative cells (Stewart 1977). *Gloeocapsa*, a non-heterocystous cyanobacteria capable of aerobic nitrogen fixation protects its nitrogenase by temporal separation of nitrogen reduction (in darkness) and photosynthesis (in light) (Tozum and Gallon 1979).

Nitrogen-fixing organisms could also protect nitrogenase by using their metabolism to reduce the oxygen concentration around nitrogenase or by destroying any toxic products generated from oxygen. *Azotobacter*, a nitrogen-fixing aerobe, has a very high respiration rate which is further increased in response to elevated pO_2 and therefore maintains a low oxygen concentration around nitrogenase. Furthermore, the respiratory chain in *Azotobacter* is branched so that at high pO_2 it is poorly coupled to ATP production (Dalton 1980).

A variation on this form of respiratory protection is seen in the metabolism of hydrogen by nitrogen-fixing organisms, a process which consumes oxygen. In pure cultures of *Frankia*, hydrogen stimulated nitrogenase activity at above-atmospheric oxygen concentrations, suggesting that hydrogenase activity provides some oxygen protection of nitrogenase (Murry and Lopez 1989).

Oxygen is toxic to living systems primarily through its reduction to superoxide ($O_2^{-\cdot}$) which in turn gives rise to H_2O_2, $OH\cdot$ and singlet oxygen. Many aerobic organisms therefore contain superoxide dismutase which converts $O_2^{-\cdot}$ to H_2O_2 and either catalase or peroxidase in order to destroy H_2O_2 (Gallon 1981). Superoxide dismutase and catalase activity have been found in *Frankia* (Puppo *et al.* 1989) as discussed in chapter five.

Some nitrogen-fixing organisms are able to use conformational change in nitrogenase to protect the enzyme from oxygen damage. Conformational protection was first proposed for *Azotobacter* (Robson and Postgate 1980) and renders the enzyme oxygen-tolerant but catalytically inactive. Studies with *Azotobacter* have shown conformational protection results from association of the enzyme complex with a third Fe - S protein which binds with nitrogenase and Mg^{2+}. However this system cannot cope with prolonged exposure to high oxygen concentrations. A similar conformational protection or switch-off mechanism has been proposed for *Anabaena* (Pienkos *et al.* 1983) and *Frankia* (Silvester and Winship 1990) in response to increases in pO_2.

A further oxygen protection mechanism simply involves synthesis of more nitrogenase enzyme to balance that inactivated by oxygen. This strategy has been shown in *Anabaena flos-aquae* (Bone 1972).

Most nitrogen-fixing organisms possess a combination of protection strategies. The following section outlines some of those mechanisms found in *Frankia* and actinorhizal nodules and compares them to the situation in *Rhizobium* and legumes.

1.6.2 Frankia vesicles and oxygen protection of nitrogenase

In 1980 it was shown *Frankia* in culture has maximum nitrogenase activity at or about atmospheric oxygen concentrations (Tjepkema *et al.* 1980). This is in direct contrast to *Rhizobium* which, while also able to grow in air, is able to express nitrogenase only when external oxygen concentration is extremely low (Tjepkema and Evans 1975). The implication from this is that *Frankia* has an intrinsic oxygen protection mechanism while *Rhizobium*, whether in culture or symbiosis, relies on external limitation of oxygen diffusion.

The vesicle has been clearly identified as the site of nitrogenase in *Frankia* in culture and in most symbioses and various studies have provided evidence supporting this role. Mian and Bond (1978) showed the occurrence of nitrogenase activity in root nodules of *Alnus glutinosa* coincides with vesicle formation and similar results have been recorded for nodules of other actinorhizal species (Murry *et al.* 1985). Correlation of vesicle formation with the onset of nitrogenase activity in nitrogen-limited *Frankia* cultures also supports the hypothesis (Murry *et al.* 1984*a*). Furthermore an increase in vesicle number was found to be concomitant with increased acetylene reduction activity in nodules (Becking 1977) and in pure culture (Tjepkema *et al.* 1980; 1981). Akkermans (1971) provided evidence for the vesicle as the site of nitrogen fixation based on the localisation of tetrazolium dye reduction in nodule vesicles and the isolation of vesicle clusters capable of acetylene reduction. More recently vesicles were shown to contain nitrogenase by characterisation of oxygen labile nitrogenase from vesicles and vesicle clusters of *Alnus* root nodules (Benson *et al.* 1979), through direct assay of permeabilised vesicles isolated in sucrose density gradients (Noridge and Benson 1986) and by ultrastructural studies using colloidal gold-labelled antibodies to nitrogenase (Meesters *et al.* 1987; Huss-Danell and Bergman 1990).

Thus oxygen protection of nitrogenase in *Frankia* may, in part, be attributed to localisation of enzyme in vesicles with the laminated structure of the vesicle envelope has

been proposed as an oxygen diffusion barrier (Torrey and Callaham 1982; Murry *et al.* 1984*a*). Use of Nile red fluorescent stain, specific for lipid, indicates the vesicle envelope is proposed of lipid monolayers (Lamont *et al.* 1988). Chemical analysis shows vesicles have a higher lipid content than vegetative cells (Tindall *et al.* 1989) and that hopanoids comprise the largest proportion of envelope lipids (Berry *et al.* 1991). The lipid structure of vesicles is discussed in chapter six.

The role of the vesicle as an oxygen diffusion barrier is further supported by observations that vesicle formation in certain strains is repressed when nitrogenase is induced under low oxygen levels. As a result these *Frankia* strains are very sensitive to inhibition by oxygen (Murry *et al.* 1985). In contrast, cultures grown at atmospheric pO_2 form vesicles and are not inhibited by atmospheric pO_2. Parsons *et al.* (1987) showed that *Frankia* cultures can fix nitrogen up to 80 kPa O_2, provided they are adapted to elevated oxygen tensions, and that adaptation to above atmospheric pO_2 is accompanied by an apparent thickening and brightening of the vesicle wall viewed under dark field microscopy. Electron microscopy of freeze fracture preparations indicated thickening of the vesicle wall was due to the addition of lipid monolayers as oxygen concentration was increased.

Further evidence of a physical barrier to gas diffusion in vesicles comes from studies of oxygen uptake by *Frankia* cultures. Respiration of cultures lacking vesicles becomes oxygen-saturated at low pO_2 values. However when vesicles are present, a much higher pO_2 is required for oxygen saturation. Such kinetics are consistent with diffusion limitation of oxygen uptake into the vesicle (Murry *et al.* 1984*a*; 1985). Further morphological and physiological evidence for the role of the vesicle in oxygen protection of nitrogenase is presented in chapter five.

1.6.3 Oxygen protection mechanisms in legume and actinorhizal nodules

Like their respective endosymbionts, the responses to oxygen of actinorhizal and legume nodules differ significantly despite both systems having oxygen-sensitive nitrogenase.

Oxygen concentrations above atmospheric enhance nitrogen fixation in legume nodules, as first demonstrated with soybean (Bergersen 1982), which is in direct contrast to the response of cultured *Rhizobium*. Oxygen has an inhibitory effect only above about 60 kPa O_2. This result suggests the presence of a barrier to oxygen diffusion maintains a low pO_2 in the central nodule tissue where the nitrogen-fixing bacteroids are located.

The oxygen concentration in the bacteroid zone of legume nodules has been estimated from percentage oxygenation of leghaemoglobin at about 1 Pa i.e. 20,000 x lower than atmospheric pO_2 (Appleby 1969; Tjepkema 1971), a figure later confirmed by oxygen microelectrode probes of soybean nodules (Tjepkema and Yocum 1974). The attainment and maintenance of this reduced oxygen level surrounding the bacteroids is the result of a balance between:

(1) oxygen entry via lenticels and intercellular spaces

(2) resistance to gas diffusion afforded by the inner cortex

(3) high respiration rate and oxygen consumption within infected tissue

(4) oxygen carrying and buffering capacity of leghaemoglobin (Bergersen 1982).

The factor most responsible for low pO_2 in legume nodules is the lack of intercellular spaces in the band of cortex cells surrounding the central tissue. Oxygen is therefore forced to diffuse through water of the cytoplasm to reach the internal air spaces, a much slower process than diffusion through air. Evidence for this layer was provided by oxygen microelectrode probes and vacuum infiltration of soybean nodules with India ink (Tjepkema and Yocum 1974) and confirmed more recently by Witty *et al.* (1987) in studies using microelectrode probes to quantify oxygen concentrations in various regions

of pea and bean nodules. Measurements of oxygen uptake in the bacteroid zone showed respiration to be severely diffusion limited which offered further support for the inner cortex functioning as a diffusion barrier (Tjepkema and Yoc um 1974).

Legume nodules also possess leghaemoglobin, which readily binds oxygen and distributes low concentrations of oxygen at a high rate within the central part of the nodule to support the respiratory needs of the bacteroids. Leghaemoglobin is located in the infected cell cytosol and the peribacteroid space and measurements indicate about 20 to 25 % of the physiologically functional leghaemoglobin is oxygenated (Appleby 1984).

A number of studies with a range of leguminous plants (Sheehy *et al.* 1983; Hunt *et al.* 1987; Weisz and Sinclair 1987; Witty *et al.* 1987) have reported that stepwise increases in external oxygen supply did not cause irreversible damage to nitrogenase. Furthermore, concomitant measurement of CO_2 evolution showed that respiratory activity was not increased, indicating the elevated level of oxygen entering the nodule was not balanced by enhanced "protective respiration". These observations have led to the idea that resistance to gas diffusion in legume nodules is not constant but varies due to some control exerted through mechanisms which "sense" the intracellular level of free oxygen. Apparent nodule diffusion resistance has been determined using a variety of methods including the lag-phase technique (Weisz and Sinclair 1987) and measurement of leghaemoglobin oxygenation using non-invasive spectroscopic techniques (Denison and Layzell 1991). Studies indicate legumes increase diffusion resistance in response to elevated pO_2 (Sheehy *et al.* 1983; Witty *et al.* 1983; Hunt *et al.* 1987; Denison and Layzell 1991; Minchin and Witty 1990), acetylene exposure (Witty *et al.* 1984; 1986; Minchin and Witty 1990), prolonged darkening (Minchin *et al.* 1985), defoliation (Hartwig *et al.* 1987) and nitrate addition (Minchin *et al.* 1986; Schuller *et al.* 1988; Sprent 1989). The nature and location of the variable diffusion resistance mechanism is discussed in detail in later chapters.

Actinorhizal nodules are generally much better aerated than legume nodules and it appears that *Frankia*-containing cells of some nodules are exposed to near atmospheric pO_2 values (Tjepkema 1979). Vacuum infiltration of nodules with India ink shows continuous air spaces occur between the rhizosphere atmosphere and part of the surface area of infected cells in most actinorhizal nodules (Tjepkema and Yocum 1974). Thus the aerated nature of actinorhizal nodules, the apparent lack of a visible diffusion barrier like that in legumes and an optimal pO_2 for nitrogenase activity close to atmospheric with higher values being inhibitory (Bond 1961), suggests an entirely different oxygen protection mechanism from that found in legumes.

Work to date indicates actinorhizal plants possess a wide range of devices for preventing oxygen inactivation of nitrogenase including both short and long-term morphological and physiological adaptations. One end of the spectrum is typified by *Alnus* where the major resistance to oxygen diffusion may be the *Frankia* vesicle envelope. This hypothesis is supported by observations that vesicles in nodules grown at and adapted to a range of different oxygen concentrations are thicker-walled at higher pO_2 levels (Silvester *et al.* 1988*b*).

Myrica typifies the intermediate group where the nodule is relatively impermeable to oxygen since it lacks lenticels, and gas exchange instead occurs via nodule roots which change in surface area according to ambient pO_2. Thus at low oxygen concentrations nodule roots are longer, often branched and relatively thick while at high pO_2 they are short and thin. Other morphological changes, such as a decrease in the degree of intercellular aeration in the nodule cortex and infected zone and a slight increase in the thickness of infected cell walls were observed in nodules adapted to high pO_2 but no change in vesicle wall structure occurred (Silvester *et al.* 1988*a*). Oxygen microelectrode probes show regions of low pO_2 occur in *Myrica* nodules associated with clusters of infected cells (Tjepkema 1983) and Berg (1983) suggests modification of infected host cell walls may play a role in diffusion resistance in *Myrica* nodules.

Coriaria nodules are structually very different from most other actinorhizal genera as outlined above. When grown at a range of oxygen concentrations little change is observed in nodule structure except for an increase in the thickness of the suberised periderm surrounding the nodule. Vacuum infiltration of nodules with India ink confirms the impermeability of this layer and there is some evidence for modification of infected cell walls at elevated oxygen concentrations suggesting the periderm together with infected cell walls are the sites of most diffusion resistance (Silvester and Harris 1989).

Casuarina in which *Frankia* lacks vesicles, represents the other end of the spectrum. In this case India ink diffuses into only the outermost layers of the cortical region and never into the zone of infected cells where there are very few air spaces (Tjepkema *et al.* 1988*a*). In *Casuarina* nodules the suberised wall of the infected host cell probably provides a significant oxygen diffusion barrier (Berg 1983).

Casuarina and *Myrica* are the only actinorhizal genera reported to contain significant levels of haemoglobin approaching those found in legume nodules. Since *Casuarina* and *Myrica* nodules are recognised as being poorly aerated relative to other actinorhizal plants, the haemoglobin is probably involved in facilitating oxygen transport from the outer surface of the cytoplasm to the respiratory sinks (*Frankia* cells and host mitochondria) (Tjepkema and Asa 1987). Thus haemoglobin may only be needed in actinorhizal nodules when the concentration of oxygen in the host cytoplasm is low because of the presence of an external diffusion barrier.

Actinorhizal plants show an optimum pO_2 for nitrogenase activity around atmospheric which is in contrast to the situation described for legumes (Wheeler *et al.* 1979; Winship and Tjepkema 1985; Rosendahl and Huss-Danell 1988; Silvester *et al* 1988*a*,*b*; Tjepkema and Murry 1989). Below atmospheric pO_2, nitrogenase activity is oxygen-limited while above atmospheric levels generally cause oxygen-inhibition of activity. However, short-term adaptation to pO_2 has been observed in several actinorhizal species as a rapid response of nitrogenase activity to stepwise increases in oxygen concentration. This

characteristic response has been termed the "oxygen transient" and is manifest as a sharp drop in activity followed by a gradual recovery when nodules are exposed to higher pO_2. The effect is rapid and activity is generally recovered to a new level within approximately 30 minutes. Oxygen transients have been shown in *Myrica gale* (Silvester *et al.* 1988*a*), *Alnus incana* (Rosendahl and Huss-Danell 1988; Silvester *et al.* 1988*b*), *Casuarina cunninghamiana* (Silvester and Winship 1990) and *Coriaria arborea* (Silvester and Harris 1989). Oxygen transients have also been reported in *Frankia* cultures and are proposed as being an expression of conformational protection of nitrogenase. It is suggested the wave of oxygen passing into the nodule following an increase in external pO_2 results in an instantaneous switch-off of nitrogenase (conformational protection). Respiration then mops up the extra oxygen and, as internal pO_2 drops back to normal, nitrogenase activity returns (Silvester and Winship 1990).

Recent work (Silvester and Harris 1989) indicates *Coriaria* is distinct from other actinorhizal genera in that it has a comparatively broad pO_2 optimum for nitrogenase activity and is able to adjust activity to a wide range of oxygen concentrations in a fashion reminiscent of legumes. Lag-phase measurements show *Coriaria* responds to increases in external oxygen concentration by increasing apparent nodule diffusion resistance. For example, an increase in pO_2 from 21 to 40 kPa resulted in a significant increase in apparent diffusion resistance within two minutes. The nature and functioning of the variable diffusion resistance mechanism in *Coriaria* is further investigated in this thesis.

1.7 This study

Clearly oxygen and its availability has a major influence on *Frankia* morphology and physiology both in culture and in symbiosis with actinorhizal species and this influence is the main focus of this study. The first half of the thesis looks at *Frankia* in culture while the second part deals more with the symbiotic situation. Early in the study a method was developed to separate the effects of environmental parameters such as oxygen on *Frankia* morphology and physiology from those changes which occur as a consequence of "normal" endogenous development in culture. The development of this method is outlined in chapter three and is used in chapters four and five to examine the effects of various nitrogen sources and oxygen concentration on *Frankia* in culture. Chapter six looks specifically at the lipid structure of the vesicle envelope, the proposed diffusion barrier in *Frankia*, and in particular the effect of oxygen on its make-up. Most work on actinorhizal symbioses used *Coriaria arborea* and attempted to further determine the nature and functioning of the variable diffusion resistance in this species. Chapter seven examines the effect of acetylene exposure on nodule activity while chapter eight looks at defoliation effects on *Coriaria* and a new technique to determine the nature of nodule diffusion resistance in several actinorhizal species.

CHAPTER TWO

MATERIALS AND METHODS

2.1 *Frankia*

2.1.1 Stock cultures

Two *Frankia* strains were used throughout this work : HFPCcI3 (catalogue number HFP020203) isolated from *Casuarina cunninghamiana* (Zhang *et al.* 1984) and HFPArI3 (catalogue number HFP013103) isolated from *Alnus rubra* (Berry and Torrey 1979). Stock cultures of HFPCcI3 were maintained on B medium (Murry *et al.* 1984*a*) with NH_4Cl and pyruvate as the respective nitrogen and carbon sources. HFPArI3 was grown in stock culture on DPM medium (Baker and O'Keefe 1984) also with NH_4Cl as the sole nitrogen source but with propionate as the carbon source. Stock cultures were grown as batch cultures at 28 °C and were stirred continuously (90 rpm). To avoid clumping, the *Frankia* was forced through a 19 gauge syringe needle, when stock cultures were transferred on to fresh medium every three weeks.

2.1.2 Experimental cultures

Frankia from seven-day-old stock cultures was homogenised prior to inoculation using a 19 gauge syringe needle and transferred to 100 mls of nitrogen-free (-N) medium to give an initial inoculum size of 20 μg protein. ml^{-1}. Cultures were grown in one-litre Schott bottles fitted with a double-layer rubber septum in the lids to allow addition and removal of material from the bottle using a syringe fitted with a 19-gauge needle. Cultures were grown at 28 °C on a gyratory shaker table (90 rpm).

2.1.3 Continuous cultures

Work presented in chapter three indicated it was preferable to grow *Frankia* in continuous culture during experiments and several trials were conducted in an effort to develop a suitable continuous culture technique. The method eventually arrived at is outlined here.

A continuous culture regime was usually initiated 72 h after inoculation, a period which allowed cultures to recover from effects of transfer to nitrogen-free medium. Sterile medium was added to the bottles via syringe through the rubber septum. At the same time an equivalent amount of culture (medium and *Frankia*) was removed thus giving the required dilution rate (D). Care was taken to ensure the material removed was a homogenous sample of the entire culture. The pH of the cultures was checked periodically and was found to remain stable throughout the experiments.

2.1.4 Dilution rate

In the continuous culture system sterile growth medium was fed into the culture vessel at a selected flow rate (f) and culture was removed at the same rate thus keeping the volume of the culture (v) constant. The contents of the vessel were well stirred to ensure complete mixing so that growth medium entering was immediately and uniformly dispersed throughout the vessel. Dilution rate (D) is calculated as f/v (Herbert *et al.* 1956) and is normally expressed as the number of complete volume changes per hour. *Frankia* however is very slow growing with a doubling time in the logarithmic phase of approximately 2.17 days (Burggraaf and Shipton 1983), therefore low dilution rates were used such that D has been expressed here as the number of complete volume changes per day (day^{-1}).

2.1.5 Growth rate

Growth rate was measured as protein, determined using a modification of the Bradford protein assay (Murry *et al.* 1984*a*). Assays were performed on replicate 1 ml subsamples removed from each experimental culture at regular time intervals. Absorbance readings of samples treated with Coomassie blue stain were converted to protein measurements using a standard curve prepared using bovine serum albumin.

2.1.6 Growth pO_2

All cultures were grown with atmospheric pO_2 (21 kPa O_2), unless otherwise stated, with 1 kPa CO_2 in the bottle headspace. A high gas headspace to liquid volume ratio in addition to the stirring ensured adequate aeration of cultures. Oxygen levels were checked regularly using a Teledyne portable oxygen analyser and the headspace regularly regassed to maintain the oxygen concentration within 10 % of the nominal value.

2.1.7 Vesicle number and morphology

Vesicles were viewed and counted under dark-field microscopy using a Reichert-Jung Polyvar photomicroscope.

Vesicle number was determined from six randomly selected grid counts. Care was taken to ensure culture subsamples were thoroughly homogenised prior to counting. Mature vesicles were scored as two, provesicles as one and the number of hyphal braches within ech square of the grid was also totalled. Thus vesicle number / unit hyphae was calculated as:

$$\frac{(\text{vesicle number} \times 2) + (\text{provesicle number} \times 1)}{\text{total hyphal branches in grid}}$$

Using dark field microscopy it is impossible to distinguish the various layers of the vesicle wall structure i.e. the cell membrane, cell wall and envelope consisting of lipid laminae. Consequently vesicle envelope thickness was measured together with the cell membrane and cell wall; a structure referred to in this thesis as the vesicle wall. Vesicle walla appear as images with varying degrees of brightness, an optical effect attributable to reflection of light from a lateral source. Previous work (Parsons *et al.* 1987) showed that while the bright image produced may not accurately define vesicle wall thickness, there is good reason to believe that it is at least proportional to wall thickness. Thus transferring the vesicle image to a high resolution TV monitor made it possible to measure the wall thickness on the screen using callipers. Data from approximately 400 vesicles was converted to give a measure of apparent vesicle wall thickness in μm.

2.2 Plants

2.2.1 Germination and nodulation of seedlings

Four actinorhizal species, *Alnus glutinosa*, *Casuarina cunninghamiana*, *Coriaria arborea* and *Myrica gale*, and a single legume species, lucerne (*Medicago sativa*), were used during this work. *Alnus* and *Casuarina* seed was collected from trees growing at the University of Waikato. *Coriaria* seed was collected from *Pinus radiata* forest near Tokoroa in the central North Island and *Myrica* seed, from a site in Massachusetts, USA, was supplied by Larry Winship. Lucerne seed was obtained commercially. Seed was germinated on pumice sand in the University of Waikato glasshouse.

Seedlings were inoculated to induce formation of nodules approximately four weeks after germination. *Alnus*, *Casuarina* and *Myrica* seedlings were inoculated by injecting the appropriate *Frankia* sp. strain i.e. ArI3, CcI3 or M+g, into the pumice sand next to the plant roots. *Coriaria* was nodulated in habitat soil collected from under mature, nodulated plants in the Tokoroa forest while lucerne was also nodulated by transferring seedlings to

a soil collected from under mature plants. To assist nodulation, seedlings were raised in the glasshouse on a heated sand bed (21 °C) under a 16 h day / 8 h night light regime. Daily watering was supplemented with half strength -N Ruakura nutrient solution (Smith *et al.* 1983) once a week.

2.2.2 Aeroponics system

Nodulated seedlings were transferred to an aeroponics system in the laboratory 3 to 4 weeks prior to use. The system was housed in a simple growth chamber and illuminated with an HLRG mercury vapour lamp and ambient daylight of 16 h per day. The chamber maintained temperatures several degrees above laboratory temperature in the 22 °C to 28 °C range but was not otherwise controlled.

Plants were grown on quarter strength -N Ruakura nutrient solution which was aerated. Nutrient solution level, conductivity and pH (pH 6) were checked regularly and adjusted accordingly. When plants became established the liquid level was lowered and plants were then maintained with only the lower roots in solution and the upper roots and nodules in an aeroponics environment created by the breaking bubbles of the aerator.

2.3 Acetylene reduction assays

2.3.1 General

In addition to reducing nitrogen (N_2) to ammonia (NH_3), nitrogenase will reduce acetylene (C_2H_2) to ethylene (C_2H_4) which can be detected using gas chromatography. This acetylene reduction assay provides a simple and rapid method of assaying nitrogenase activity and is now widely used for measuring activity in both free-living and symbiotic nitrogen-fixing systems.

The reactions for nitrogen and acetylene reduction, excluding hydrogen evolution, may be designated as follows:

$$N_2 + 6H^+ + 6e^- \text{ ----> } 2NH_3$$

$$C_2H_2 + 2H^+ + 2e^- \text{ ----> } C_2H_4$$

Thus the theoretical ratio for acetylene : nitrogen reduction based on substrates reduced is 3 : 1. However those nitrogen-fixing systems tested showed a ratio ranging from 2.3 to 3.8 (Masterton and Murphy 1980). Therefore, while acetylene reduction provides an indication of nitrogenase activity, absolute values for activity are better determined using ^{15}N enrichment techniques.

2.3.2 Acetylene

Acetylene was generated from calcium carbide and used at a concentration of 10 kPa. This gas is generally "cleaner" than the commercial product although it may contain traces of methane and ethylene. All gas mixtures were therefore tested for background ethylene.

2.3.3 Batch assays

In most experiments using *Frankia*, nitrogenase activity was measured using a batch assay technique. Batch assays were conducted in 7 ml bijou bottles containing 1 ml of culture. Acetylene was injected to give a final concentration of 10 kPa and samples incubated at 28 ^oC at the growth pO_2 of the culture. Gas samples (0.1 ml) were taken after a recorded time period, usually 45 minutes and again at 90 minutes, and ethylene concentration measured using gas chromatography. Replicate acetylene reduction rates were usually in close agreement so that any error bars which could be shown on graphs would be largely obscured by the symbols.

2.3.4 Continuous flow assays

Batch assay of acetylene reduction activity often provides an erroneous measure of nitrogenase activity because of a number of factors including the effects of a decrease in oxygen concentration in the incubation vessel, build-up of inhibitors, repression of enzyme activity products of nitrogen fixation, effects of using detached nodules and acetylene induced declines in activity. Consequently a continuous flow system was used to measure nitrogenase activity whenever possible.

The continuous flow system was based on a technique previously described by Minchin *et al.* (1983) and Silvester *et al.* (1988*a*). Gas mixtures containing various concentrations of oxygen, with either nitrogen or argon as the balance gas, were prepared using a Tylan mass flow controller and collected in 20 l PVC beach balls. The beach balls were impermeable to gas and no change in gas content could be detected over the course of the experiments. Rubber septa fitted into the balls allowed gas samples to be taken by syringe. Oxygen level was checked using a Teledyne portable oxygen analyser and all gas mixtures were water saturated to reduce dehydration of the plants. Beach balls were connected to a low volume manifold so that gas mixtures could be switched in rapid succession and gas was pumped at a set flow rate (usually 45 ml. min^{-1}) through a two-channel peristaltic pump.

Plants were removed from the laboratory growth chamber and inserted into a cuvette adapted from a 60 ml syringe with the head removed and a split rubber bung inserted to hold the plant. The cuvette was also fitted with an inflow gas tube at the base and an exit tube to the GC at the top. Moist filter paper was placed in the bottom of the cuvette to reduce plant dehydration and the syringe plunger adjusted to give a cuvette volume of approximately 20 mls. Thus the flushing rate of the cuvette was approximately 27 seconds. Throughout the experiment the plant was maintained at 25 ^{o}C by placing the cuvette in a water bath.

The continuous flow system could also be modified to measure nitrogenase activity in *Frankia* cultures (section 4.5.1) and for use in lag-phase measurements (sections 7.6 and 8.3).

2.3.5 Gas chromatography

Ethylene concentration was measured using a Shimadzu GC-8A gas chromatograph with nitrogen as the carrier gas and hydrogen and air as the combustion gases. Results were automatically analysed by an integrator (Shimadzu Cr-3A) and logged directly to a computer using Shimadzu Human Interface Tool software developed by John Curtis. Results from batch assays of acetylene reduction in *Frankia* were expressed as nmol C_2H_4. mg protein^{-1}. h^{-1}.

When using the continuous flow system, effluent gas was led via small bore tubing to an automatic switching valve that introduced 0.2 ml of gas into the GC every 75 seconds. Results were expressed as μmol C_2H_4. g dwt nodule^{-1}. min^{-1}.

2.3.6 Respiration

Root and nodule respiration was measured as CO_2 evolution in conjunction with acetylene reduction activity. Waste gas from the GC switching valve was fed to an ADC Series 225 Infra Red Gas Analyser (IRGA) using small bore tubing and the mean rate of CO_2 evolution over each 75 second period logged on computer. Prior to the experiment, CO_2 was injected into the gas bags to give between 350 and 400 ppm CO_2 which was measured on the IRGA at the beginning and end of the experiment. By subtracting the background CO_2 level of the bags and calculating the proportion of CO_2 evolution due to nodule activity (section 8.2) the results could be expressed as μmol CO_2. g dwt nodule-1. min-1.

CHAPTER THREE

FRANKIA IN BATCH AND CONTINUOUS CULTURE

3.1 Introduction

Since the first isolation and culture of *Frankia* from root nodules of *Comptonia peregrina* (Callaham *et al.* 1978) there has been considerable interest in defining optimum conditions for growth of *Frankia in vitro*. Various research groups have focussed on determining factors such as the most desirable inoculum size (Murry *et al.* 1984*b*), media components (Blom *et al.* 1980; Blom 1981; Shipton and Burggraaf 1982; Akkermans *et al.* 1983; Murry *et al.* 1984*b*;; Zhang *et al.* 1986), temperature (Burggraaf and Shipton 1982; Murry *et al.* 1984*b*; Zhang *et al.* 1986) and pH (Burggraaf and Shipton 1982; Murry *et al.* 1984*b*; Zhang *et al.* 1986) for growth of a number of *Frankia* strains in culture.

Frankia has been grown, until now, in batch culture which is an example of a closed system since the essential components of the system cannot enter or leave it. In a closed system the growth rate of the biomass must tend towards zero, either because of lack of an essential nutrient or because accumulation of a product can no longer be tolerated (Pirt 1975).

Most bacteria follow the normal growth cycle for a bacterial population in a batch culture system which is divided into four stages: lag phase, growth phase, maximum stationary phase and death phase (Drew 1981). However it has been shown (Murry *et al.* 1984*b* ; Zhang *et al.* 1986) that *Frankia* growth in batch culture, both with a source of combined nitrogen (Murry *et al.* 1984*b*) and without (Murry *et al.* 1984*b* ; Burggraaf and Shipton 1983), deviates from this pattern. Both growth rate (protein level) and nitrogenase activity in derepressed cultures (cultures induced to synthesise nitrogenase by transfer to

medium lacking a source of combined nitrogen) decline rapidly after a short stationary phase as cultures begin to lyse and vesicles degenerate ("ghost" vesicles) (Fontaine *et al.* 1984; Murry *et al.* 1984*b*). Thus *Frankia* grown in batch culture does not display the stationary phase typical of most other bacteria.

Since *Frankia* in batch culture has a continually changing growth rate and physiology, it is difficult to distinguish the effects of external environmental changes from those due to normal, endogenous development. Unequivocal evidence of environmental influences on *Frankia* morphology and physiology could be obtained if a steady state of growth and nitrogenase activity were maintained. Such conditions can be achieved in "open" systems, for example continuous flow cultures. However, previous attempts to prolong a state of stable growth and nitrogenase activity in *Frankia* cultures have been unsuccessful (Murry *et al.* 1984*b*).

The aim of this work was firstly to confirm the behaviour of *Frankia* in batch culture and secondly to test several simple methods of prolonging the maximum stationary phase in batch culture with the overall objective being to develop a technique for maintaining *Frankia* in continuous culture.

3.2 *Frankia* in batch culture

Numerous studies have focussed on determining the most favourable cultural conditions for growing various *Frankia* strains in batch culture, first with complex media and then with defined media. For example, trials conducted with *Frankia* sp. strain HFPCcI3 on various complex media (Baker and Torrey 1979, Lalonde and Calvert 1979) failed to produce rapid growth and vegetative filaments frequently appeared abnormal. However synthetic media, such as BAP (Murry *et al.* 1984*b*), with defined carbon and nitrogen sources, gave excellent growth rates. Zhang *et al.* (1986) achieved maximum growth with a doubling time of approximately 24 h by culturing CcI3 at 33 ^{o}C and pH 6.3 in

BAP using pyruvate as the primary carbon source and NH_4Cl as the sole nitrogen source. *Frankia* sp. strain HFPArI3 however shows fastest growth on DPM medium (Baker and O'Keefe 1984), a defined medium with propionate and NH_4Cl as the sole carbon and nitrogen sources respectively. Growth of ArI3 in air-sparged, stirred cultures at 28 °C yielded a high growth rate with a doubling time of less than 48 h (Murry *et al.* 1984*b*).

Although *Frankia*, in contrast to the majority of bacteria, is particularly slow growing, the growth rate achieved by Zhang *et al.* (1985) for CcI3 is relatively fast compared with the results from other studies on *Frankia*. Nutritional studies (Blom 1982, Lechevalier *et al.* 1983, Shipton and Burggraaf 1983, Murry *et al.* 1984*b*, Zhang *et al.* 1985) have shown doubling times may vary from one to four days for different *Frankia* strains depending on growth conditions.

The aim of this experiment was to determine the kinetics of growth, nitrogenase activity and vesicle development of *Frankia* sp. strains HFPCcI3 and HFPArI3 in batch culture under the growth conditions described below.

CcI3 was established in six replicate batch cultures on nitrogen-free B medium. An inoculum was added to each 100 ml medium to give an initial protein level of 20 $\mu g.\ ml^{-1}$. Duplicate subsamples were removed from cultures at regular intervals for measurement of nitrogenase activity, protein level and observation of vesicles.

For a 78 h period immediately following inoculation CcI3 showed a relatively slow growth rate i.e. lag phase (Fig. 3.1). However after 78 h the growth rate increased rapidly to reach a maximum around 96 h. During this exponential growth phase the doubling time was approximately 38 h but if the lag period is included the doubling time was closer to 96 h. Following the exponential growth phase, protein level increased only slowly until about 332 h whereupon the protein level decreased (Fig. 3.1). This was presumably an indication that autolysis occurred in cultures.

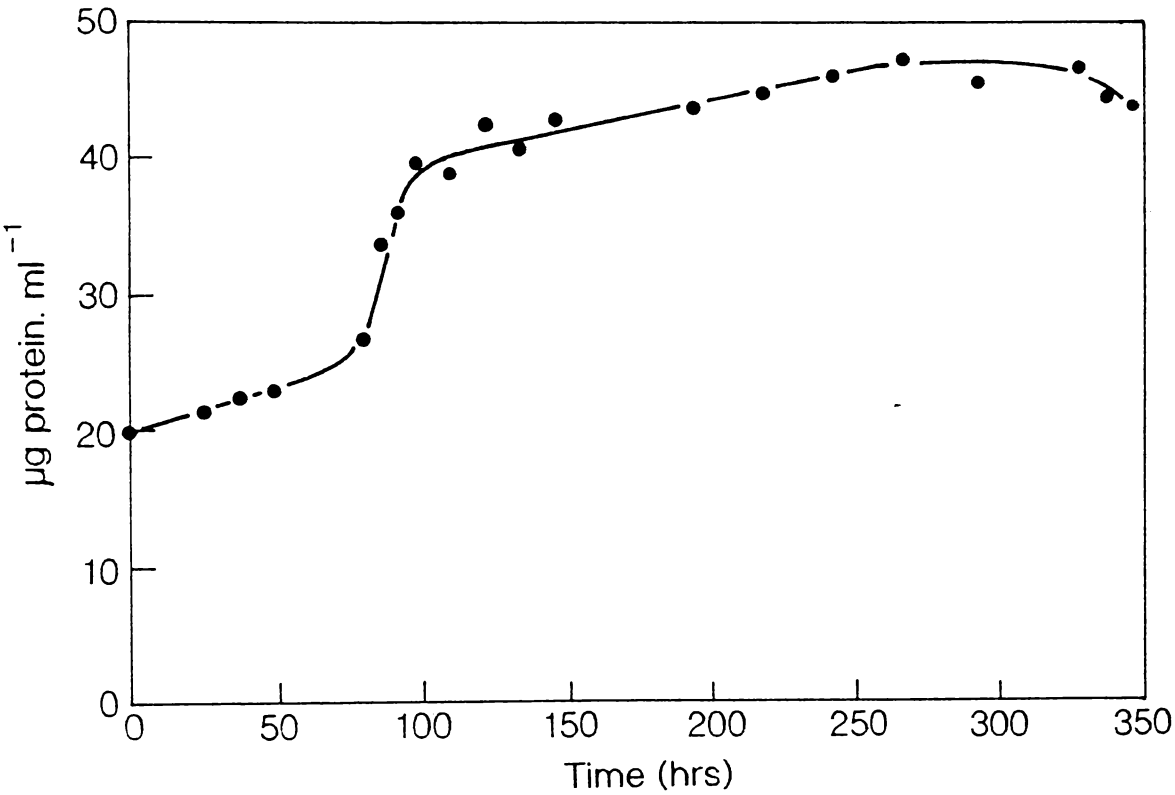


Figure 3.1 Growth (protein level) of *Frankia* sp. strain HFPCcI3 grown in batch culture.

In response to removal of combined nitrogen from the medium at inoculation CcI3 formed vesicles at terminal positions of some hyphae. Thin-walled provesicles, approximately 2 μm in diameter (Fig. 3.2 a), first appeared after 42 h. A rapid increase in the number (Fig. 3.3) of vesicles was then observed for the next 90 h. During this period vesicles matured, increasing in size to reach 2-3μm diameter and developing thicker walls which appeared much brighter than the hyphal walls under dark-field microscopy (Fig. 3.2 b). After 130 h vesicle number remained relatively constant (Fig. 3.3) although some vesicles started to appear structually damaged i.e. ghost vesicles (Fig. 3.2 c) around 225 h. Towards the end of the experiment vesicle number decreased (Fig. 3.3), presumably as lysis of vesicles occurred.

Induction of nitrogenase activity showed a lag of approximately 75 h with no nitrogenase activity detected in any cultures prior to this despite the presence of provesicles (Fig. 3.4). The subsequent onset and rise in nitrogenase activity coincided with the formation of mature vesicles. Activity increased exponentially to reach a maximum of 139 nmol C_2H_4. mg protein^{-1}. h^{-1} at 206 h. However CcI3 was unable to maintain a high rate of nitrogenase activity in batch culture and activity declined rapidly so that after 280 h no further activity could be detected in any cultures.

Frankia sp. strain HFPArI3 was also grown in batch culture on DPM medium under the same conditions as described for CcI3.

The kinetics of growth, nitrogenase activity and vesicle development were similar to those detailed for CcI3. Growth (Fig. 3.5) and nitrogenase activity (Fig. 3.6) showed an initial lag phase of approximately 72 h following inoculation. As in CcI3 culturesthe onset and rise in nitrogenase activity coincided with the appearance of and increase in the number of mature vesicles. A maximum steady state rate of activity could not be maintained however and after 190 h nitrogenase activity rapidly declined (Fig. 3.6). Like CcI3, ArI3 showed a decline in protein level towards the end of the experiment although the decline shown by ArI3 was considerably larger.

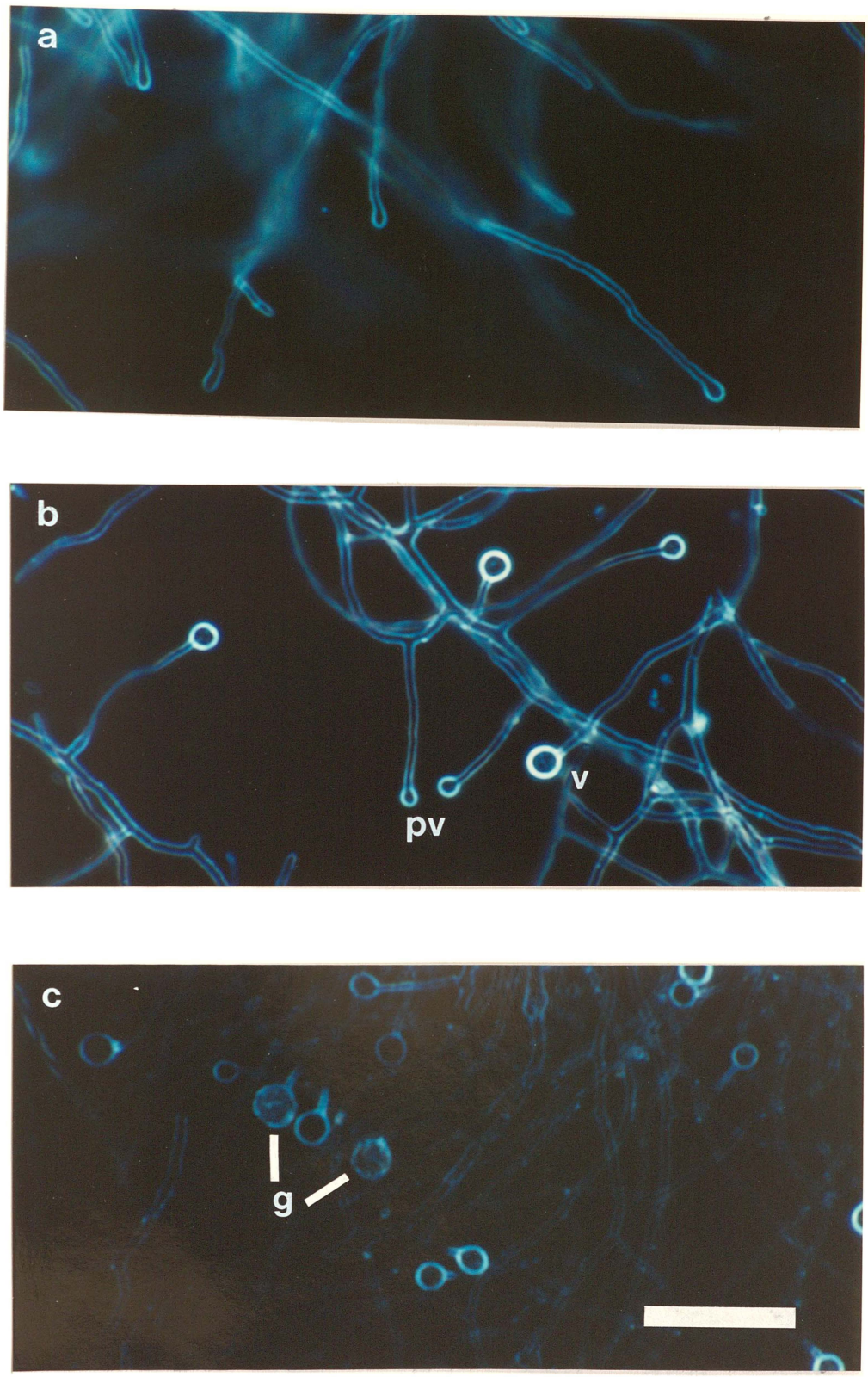


Figure 3.2 Stages of vesicle development in *Frankia* grown in batch culture viewed under dark field microscopy: (a.) early provesicles; (b.) provesicles (pv) and mature vesicles (v); (c.) "ghost" vesicles (g). Scale bar = 10 μm.

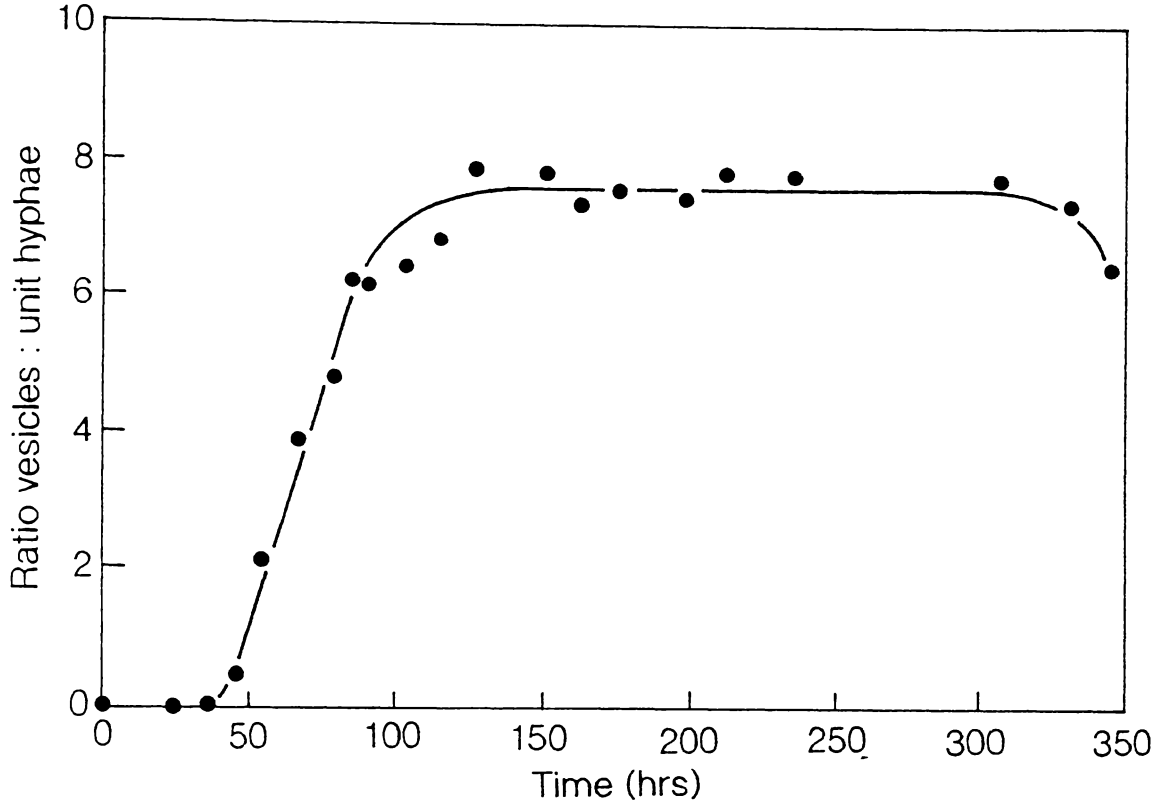


Figure 3.3 Change in vesicle number (see section 2.1.7) in *Frankia* sp. strain HFPCcI3 grown in batch culture.

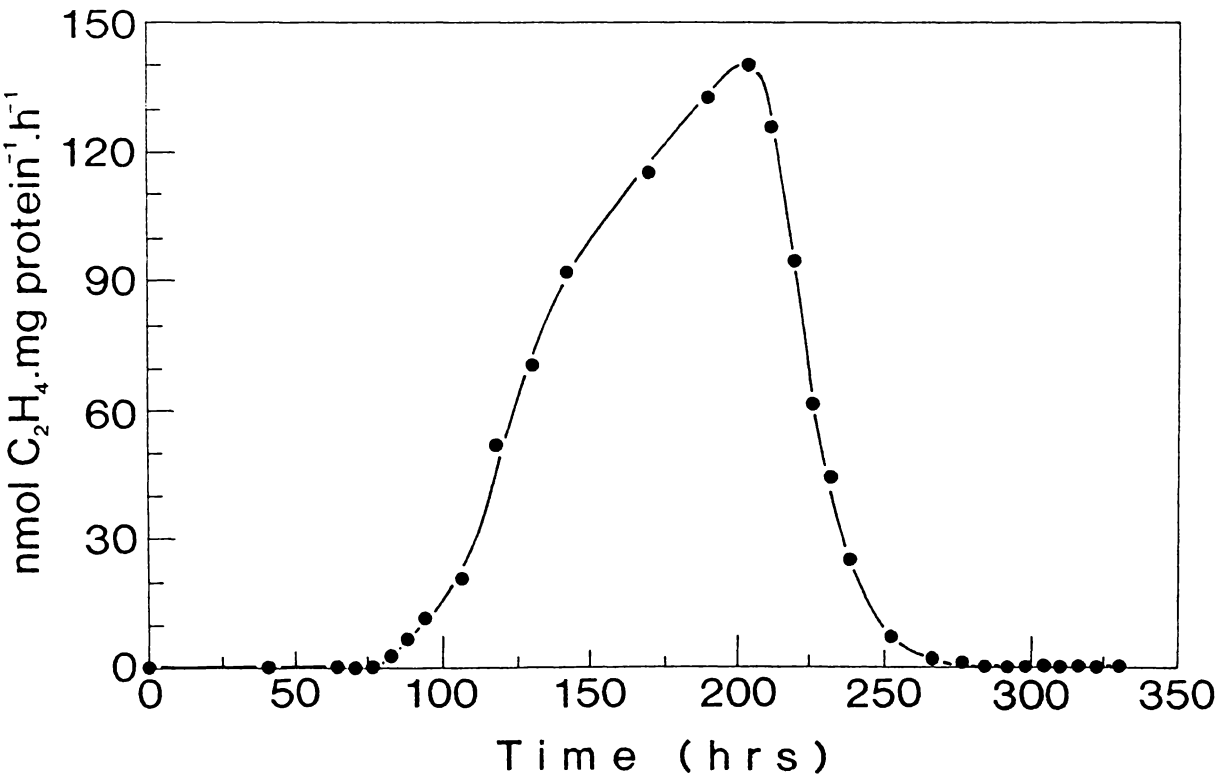


Figure 3.4 Kinetics of nitrogenase activity of *Frankia* sp. strain HFPCcI3 grown in batch culture.

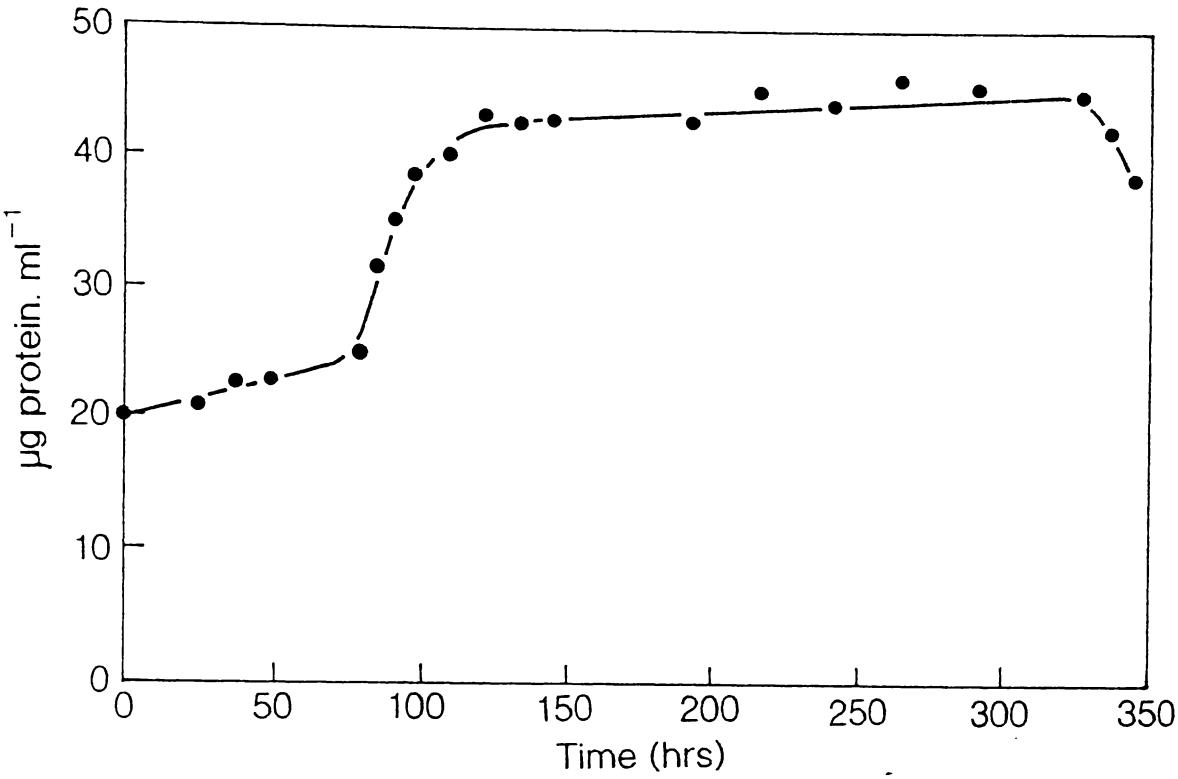


Figure 3.5 Growth (protein level) of *Frankia* sp. strain HFPArI3 grown in batch culture.

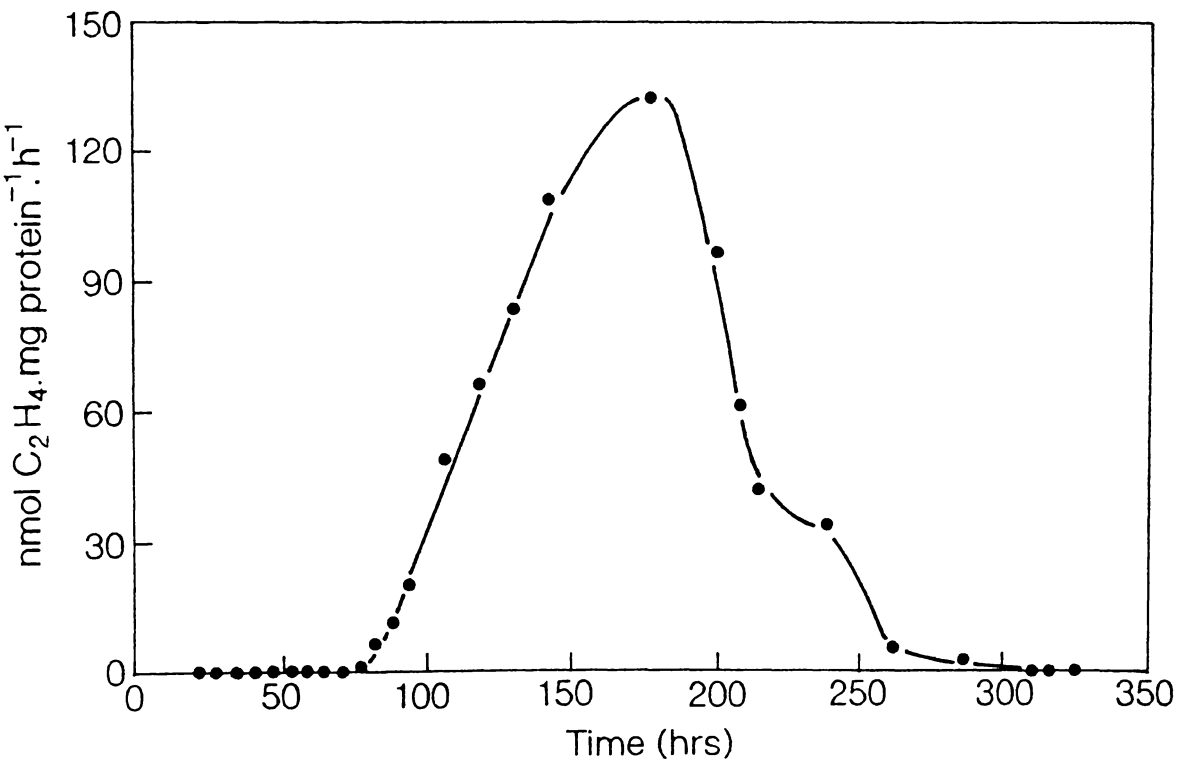


Figure 3.6 Kinetics of acetylene reduction activity of *Frankia* sp. strain HFPArI3 grown in batch culture.

3.3 Effect of addition of carbon on batch cultures

One factor contributing to the decline in growth rate and nitrogenase activity of *Frankia* in batch culture may be a limitation in the amount of available carbon. It was therefore decided to investigate whether maintaining the carbon level in cultures would help to prevent the decline in activity of ArI3 growing on DPM medium.

Cultures were established as described above and sodium propionate (5 mM), the carbon and energy source in DPM medium, added by syringe through the rubber septum at 190, 258 and 316 h. Several batch cultures were also established to act as controls and these gave profiles of acetylene reduction activity similar to Figure 3.6. Addition of sodium propionate at 190 h was followed by the return of almost all the acetylene reduction activity, giving a second peak at 250 h (Fig. 3.7). An increase in the number of provesicles and vesicles was also observed after sodium propionate was added. Although subsequent additions of sodium propionate at 258 and 316 h were followed by short-term increases in activity, the maximum rate was never regained and activity eventually declined to zero (Fig. 3.7).

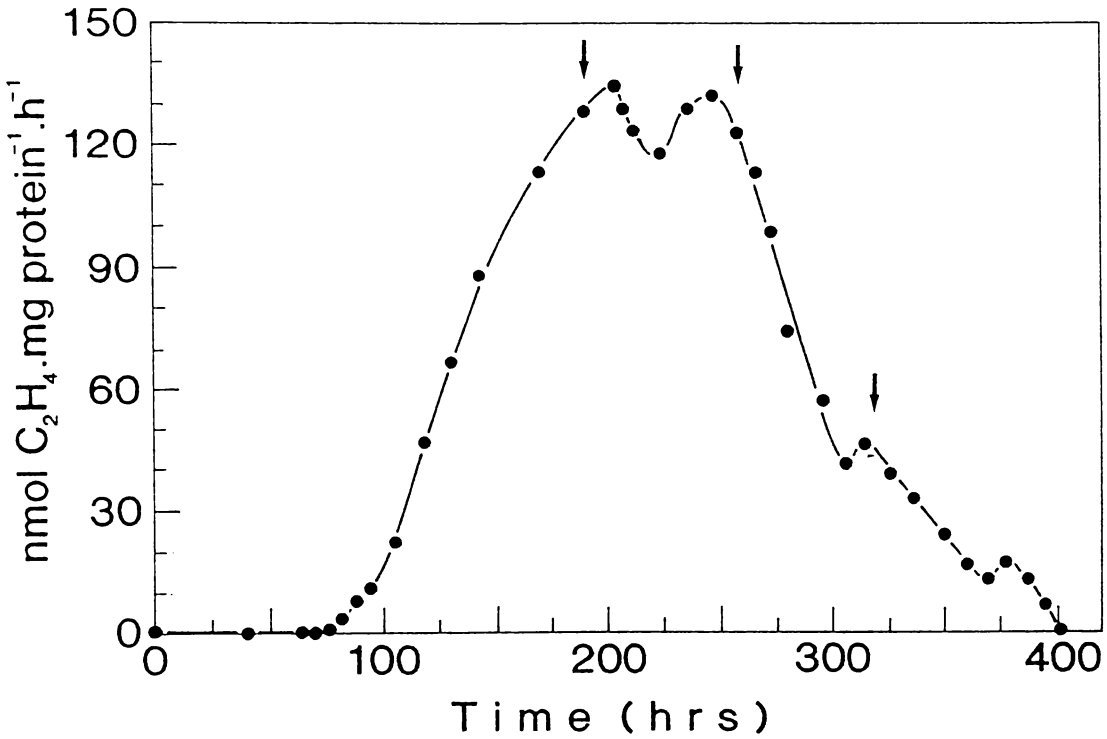


Figure 3.7 Effect of addition of 5 mM propionate (↓) on acetylene reduction activity of *Frankia* sp. strain HFPArI3 grown in batch culture.

3.4 Effect of transfer to fresh medium

Depletion of medium components other than the carbon source may also contribute to the decline in growth rate and nitrogenase activity observed in batch culture. Experiments were therefore conducted to test whether transferral of already derepressed cultures to fresh medium would allow a maximum level of growth and nitrogenase activity to be maintained.

Cultures of CcI3 were set up as described earlier and transfer of *Frankia* to fresh B medium was accomplished by inverting the bottles to allow the *Frankia* to settle out, removing the old medium through the rubber septum in the bottle lid using a syringe and adding the equivalent volume of fresh medium in the same fashion.

Transfer of *Frankia* already growing on nitrogen-free medium, to fresh medium resulted in recovery of acetylene reduction activity although the extent of recovery appeared to be dependent on the time of transfer (Fig. 3.8). Cultures transferred to fresh medium after 180 h, at which time acetylene reduction activity was near maximum, suffered only a slight decrease in activity before maximum rate was regained. Cultures transferred to fresh medium after 220 h, approximately half-way through the decline phase, recovered approximately 70 % of their maximum activity. This second peak in nitrogenase activity was reached approximately 105 h after transfer to fresh medium, a considerably shorter time period than that from inoculation to the first peak in acetylene reduction activity. Cultures transferred at 300 h, after 20 h with no nitrogenase activity, recovered only 11 % of the maximum activity level. This second, smaller peak was reached 190 h after transfer to new medium, a similar time period to that taken to reach the first peak in activity following inoculation.

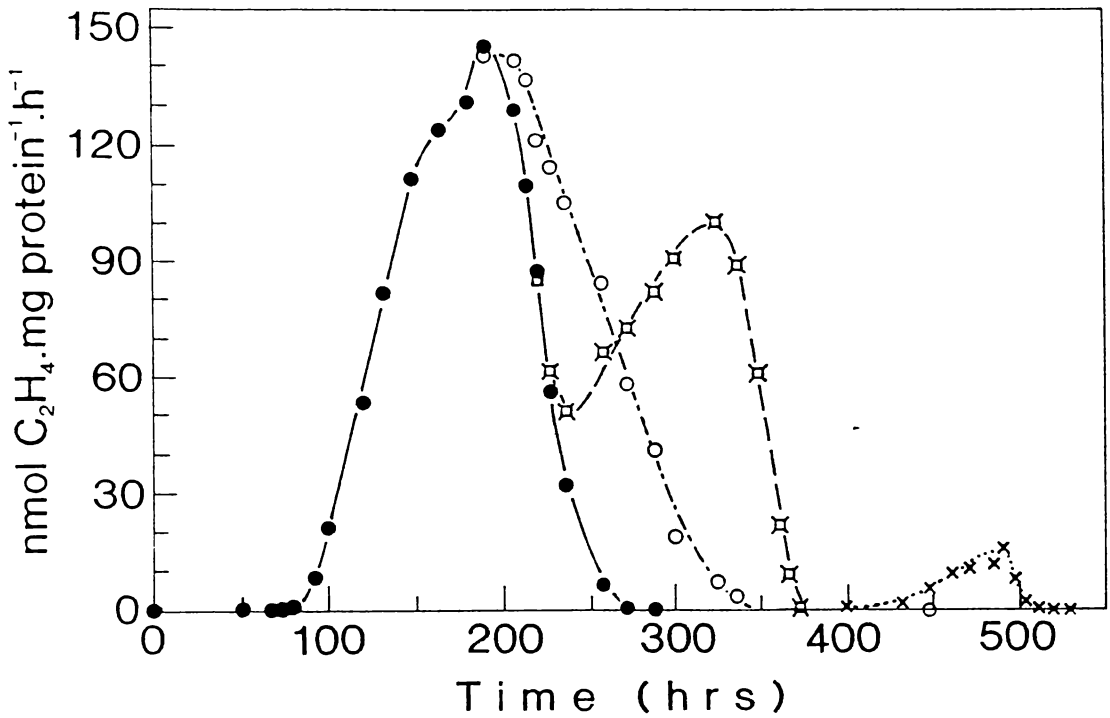


Figure 3.8 Nitrogenase activity of *Frankia* sp. strain HFPCcI3 in batch culture (—●—) and following transfer to fresh B medium after 180 h (-·-o-·-), 220 h (—⋈—) and 300 h (-----×-----).

3.5 *Frankia* in continuous culture

The previous experiment suggested nitrogenase activity could be maintained by regular transferral of *Frankia* to fresh medium. However this technique would probably be of little practical use since transfer usually involves some loss of the *Frankia* population or disturbance of environmental conditions. Instead maximum nitrogenase activity could be sustained, without incurring the above practical difficulties, by regular addition of fresh sterile medium and removal of an equivalent amount of culture, i.e. by establishing a continuous culture regime. Initial trials showed addition of fresh medium sustained a higher level of acetylene reduction activity for longer periods of time than when only the carbon source was added or when *Frankia* was grown in batch culture.

3.5.1 Effect of dilution rate

A number of continuous cultures were set up to determine the effect of dilution rate (D) on the growth (protein level) and nitrogenase activity of *Frankia* sp. strain HFPArI3. Nitrogen-free medium was added once daily to give dilution rates ranging from 0.031 day^{-1} to 0.250 day^{-1} and equivalent volumes of culture removed to prevent dilution of cultures.

Higher dilution rates (D=0.0165 day^{-1} and D=0.250 day^{-1}) caused a steady decline in protein level and eventually led to extreme dilution or washout of cultures (Fig. 3.9). However more stable protein levels of between 0.035 to 0.055 mg protein. ml^{-1} were maintained in cultures with dilution rates of 0.125 day^{-1}, 0.063 day^{-1} and 0.031 day^{-1} (Fig. 3.9).

Dilution rate also had a significant effect on nitrogenase activity. Cultures took approximately 230 h to reach maximum activity irrespective of dilution rate (Fig. 3.10). However a maximum specific activity rate of approximately 150 nmol C_2H_4. mg

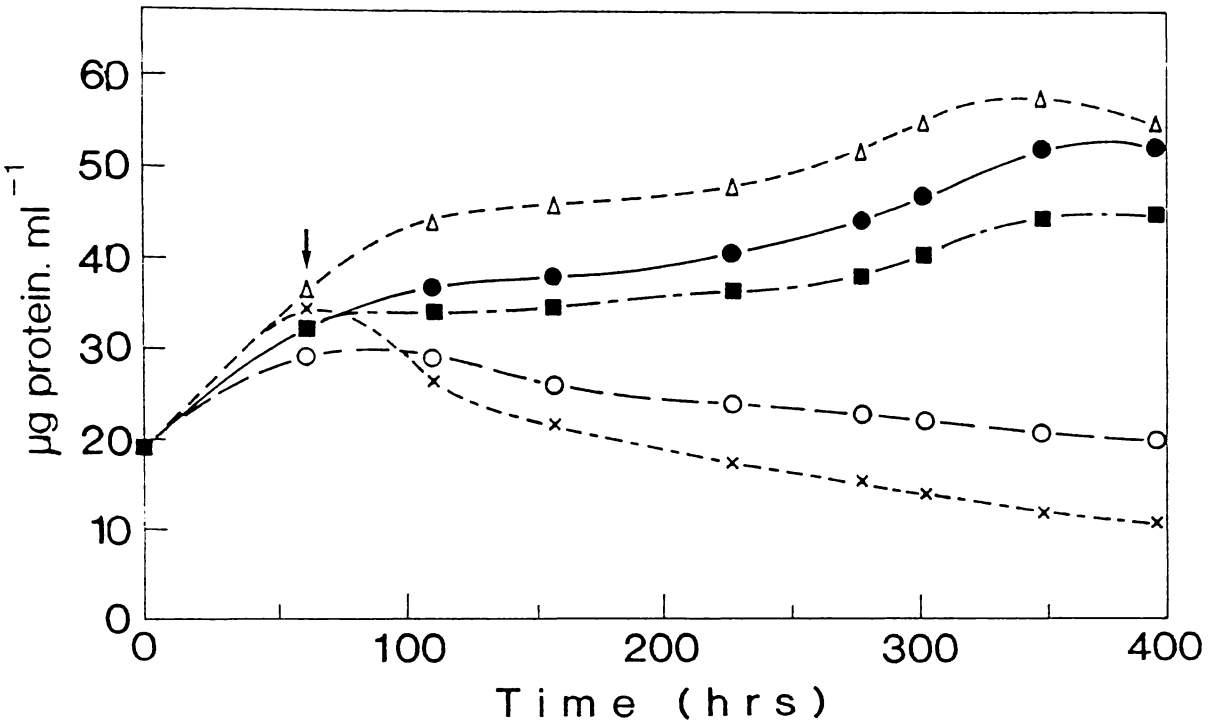


Figure 3.9 Effect of dilution rate (D) on growth (protein level) of *Frankia* sp. strain HFPArI3 grown in continuous culture. D = 0.031 day^{-1} (—Δ—), 0.063 day^{-1} (—●—), 0.125 day^{-1} (—■—), 0.165 day^{-1} (—○—) and 0.250 day^{-1} (—×—).

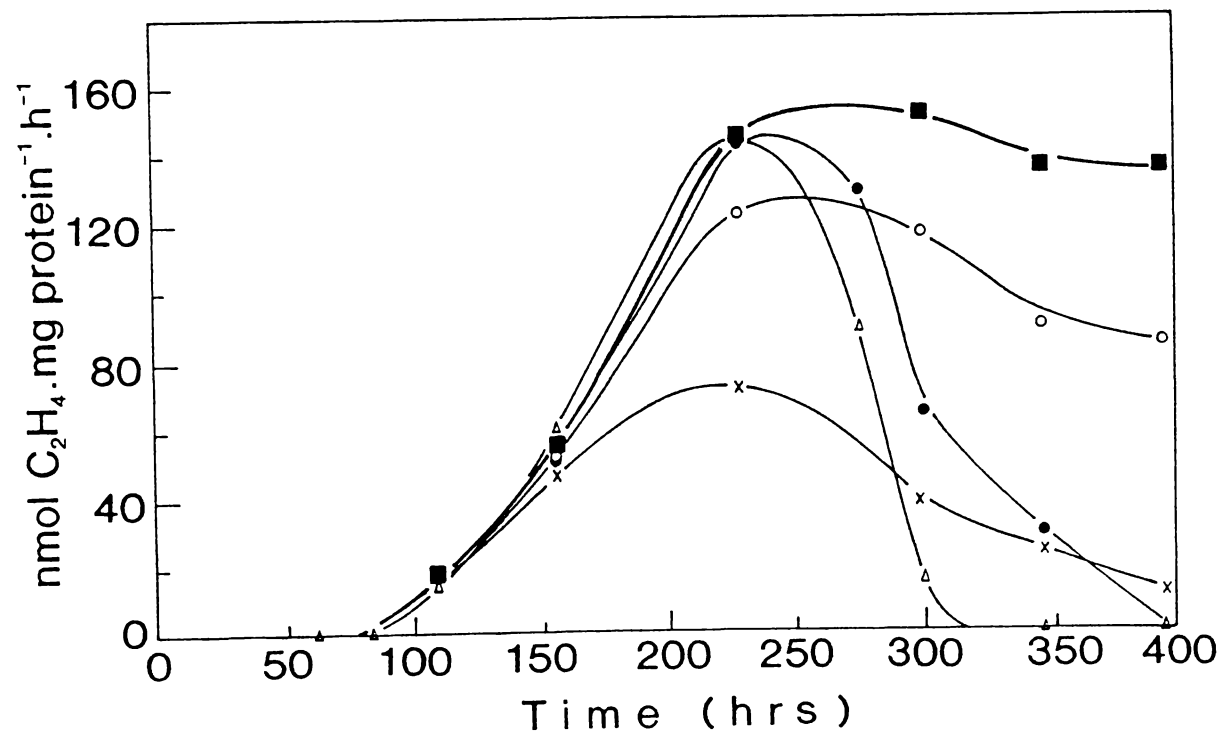


Figure 3.10 Effect of dilution rate on nitrogenase activity of *Frankia* sp. strain HFPArI3 grown in continuous culture. D = 0.031 day^{-1} (—Δ—), 0.063 day^{-1} (—●—), 0.125 day^{-1} (—■—), 0.165 day^{-1} (—○—) and 0.250 day^{-1} (—×—).

protein^{-1}. h^{-1} was maintained only with a dilution rate of 0.125 day^{-1}, i.e. 12.5 mls of medium were added per 100 mls of culture per day and 12.5 mls of culture removed. At higher dilution rates activity was generally lower and decreased further as cultures suffered washout. Nitrogenase activity in cultures with low dilution rates rapidly decreased, similar to the decline in activity observed in batch cultures (Fig. 3.10). The effect of dilution rate on total activity levels (nmol C_2H_4. 100 mls culture^{-1}. h^{-1}) was similar (data not shown) with a stable level of activity maintained only at a dilution rate of 0.125 day^{-1}.

Similar experiments, using *Frankia* sp. strain HFPCcI3, indicated this strain is best maintained in continuous, derepressed culture at a slightly lower dilution rate of 0.100 day^{-1}, i.e. 10 mls of medium added per 100 mls of culture per day and 10 mls of culture removed.

3.5.2 Frequency of feeding in continuous culture

A further experiment was conducted to determine whether continuous, derepressed cultures could be maintained if fresh medium was added only once per day or whether more regular addition of medium, at the same dilution rate, would give a more stable level of growth and nitrogenase activity.

Frankia sp. strain HFPArI3 was grown in continuous culture as previously described, at a dilution rate of 0.120 day^{-1} (12 mls of medium added per 100 mls culture). Three treatments were set up so that either 12 mls of medium were added every 24 h, 6 mls of medium added every 12 h or 3 mls of medium added every 6 h. Acetylene reduction activity (Fig. 3.11) and growth rate (protein level) were not significantly different for each of the three treatments and a stable level of growth and nitrogenase activity was reached after approximately 200 h regardless of how frequently fresh medium was added to the cultures.

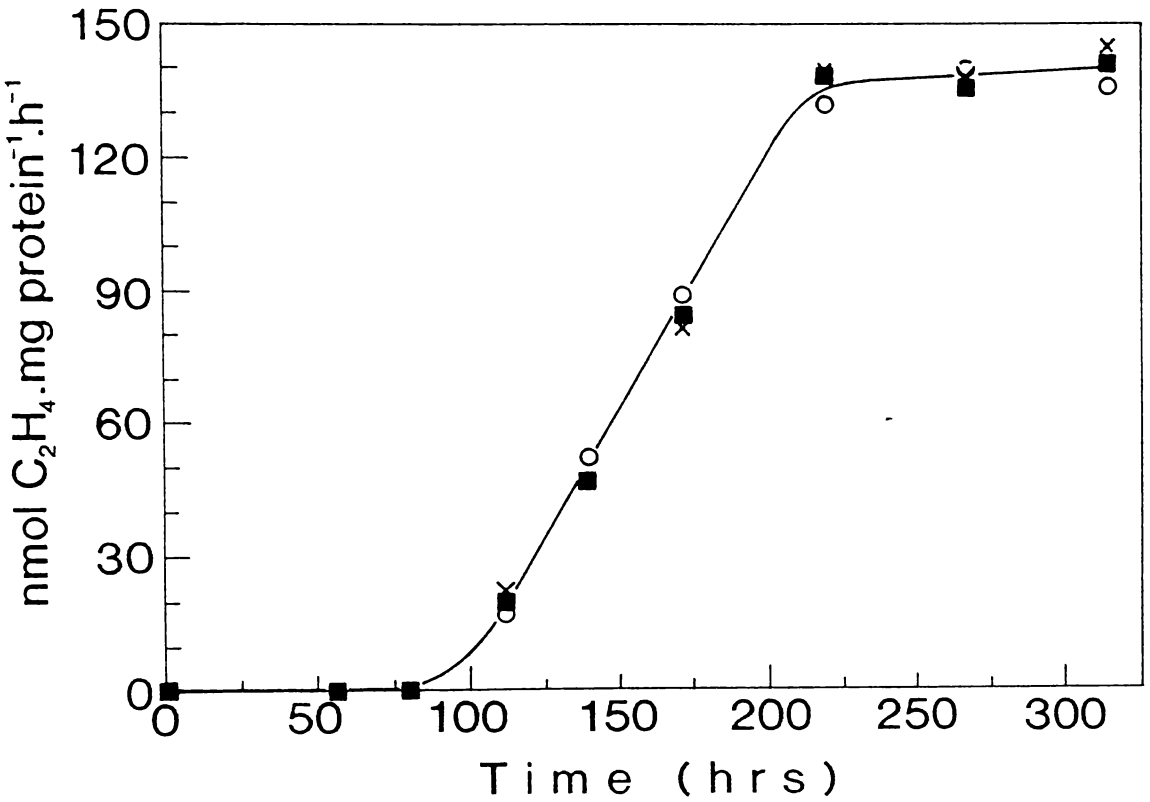


Figure 3.11 Effect of frequency of addition of medium on nitrogenase activity of *Frankia* sp. strain HFPArI3 grown in continuous culture (D=0.120 day^{-1}). Treatments were : 12 mls added every 24 hours (—■—), 6 mls added every 12 hours (—O—) or 3 mls added every 6 hours (—×—).

3.6 Vesicle populations in batch and continuous culture

Examination of ArI3 vesicles indicated a significant difference in the relative proportions of provesicles, mature vesicles and "ghost" vesicles between batch and continuous cultures (D=0.125 day^{-1}) (Fig. 3.12). The composition of vesicle populations at 48, 80 and 120 h was similar in batch and continuous culture and under both regimes the proportion of provesicles decreased from approximately 40 % at 48 h to 11 % at 120 h as vesicles matured. In continuous culture the proportion of provesicles remained relatively constant for the remainder of the experiment and balanced the number of vesicles lost from the active population as "ghost" vesicles. In batch culture however the proportion of provesicles continued to decrease while "ghost" vesicles rapidly increased in number after 120 h. The resultant decrease in the size of the mature vesicle population is correlated with the decline in nitrogenase activity observed in batch culture (Fig. 3.6).

3.7 Discussion

Growth kinetics of *Frankia* sp. strains HFPCcI3 and HFPArI3 in batch culture closely resemble those of other filamentous bacteria and fungi and are typical of most *Frankia* strains grown under similar conditions. The overall doubling time calculated for CcI3 in this study was typical of that for most *Frankia* strains although Zhang *et al.* (1986) achieved a faster doubling time for CcI3 by increasing the growth temperature to 33 °C rather than 28 °C as was used here. Medium pH and carbon source have also been shown to have an effect on growth rate (Shipton and Burggraaf 1983; Zhang *et al.* 1984, 1986; Tisa and Ensign 1987). In this study the optimum pH and carbon source i.e. pyruvate for CcI3 and propionate for ArI3, were used. Zhang *et al.* also showed growth was slower when cultures were stirred on a rotary shaker rather than air-sparged. In this study several attempts made to air-sparge cultures were unsuccessful largely due to contamination problems. However some form of mixing and aeration of cultures is

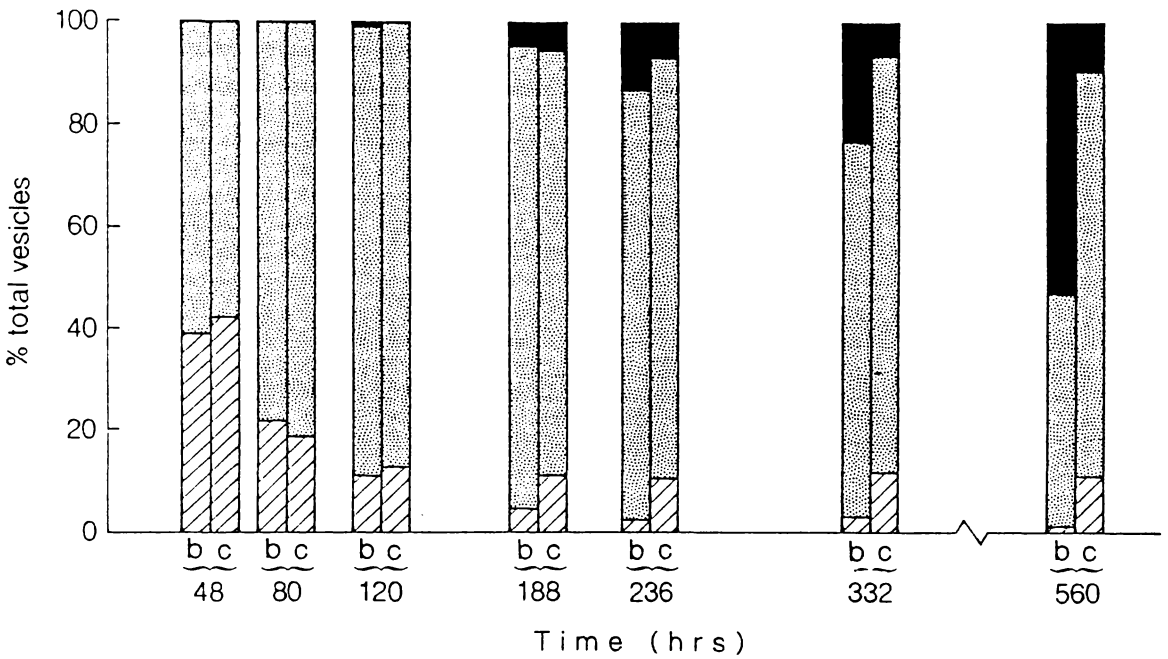


Figure 3.12 Change in proportion, expressed as percent of total vesicles, of provesicles (▨), mature vesicles (▩) and "ghost" vesicles (■) in batch (b) and continuous (c) cultures of *Frankia* sp. strain HFPArI3 sampled at different times in culture.

necessary to maintain a reasonable growth rate since, without agitation, cultures become oxygen limited.

CcI3 and ArI3 grown under derepressing conditions both showed a lag period in growth (protein level) of approximately 70 h before the start of the exponential growth phase. A similar lag phase was observed prior to the onset of nitrogenase activity. This lag is largely due to the time taken to form mature vesicles and induce nitrogenase (Fontaine *et al.* 1984; Murry *et al.* 1984*b*) The fixed nitrogen resulting from nitrogenase activity then contributes to the sudden rise in growth rate. Thus the onset of exponential growth, nitrogenase activity and the formation of mature vesicles are all interrelated.

Lag phases of variable length have also been observed in other *Frankia* strains in culture on both +N and -N media. *Frankia* sp. strain AvcI1 for example had a lag phase of 48 h under derepressed conditions while LDMg1 showed a considerably longer lag phase of 19 days under similar conditions (Burggraaf and Shipton 1983). Murry *et al.* (1984*b*) report the lag phase following inoculation can be minimised by use of a large inoculum (10 to 20 % v/v). A similar result has been shown by Burggraaf and Shipton (1983). Murry *et al.* (1984*b*) also showed the composition of the inoculum has an effect on the length of the lag phase and on subsequent nitrogenase activity. A short lag phase, maximum rates of acetylene reduction and the longest duration of activity resulted when exponential phase cells were used as the inoculum. Lower, but still substantial rates were obtained when late, lag phase cells were used but inocula composed of declining phase cells gave little activity. The inocula used throughout this study was obtained from five-to-seven-day old ammonia-grown cultures and so was composed mainly of exponential phase cells. However, the inoculm was not particularly large and therefore a lag phase was still observed although Murry *et al.* (1984*b*) report that inocula of the size used in this study (give an initial protein level of approximately 20 μg protein. ml^{-1}) generally gave maximum specific acetylene reduction rates. Thus the composition of the inoculum is of great importance to the metabolic behaviour of *Frankia* in successive cultivations, a situation common among mycelial organisms (Gottleib and van Etten 1964).

Neither CcI3 nor ArI3 was able to maintain a maximum rate of nitrogenase in batch culture for more than a few hours. Protein level also decreased toward the end of the experiment indicating autolysis was occurring in the cultures. Autolysis in batch culture has been previously reported for ArI3 (Murry *et al.* 1984*b*) and other *Alnus* isolates and has been shown to occur irrespective of the protein or pH level reached in cultures (Burggraaf and Shipton 1983). However Zhang *et al.* (1986) observed no autolysis in batch cultures of CcI3 and cells remained viable for at least seven days under the growth conditions they described. Although CcI3 did show evidence of the occurrence of autolysis, the decrease in protein level was not as significant as the decrease exhibited by ArI3 cultures. This suggests CcI3 may have a lower susceptibility to autolysis than some other *Frankia* strains .

Reasons for rapid decline in growth and nitrogenase activity and eventual lysis of *Frankia* in batch culture are still not clear. Autolysis in response to carbon limitation has been observed for other bacteria (Drew 1981) and is a possible explanation for the lack of a stationary phase in *Frankia*. Murry *et al.* (1984*b*) suggested other reasons may be involved such as induction of an activation factor upon reaching critical cell mass. Autolysis may also be an inherent feature of the *Frankia* life cycle since hyphal disintegration has been observed in aging nodules (Newcomb *et al.* 1978). If carbon limitation is the sole factor causing decline, addition of sodium propionate at regular intervals would be expected to have a more significant effect on maintaining a maximum rate of acetylene reduction activity. Murry *et al.* (1984*b*) found that addition of 5 mM sodium propionate to eight-day-old cultures caused a second peak in both vesicle number and specific nitrogenase activity but only at low cell densities. In work presented here, only the first increment of additional carbon resulted in a significant increase in acetylene reduction activity approximately 58 h later. The minimal response following two further carbon additions may have resulted from the *Frankia* cell mass being higher than at the time of the first addition. An alternative explanation for the low response could be that further addition of carbon caused the propionate level to become inhibitory. Murry *et al.* (1984*b*) found ArI3 respiratory activity is inhibited by propionate levels above 5.5 mM

and is abolished at 12.5 mM. If regular addition of further carbon source was to be used as a technique for maintaining *Frankia* cultures, it would be essential to monitor the level of carbon and other nutrients present in the medium to keep them balanced. It is therefore more practical to add complete medium.

Transfer of *Frankia* to fresh medium also resulted in recovery of acetylene reduction activity although the extent of recovery appeared to be dependent on when cultures were transferred. For example, *Frankia* transferred to fresh medium 20 h after losing nitrogenase activity only recovered a low level of activity. This may have been because the *Frankia* transferred contained only a small proportion of live cells. *Frankia* within nodules of *Alnus incana* (L.) plants kept in the dark for two- and four-day periods showed signs of degradation and had a reduced level of acetylene reduction activity. These changes were probably triggered by carbon limitation. On return to a normal light-dark cycle a proportion of the infected cells and *Frankia* showed signs of recovery indicating that, in some cells at least, the effect of carbon limitation was irreversible (Vikman *et al.* 1990). Thus it is unlikely the nitrogenase enzyme in *Frankia* vesicles transferred to fresh medium recovered to give a second peak in activity. In fact the lag from the time of transfer at 300 h to the second small peak at 490 h is comparable to the time taken to reach the initial peak in activity following inoculation. This fact indicates recovery of activity following the final transferral probably involved formation of new vesicles and synthesis of more nitrogenase enzyme.

By comparison, *Frankia* transferred at mid-decline recovered proportionately more activity, probably because the culture contained a larger percentage of active vesicles. Since the lag time to the second peak in activity of *Frankia* transferred at mid-decline phase was considerably shorter than the initial time lag, it is possible that recovery of acetylene reduction activity involved only reactivation of nitrogenase and synthesis of further enzyme in existing vesicles rather than formation of new vesicles as well.

An increase in the level of inhibitory waste products present may also have contributed to lower levels of recovery in older cultures. Upon transfer, the *Frankia* remained in contact with small volumes of the old medium and cells were not washed with fresh medium.

The changes in nitrogenase activity correlated closely with morphological development of vesicles in batch culture. Under such conditions a single cohort of provesicles arises and undergoes a series of morphological, physiological and biochemical transformations through to mature vesicles which then degenerate to form ghost vesicles. No acetylene reduction activity is associated with the thin-walled provesicles. TEM work by Fontaine *et al.* (1984) showed these vesicles to be relatively unspecialised with dense cytoplasm and very few internal septa. Vesicles which matures past the provesicle stage increased in size and developed a thicker wall. The cross-wall delineating the vesicle from the subtending hypha also appeared much clearer under dark field microscopy. TEM microscopy (Fontaine *et al.* 1984) revealed the degree of internal compartmentalisation of mature vesicles increased with the formation of internal septations. The decline in nitrogenase activity in batch culture was accompanied by an increase in the proportion of ghost vesicles. Ghost vesicles typically appear empty and Fontaine *et al.* (1984) showed they exhibit a breakdown of the cytoplasm and internal septa at the ultrastructural level. Immunolocalisation of the Fe- and Mo- proteins of nitrogenase showed that old, degenerating vesicles from *Alnus incana* (L.) root nodules contain approximately half as much nitrogenase as mature vesicles (Huss-Danell and Bergman 1990).

Daily addition of fresh sterile medium to derepressed *Frankia* cultures clearly provides a simple means of maintaining a maximum level of growth and nitrogenase activity. In continuous culture, once steady state conditions are reached, composition of the vesicle population remains relatively stable, indicating new provesicles enter the active vesicle population at approximately the same rate "ghost" vesicles are formed. Hence, the vesicle population is continuously added to and contains vesicles covering the full spectrum of stages of vesicle development rather than a single cohort undergoing synchronous development, as occurs in batch cultures.

As discussed above, daily addition of fresh medium at a predetermined dilution rate, and removal of an equivalent volume of culture, proved the most satisfactory method for maintaining *Frankia* in continuous culture. *Frankia*'s slow growth rate means a very low dilution rate is necessary in contrast to most other bacteria raised in continuous culture. Nitrogen-fixing cultures of *Azospirillum brasilense* for example have been maintained in continuous culture with a dilution rate of 0.117 h^{-1} (Nelson and Knowles 1978) while *Azotobacter chroococcum* is typically grown in continuous culture with a dilution rate of approximately 0.25 h^{-1} (Hine and Lees 1976). The slow growth rate of *Frankia* also means addition of a small volume of medium every 24 h gives much the same level of growth and nitrogenase activity as more frequent addition of medium. Consequently, sophisticated continuous culture systems and equipment commonly used for other bacteria are unnecessary for maintaining *Frankia*. Using this technique, continuous cultures of *Frankia* have been maintained with a stable level of growth and acetylene reduction activity for periods of more than 30 days.

This method of continuous culture outlined above provides *Frankia* populations where the effects of environmental factors can be investigated without having to consider the state of vesicle development or having to overcome problems associated with a changing rate of growth and nitrogenase activity. *Frankia* from these cultures for example has been used to investigate the effects of nitrogen source (chapter four) and pO2 (chapter five) on growth, nitrogenase activity and vesicle development.

CHAPTER FOUR

FRANKIA GROWTH, NITROGENASE ACTIVITY AND VESICLE DEVELOPMENT ON NITRATE

4.1 Introduction

Nitrogen metabolism is central to the actinorhizal symbiosis and, using other bacterial systems as a guide, a general outline of nitrogen metabolism in *Frankia* has been developed (section 1.5.5). In addition to determination of the pathway of nitrogen assimilation in symbiosis (Wheeler and Bond 1970; Schubert *et-al.* 1981; Hirel *et al.* 1982; Schubert 1986) and in cultured *Frankia* (Edmands *et al.* 1987; Tsai and Benson 1989; Schultz and Benson 1990), several studies have focussed on the effect of various nitrogen compounds on *Frankia* growth and nitrogenase activity in vitro. Strains vary in their ability to grow on media containing different nitrogen compounds but some definite trends in nitrogen utilisation do exist among *Frankia*. Almost all strains prefer NH_4Cl as the sole nitrogen source (Shipton and Burggraaf 1982, 1983; Lechevalier *et al.* 1983) although some isolates also show a growth response on amino acids such as aspartate, glutamate, glutamine and asparagine as well as urea, adenine, uracil and nitrate (Shipton and Burggraaf 1983; Lamont et el 1985). Some, if not all, *Frankia* can grow on N_2, although growth rates are generally low (Shipton and Burggraaf 1982; Fontaine *et al.* 1984). However a few strains apparently prefer N_2 over ammonia (D. D. Baker unpublished results). Absence of a source of combined nitrogen appears to act as a signal for vesicle formation. When grown on nitrogen-free medium, *Frankia* forms vesicles containing active nitrogenase (Tjepkema *et al.* 1980; Murry *et al.* 1984*a*,*b*). Several studies have shown the presence of combined nitrogen in medium represses formation of vesicles and reduction of nitrogen by *Frankia* (Gauthier *et al.* 1981; Tjepkema *et al.* 1981; Fontaine *et al.* 1984). However recent work (Meesters *et al.* 1985; Tisa and Ensign 1987) has shown repression of vesicle formation and nitrogenase activity by combined

nitrogen is not common to all *Frankia* strains. Such observations immediately raise speculation regarding the role of vesicles formed under conditions where combined nitrogen is available. The signals thought to trigger vesicle formation and nitrogenase synthesis and whether these two processes are in fact synonymous, may also need to be re-examined in light of the findings of Meesters *et al.* (1985) and Tisa and Ensign (1987). With these questions in mind, a study was made of the ability of one *Frankia* sp. strain, HFPCcI3, to utilise a range of nitrogen compounds and in particular the effect of nitrate on growth, nitrogenase activity and vesicle development.

4.2 Growth on various nitrogen sources

Frankia sp. strain HFPCcI3 is normally grown on BAP medium (Murry *et al.* 1984*a*) either with NH_4Cl (5 mM) as the sole nitrogen source (+N) or without a source of combined nitrogen (-N). When grown on NH_4Cl, vesicle formation and nitrogen fixation are repressed while removal of nitrogen from the medium (-N) derepresses both (Zhang *et al.* 1986). This experiment investigated the effect of other nitrogen compounds on the growth, nitrogenase activity and vesicle development of CcI3 to ascertain whether they, like NH_4Cl, repress vesicle formation and nitrogenase activity.

CcI3 was grown in batch culture on BAP medium with either NH_4Cl (5 mM), aspartate (5 mM), glutamate (5 mM), glutamine (5 mM) or KNO_3 (5 mM) as the sole nitrogen source or on -N medium. Four replicate batch cultures were established on 100 ml medium (innitial protein level = 15 μg protein. ml^{-1}) for each nitrogen source. Growth (section 2.1.5) of cultures was determined after seven days and nitrogenase activity (section 2.3.3) measured on a daily basis. Vesicles were viewed using dark field microscopy (section2.1.7).

CcI3 grew to some extent on all the nitrogen compounds trialed (Table 4.1) although highest growth was measured on NH_4Cl. Microscope examination of cultures showed

new hyphal growth occurred on all nitrogen sources and on -N medium. Although -N medium supported the lowest rate of growth it was the only treatment that showed acetylene reduction activity. In correlation with the presence of nitrogenase activity, vesicles were observed on -N medium (Table 4.1). Low numbers of vesicles were also present in nitrate grown cultures despite the apparent lack of acetylene reduction activity. Although nitrate-grown vesicles appeared identical to vesicles formed on medium lacking a source of combined nitrogen, vesicle numbers were only 20 % of those on -N medium.

Table 4.1 Effect of nitrogen source on growth, nitrogenase activity and vesicle development of *Frankia* sp. strain HFPCcI3. Results show mean and (standard deviation; sample size).

Nitrogen source (5 mM)	Protein level [a] (μg protein. ml^{-1})	Nitrogenase activity [b] (nmol C_2H_4. mg protein^{-1}. h^{-1})	Vesicles (vesicles / hyphae) [c]
NH_4Cl	41.84 (0.46; 12)	0	0
KNO_3	38.51 (0.59; 12)	0	1.75
aspartate	30.42 (0.61; 12)	0	0
glutamate	31.77 (0.50; 12)	0	0
glutamine	34.90 (0.63; 12)	0	0
-N	27.41 (0.42; 12)	169.27 (7.61; 12)	7.63

a. Measured after 7 days. Initial protein level (at 0 days) was 15 μg protein. ml^{-1}.

b. Measured after 5 days.

c. Measured after 7 days. See section 2.1.7 for details.

4.3 Utilisation of nitrate as a nitrogen source

Nitrate is clearly able to support growth of CcI3 at a level comparable to growth on ammonium (Table 4.1). However the presence of vesicles in nitrate-grown cultures appears to be somewhat of an anomaly particularly since no nitrogenase activity was detected using acetylene reduction assay. It is possible that vesicles in nitrate-grown cultures contain no active nitrogenase and instead CcI3 assimilates nitrogen from the medium rather than fixing nitrogen. The aim of this experiment was to use stable isotope techniques to test whether CcI3 utilises nitrate in the medium for growth or whether vesicles were actively fixing nitrogen.

Determination of nitrate utilisation by CcI3 involved raising continuous cultures (section 2.1.3) on medium containing ^{15}N labelled KNO_3. Ten replicate cultures were established on BAP medium containing 5 mM KNO_3 - 2 % ^{15}N atom % excess and ten control cultures were also established on BAP medium containing unlabelled KNO_3. Cultures were fed with the appropriate labelled or unlabelled media. Two replicate cultures were harvested at the start of the experiment and then after 130 h, 192 h, 240 h and 288 h in continuous culture. Harvesting involved passing the cultures through a glass fibre filter (Watman GF/C) and then drying the filters at 80°C for twelve hours. The dried *Frankia* and filters were then folded and stored in pure tin capsules for analysis using an automated Dumas GC.MS system (Waikato Stable Isotope Unit, Ruakura Agricultural Cantre, Hamilton) Samples were run against a DPC reference. Acetylene reduction assays (section 2.3.3) were also performed at the harvest times while measurement of protein level (section 2.1.5) and observation of vesicles (section 2.1.7) was done on 1 ml samples after the final harvest.

All cultures grew on nitrate-containing medium reaching a final protein level of 38 µg protein. ml^{-1} at 288 h. Vesicles were present in cultures at this time although no nitrogenase activity was detected, using acetylene reduction assays, at any time during the

experiment. This included assays performed on 10 ml subsamples of culture in 30 ml universal bottles incubated for four hours.

Results from stable isotope enrichment analysis are shown in table 4.2. Cultures grown on unlabelled nitrate showed only slight enrichment above the reference but cultures grown on labelled nitrate showed considerably greater enrichment reaching a level almost the same as the added $^{15}NO3$ i.e. 2 % ^{15}N atom % excess. The atom % excess ^{15}N increased with culture age presumably as further nitrate was taken up from the medium. Although the data is limited, it appears most of the $^{15}NO_3$ was assimilated within the first 130 h. After 288 h atom % ^{15}N from the labelled cultures had reached 1.73 atom % excess indicating approximately 87 % of the $^{15}NO_3$ in the medium had been assimilated. In fact this observed figure of 87 % was probably lower than the true level since addition of the *Frankia* inoculum and vitamins to the medium would have had a slight dilution effect at the start of the experiment.

Table 4.2 ^{15}N content of *Frankia* sp. strain HFPCcI3 cells grown on labelled or unlabelled KNO_3. Results show mean and (standard deviation; sample size).

Time (h)	Unlabelled KNO_3 (atom % excess ^{15}N)	Labelled KNO_3 (atom % excess ^{15}N)
0	0.3681 (0.0014; 2)	0.3681 (0.0014; 2)
130	0.3714 (0.0023; 2)	1.4894 (0.0036; 2)
192	0.3734 (0.0024; 2)	1.6345 (0.0049; 2)
240	0.3769 (0.0011; 2)	1.6679 (0.0042; 2)
288	0.3728 (0.0032; 2)	1.7333 (0.0062; 2)

4.4 Nitrogen fixation during growth on nitrate

^{15}N isotope techniques were also used to determine whether CcI3 growing in culture with nitrate as the sole nitrogen source was also fixing nitrogen. Eighteen replicate batch cultures were established on BAP medium with either ammonium or nitrate as the sole nitrogen source or on -N medium. An inoculum was added to 20 ml medium in 30 ml universal bottles to give an initial protein level of 20 μg protein. ml^{-1}. After 130 h four replicate cultures from each treatment were regassed and 50 % v/v $^{15}N_2$ (63.9 atom % ^{15}N) introduced into the bottle headspace to give an atom % ^{15}N of 30.42 at a pO_2 of 21 kPa O_2. The remaining two replicates from each treatment were used for protein determinations (section 2.1.5). Universal bottles containing 20 ml medium but no *Frankia* with 50 % v/v $^{15}N_2$ in the headspace functioned as controls-for each treatment.

After 178 h (48 h with $^{15}N_2$) three replicate 0.4 ml samples of the headspace gas were analysed for $^{15}N_2$ using a AEI MS 10 Mass Spectrometer. Cultures were then harvested as described earlier (section 4.2) and the ^{15}N atom % excess level of the *Frankia* measured.

The ^{15}N atom % excess level of cultures growing on -N medium was significantly lower than the controls (Table 4.3) indicating nitrogen fixation had occurred. This result was supported by the ^{15}N enrichment found in the *Frankia* (Table 4.3). No fixation occurred in the cultures grown with ammonia as the sole nitrogen source since the ^{15}N atom % excess levels of the headspace gas and the ^{15}N enrichment levels of the *Frankia* cells were close to the control values. Some nitrogen fixation appears to have occurred in the nitrate-grown cultures although the level of activity was extremely low since ^{15}N atom % excess values show a barely significant difference from the control values (Table 4.3).

Table 4.3 Incorporation of $^{15}N_2$ by cultures of *Frankia* sp. strain HFPCcI3 grown on various nitrogen sources. Results show mean and (standard deviation, sample size).

Nitrogen source	*Frankia* cells (atom % excess ^{15}N)	Headspace gas (atom % excess ^{15}N)
control	0.3659	30.40
NH_4Cl	0.3632 (0.0063; 4)	30.38 (0.09; 4)
KNO_3	0.3710 (0.0024; 4)	30.09 (0.16; 4)
-N	0.6349 (0.0064; 4)	27.45 (0.39; 4)

4.5 Effect of ammonia and nitrate on vesicle development and nitrogenase activity

Acetylene reduction assays and ^{15}N stable isotope analyses indicate vesicles in nitrate-grown cultures carry out very little, if any, nitrogen fixation. The vesicles are identical in appearance however to those formed on -N medium. This raises a question regarding the function of vesicles in nitrate-grown cultures when CcI3 appears to obtain most of its nitrogen requirements from nitrate in the medium. The purpose of the following experiments was to examine more closely the effects of nitrate on vesicle development and function.

4.5.1 Short-term effect of ammonia on nitrogenase activity

The first experiment investigated the short-term effect of NH_4Cl on acetylene reduction activity of derepressed *Frankia* cells. A 7 ml bijou bottle containing 4 ml of CcI3 (0.036 mg protein. ml^{-1}) in BAP (-N) medium was incorporated into the continuous flow system (section 2.3.4). The bijou bottle was mounted on a rotary shaker (60 rpm), to facilitate

gas mixing, in a constant temperature water bath. The experiment was conducted at a constant pO_2 of 21 kPa O_2 with a gas flow rate of 8 ml. min-1. After 30 minutes NH_4Cl was added to the bijou bottle to give a final concentration of 5 mM. A control culture was also assayed and KCl (5 mM) added to this culture after 30 minutes.

Before addition of NH_4Cl, acetylene reduction activity of the culture was relatively constant at about 1.8 nmol C_2H_4. mg protein^{-1}. min^{-1} (Fig. 4.1) which is comparable to activity rates for *Frankia* assayed in batch culture. Although activity did decline slightly throughout the experiment addition of NH_4Cl had no noticeable effect on acetylene reduction activity. A decrease in activity of similar proportions was also observed in the control culture following addition of KCl (5 mM) (Fig. 4.1).

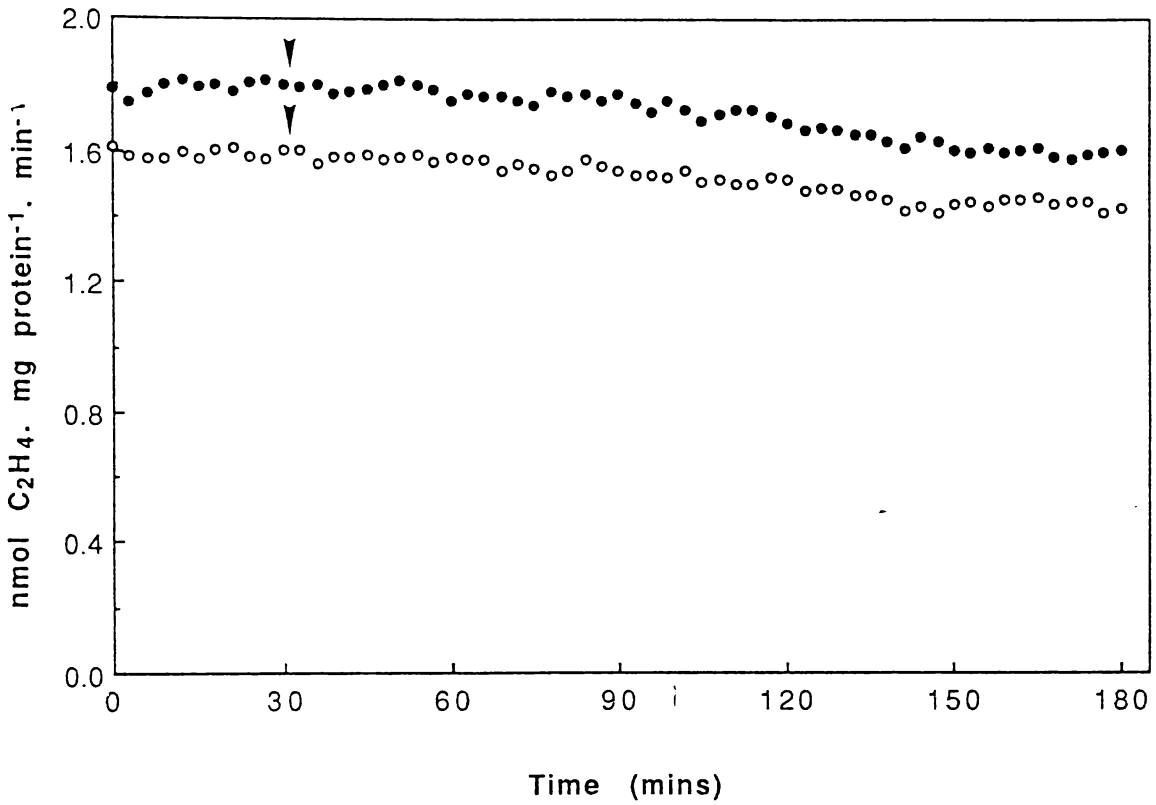


Figure 4.1 Short-term effect of added NH_4Cl on nitrogenase activity of *Frankia* sp. strain HFPCcI3 assayed using a continuous flow system. NH_4Cl (●) (final concentration 5 mM) was added after 30 minutes (▼). Activity in a control culture exposed to KCl (o) (final concentration 5 mM) was also assayed.

4.5.2 Long-term effect of ammonia and nitrate on nitrogenase activity and vesicle decelopment

The second experiment examined the time course of repression of nitrogenase activity in existing -N-grown vesicles following addition of NH_4Cl or KNO_3. Since addition of NH_4Cl appeared to have no short-term effect on nitrogenase activity, this experiment used batch assay techniques to study the longer-term response to ammonium addition.

Nine replicate continuous cultures of CcI3 were established on -N medium (section 2.1.3). Acetylene reduction assays (section 2.3.3) were performed on three replicate subsamples of cultures at regular intervals. After 215 h in continuous culture the -N medium was drained from four of the cultures and replaced with BAP medium containing either NH_4Cl (5mM) or KNO_3 (5mM). In the three remaining cultures the medium was replaced with fresh -N BAP medium. Vesicles from each treatment were observed after 359 h (144 h after addition of combined nitrogen) using dark field microscopy (section 2.1.7) for signs of structural damage.

Cultures grown on -N medium throughout the experiment maintained a relatively stable level of nitrogenase activity after reaching a maximum rate of 140.20 nmol C_2H_4. mg protein^{-1}. h^{-1}. at approximately 180 h (Fig. 4.2). Transfer to medium containing either NH_4Cl or KNO_3 caused a gradual decline in acetylene reduction activity (Fig. 4.2). Addition of ammonia resulted in a decrease in activity within 15 h and complete repression of nitrogenase activity after 65 h. By comparison the effect of nitrate on nitrogenase activity was slightly delayed. Acetylene reduction activity showed no decrease until 21 h after transfer to nitrate-containing medium and complete inhibition of activity occurred after 72 h (Fig. 4.2). This delay in the effect of nitrate in repressing nitrogenase activity, compared with the effect of ammonia, was evident in all replicate cultures.

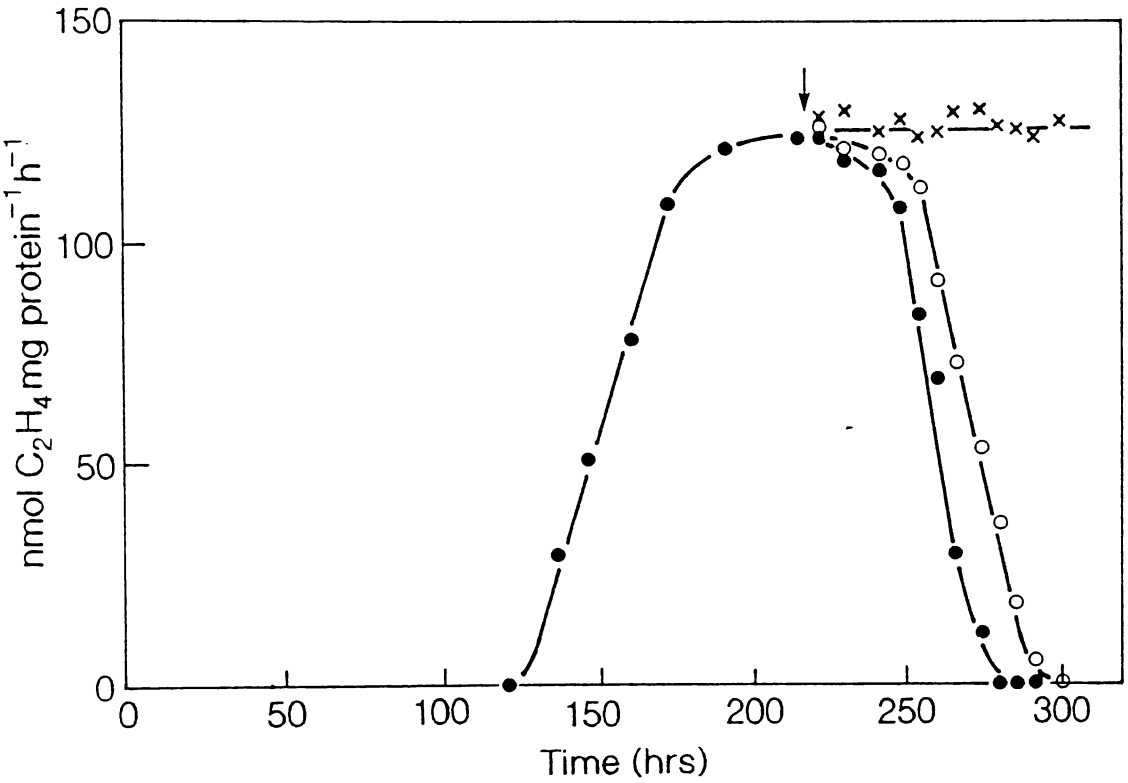


Figure 4.2 Effect of addition (↓) of 5 mM NH_4Cl (—●—) or 5 mM KNO_3 (—○—) on nitrogenase activity in *Frankia* sp. strain HFPCcI3 grown in continuous culture. Control cultures (—×—) were maintained on nitrogen-free medium throughout the experiment.

Damaged vesicles were observed in cultures from all three treatments after 359 h although at this time only cultures on -N medium still had active nitrogenase. It was impossible to detect any difference between 'ghost' vesicles, which are a normal feature of *Frankia* cultures grown under both batch and continuous conditions, and those vesicles which may have been structurally damaged due to addition of combined nitrogen. However the percentage of damaged or 'ghost' vesicles in cultures which received combined nitrogen was significantly higher than in the control cultures on -N medium (Table 4.4). The percentage of damaged vesicles in cultures which received NH_4Cl was also slightly higher than in those cultures which received nitrate but the difference was barely significant.

Table 4.4 Frequency (%) of damaged vesicles from *Frankia* strain HFPCcI3 after 359 h of various treatment.

Nitrogen treatment	% damaged vesicles
-N / -N	7.8; n=302
-N / NH_4^+	32.8; n=274
-N / NO_3	29.7; n=256

4.5.3 Nitrogenase and vesicle induction following growth on NH_4Cl and KNO_3

A third experiment investigated the comparative time course of nitrogenase induction when CcI3, grown with either ammonium or nitrate as the sole nitrogen source, was transferred to medium lacking a source of combined nitrogen. Six replicate continuous cultures were set up, as described above, on BAP medium containing either NH_4Cl (5 mM) or KNO_3 (5 mM). After 144 h the medium was drained from the bottles and replaced with -N BAP medium. Acetylene reduction assays (section 2.3.3) and protein

level assays (section 2.1.5) were carried out on three replicate subsamples from each culture at regular intervals. Vesicle development was also followed using dark field microscopy (section 2.1.7).

Prior to removal of combined nitrogen from the growth medium at 144 h (time 0 h on Fig.4.3) no acetylene reduction activity was detected in any culture. However a small population of vesicles (1.69 vesicles / unit hyphae) was observed in nitrate-grown cultures after 96 h (-48 h). No vesicles were present in ammonium-grown cultures.

After transfer to -N medium, cultures from both NH_4Cl- and KNO_3-containing medium showed a gradual increase in acetylene reduction activity. In both treatments there was a lag phase between the time of removal of the combined nitrogen source and the beginning of acetylene reduction activity (Fig. 4.3). However the lag phase shown by *Frankia* previously grown on nitrate (NO_3^- / -N) was considerably shorter than that for ammonium (NH_4^+ / -N) grown *Frankia*. While acetylene reduction activity in NO_3^- / -N cultures was first detected 68 h after transfer the lag phase in NH_4^+ / -N cultures was approximately 120 h long. Although NO_3^- / -N cultures reached a maximum level of nitrogenase activity earlier than NH_4^+ / -N cultures, the rates of acetylene reduction activity in each treatment at the conclusion of the experiment were approximately the same.

Vesicle development in NH_4^+ / -N cultures showed a similar pattern to that outlined above (section 3.2) with provesicles appearing after approximately 42 h followed by a gradual increase in the number of mature vesicles concurrent with the rise in nitrogenase activity. Although nitrate-grown *Frankia* already had a small vesicle population, transfer to -N medium resulted in a significant increase in vesicle number so that, by the end of the experiment, vesicle number was similar in the two treatments.

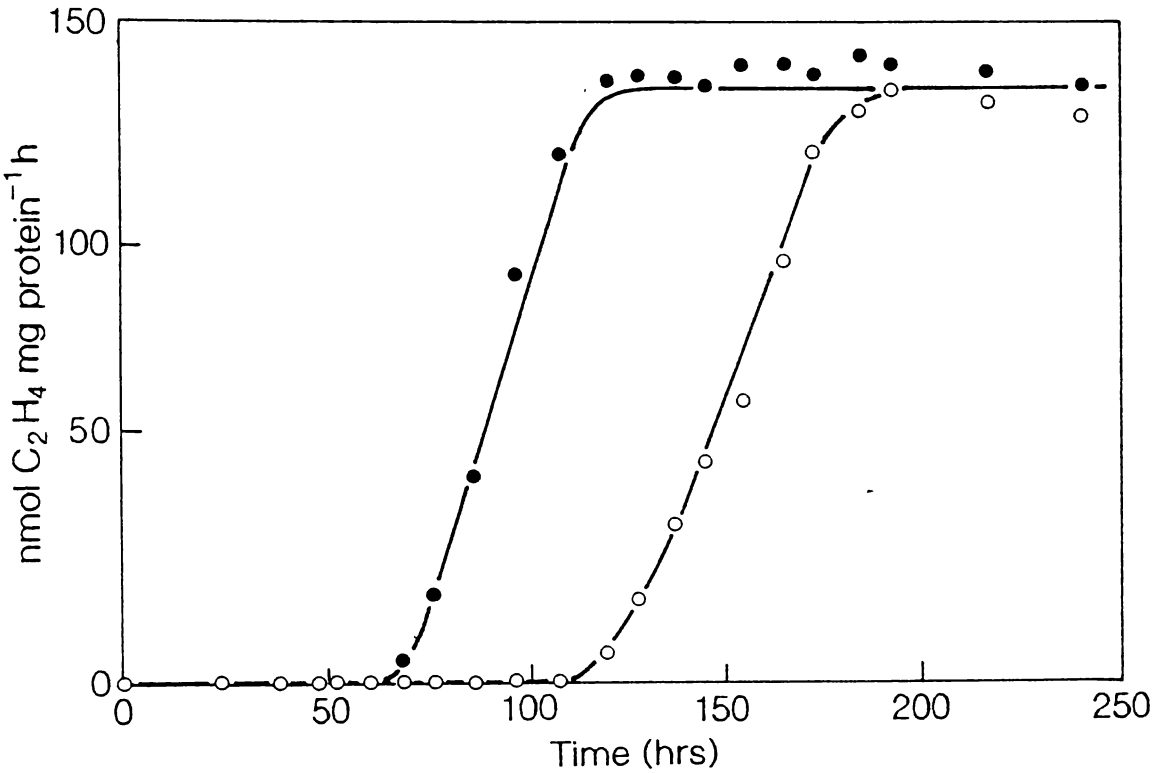


Figure 4.3 Induction of nitrogenase activity in continuous cultures of *Frankia* sp. strain HFPCcI3 following transfer to nitrogen-free medium at time 0 h. Cultures were previously grown on either 5 mM NH_4Cl (—○—) or 5 mM KNO_3 (—●—).

4.6 Discussion

Nitrogen utilisation and the effects of different sources of combined nitrogen on growth, nitrogenase activity and vesicle development among *Frankia* strains are relatively consistent. Almost all *Frankia* strains grew best with NH_4Cl as the sole nitrogen source although some strains will also utilise a range of other nitrogen compounds (Table 4.5). *Frankia* are also capable of growing in the absence of combined nitrogen i.e. -N medium. Under such conditions vesicles form and derepression of nitrogenase occurs (Tjepkema *et al.* 1980, Murry *et al.* 1984*a*,*b*). It is generally accepted the presence of vesicles is indicative of nitrogenase activity and vice versa. In most strains the formation of vesicles and reduction of nitrogen *in vitro* is repressed by the presence of combined nitrogen in the medium (Fontaine *et al.* 1984). However a few *Frankia* strains have been shown to form vesicles and fix nitrogen in the presence of combined nitrogen (Meesters *et al.* 1985; Tisa and Ensign 1987). *Frankia* sp. strain HFPCcI3, tested here, showed a typical response to a range of nitrogen compounds but differed from the majority of *Frankia* strains in its ability to form vesicles in the presence of nitrate.

CcI3 consistently showed highest growth on NH_4Cl (5 mM). In comparison, growth on -N medium was considerably lower. Like other *Frankia* strains grown on medium lacking a source of combined nitrogen, CcI3 fulfilled its nitrogen requirements through nitrogen fixation, as was indicated by the incorporation of $^{15}N_2$ by cultures as well as acetylene reduction assays. Mazzucco and Benson (1984) studied ^{14}C labelled methylammonium transport in *Frankia* strain CpI1 and showed entry of ammonium into *Frankia* cells *in vitro* may occur by two different routes depending on the concentration of ammonium. In media with a high ammonium concentration, ammonium enters the cells by passive transport while in nitrogen-free media an inducible active transport permease mediates the concentrative uptake of ammonium formed as a result of nitrogen fixation. Use of an active transport system obviously necessitates a higher energy cost for an organism. This factor, together with the energy costs involved in nitrogen fixation, may contribute to the lower growth rate of *Frankia* on -N media.

Table 4.5 Summary of nitrogen utilisation by various *Frankia* isolates.

Nitrogen compound	Number strains tested	Number strains showing growth
NH_4Cl	18	17 (94 %)
NH_4NO_3	6	3 (50 %)
KNO_3	10	6 (60 %)
alanine [a.]	3	2 (67 %)
aspartate	12	9 (75 %)
glutamate	8	5 (63 %)
glutamine	3	2 (67 %)
guanine [a.]	3	0 (0 %)
thymine	6	0 (0 %)
uracil	6	3 (50 %)
urea	6	4 (67 %)

[a.] Data obtained from only one study.

Data compiled from : Blom 1981; Gauthier *et al.* 1981; Tjepkema *et al.* 1981; Blom 1982; Shipton and Burggraaf 1982; Akkermans *et al.* 1983; Lechevalier *et al.* 1983; Shipton and Burggraaf 1983; Lamont *et al.* 1985; Tisa and Ensign 1987.

A parallel may be drawn with the growth of actinorhizal plants either supplied with combined nitrogen or reliant solely on nitrogen fixation. For example, growth rates of nodulated *Alnus incana* seedlings supplied with NH_4NO_3 at the optimum rate was almost double the growth rates of plants that received no combined nitrogen (Ingestad 1980). Theoretical estimates indicate the energy costs of assimilation of fixed nitrogen are greater than for assimilation of ammonium or nitrate (Huss-Danell 1990).

Although NH_4Cl was supplied to CcI3 in this study at a concentration of 5 mM, the concentration of combined nitrogen in media used in other studies ranges from 2 mM (Burggraaf and Shipton 1983) to 30 mM (Shipton and Burggraaf 1983). However a number of *Frankia* strains tested have shown no clear optimum concentration for satisfactory growth of *Frankia* on NH_4Cl. Shipton and Burggraaf (1982) grew three

strains of *Frankia* on propionic acid medium with different NH_4Cl concentrations. They found satisfactory growth occurred within the range of C : N ratios of 1 : 1 to 20 : 1 although wider ratios, such as 50 : 1 and 100 : 1, resulted in a marked reduction in growth.

Despite ammonium concentration having little effect on growth of *Frankia*, NH_4Cl, even at very low concentrations, has an inhibitory effect on vesicle formation and acetylene reduction in most *Frankia* strains. CcI3, tested here, showed no evidence of vesicle formation or nitrogenase activity when grown on 5 mM NH_4Cl. Tjepkema *et al.* (1981) showed the effect of NH_4Cl on vesicle formation and acetylene reduction in *Frankia* is concentration dependent but even concentrations as low as 1 mM almost completely inhibited acetylene reduction activity and suppressed vesicle formation.

Addition of NH_4Cl (5 mM) had no significant short-term effect on acetylene reduction activity of derepressed CcI3 assayed in a continuous flow system. However Fontaine *et al.* (1984) showed an inhibitory effect on nitrogenase activity of *Frankia* sp. strain HFPArI3 within two hours of addition of NH_4Cl to the medium. This effect was concentration dependent and, after two hours, 72.1 % of acetylene reduction activity was abolished by 20 mM NH_4Cl compared with 42.6 % inhibition at 5 mM NH_4Cl.

Derepressed CcI3 did however show a long-term response to NH_4Cl. Addition of 5 mM NH_4Cl had little initial effect on acetylene reduction activity, which is consistent with the lack of a short-term effect in the continuous flow experiment. However activity was completely suppressed within 65 h. A similar result was shown by Tjepkema *et al.* (1981). They found 1 mM NH_4Cl added to derepressed CpI1 cultures had no effect on acetylene reduction activity during the 24 h period immediately following addition but on successive days there was complete inhibition of activity. An explanation for the apparent delay in effect could be that ammonium in the medium primarily blocks vesicle formation and the inhibition of nitrogenase synthesis and activity is a secondary effect. No direct inhibitory effect of exogenous ammonium on nitrogenase activity was observed for

bacteroids of *Rhizobium leguminosarum* (Laane *et al.* 1980) nor for nitrogen-fixing cells of *Bacillus polymyxa* (Nambier and Shethna 1977). However, nitrogenase activity of some Rhodospirillaceae members is inhibited by addition of NH_4Cl (Howard *et al.* 1983; Kanemoto and Ludden 1984). Since NH_4Cl appears to have no direct, short-term inhibitory effect on *Frankia* nitrogenase activity, existing vesicles would remain actively fixing nitrogen but no new vesicles would be formed. Gradually the rate of nitrogenase activity would decline as the added ammonium had a detrimental effect on the structure of existing vesicles. Structural damage of vesicles in response to ammonium addition was evident in this study and has also been shown in *Frankia* strain HFPArI3 (Fontaine *et al.* 1984) and in vesicles in nodules of Alnus incana (Huss-Danell *et al.* 1982). This indirect effect of ammonium addition on nitrogenase activity, through damage to existing vesicles and inhibition of formation of new vesicles, may account for the absence of a short-term effect of NH_4Cl on acetylene reduction activity.

Other nitrogen compounds, such as glutamate, glutamine and aspartate, also had an inhibitory effect on nitrogenase and vesicle induction in CcI3. However all the nitrogen compounds tested supported growth of this strain to some extent. Suppression of nitrogenase activity by various nitrogen compounds has been demonstrated for a number of *Frankia* strains. Concentrations of glutamine above 0.54 mM suppressed nitrogenase activity in two strains of *Frankia* isolated from Casuarina equisetifolia (Gauthier *et al.* 1981) while glutamate (1.8 mM) reduced the number of vesicles and nitrogenase activity in *Frankia* strain CpI1 by half (Tjepkema *et al.* 1981). The inhibitory effects of nitrogen compounds such as glutamate and glutamine are not surprising since synthesis of nitrogenase appears to be regulated by glutamine synthetase (GS), glutamate synthase (GOGAT) or their products. Such a feedback mechanism involving GS and GOGAT in the regulation of nitrogenase synthesis in *Frankia* was first proposed by Gauthier (1983) who showed synthesis of nitrogenase occurs in the presence of ammonium when GS and GOGAT were inhibited by methionine sulfoximine. Although nitrogen compounds generally inhibit vesicle formation and nitrogenase activity, Lamont *et al.* (1985) reported

induction of vesicle formation and nitrogenase in cultures of *Frankia* strain CpI1 grown on 5 mM asparagine.

Most *Frankia* strains tested so far are able to grow with nitrate as the sole nitrogen source. Two *Frankia* strains isolated from *Casuarina equisetifolia* (D11 and G2) (Gauthier *et al.* 1981) as well as isolates from *Comptonia peregrina* (CpI1), *Alnus viridis* ssp. *crispa* ($ACN1^{AG}$), *Elaeagnus augustifolia* ($EAN1_{pec}$), *Elaeagnus umbellata* ($EUN1_f$) (Tisa and Ensign 1987), *Hippophae rhamnoides* (LDHr1) and *Myrica gale* (LDMg1) (Shipton and Burggraaf 1983) all show relatively high growth rates on KNO_3 although two *Frankia* strains isolated from *Alnus incana* ssp. *rugosa* (AirI1 and AirI2) cannot utilise nitrate (Lechevalier *et al.* 1983). CcI3, tested here, also showed a high rate of growth on nitrate comparable with that on NH_4Cl. Isotope enrichment experiments confirmed the *Frankia* cells utilised the nitrate in the medium. Nitrate supresses vesicle formation and nitrogenase activity in the majority of *Frankia* strains tested (Gauthier *et al.* 1981; Shipton and Burggraaf 1983; Tisa and Ensign 1987) although Tisa and Ensign (1987) showed *Frankia* strains $EAN1_{pec}$ and $EUN1_f$ did form vesicles and fix nitrogen when grown on nitrate. However the level of nitrogenase activity was lower than that on -N medium and decreased further as KNO_3 concentration was increased over the range 1 to 10 mM. CcI3 also formed vesicles on KNO_3 (5 mM) although the number of vesicles was significantly reduced compared with vesicle numbers on -N medium. Despite the existence of vesicles on nitrate, acetylene reduction assays and $^{15}N_2$ isotope experiments indicated only a very low level of nitrogenase activity, if any. This is in contrast to *Frankia* sp. strains $EAN1_{pec}$ and $EUN1_f$. Interestingly $EAN1_{pec}$ and $EUN1_f$ also form vesicles on 10 mM NH_4Cl but no nitrogenase activity is exhibited (Tisa and Ensign 1987). Similarly a *Frankia* isolate from *Colletia cruciata* (Cc1.17) also forms vesicles in the presence of NH_4Cl (4 mM) (Meesters *et al.* 1985). In all cases however the number of vesicles formed on NH_4Cl was lower than vesicle counts for cultures grown under nitrogen-free conditions. In contrast, CcI3 did not form vesicles on NH_4Cl.

A question therefore arises regarding the role of vesicles formed by CcI3 grown on medium containing nitrate. Clearly the *Frankia* cells are not reliant on nitrogen fixation to support growth when grown on nitrate. Nitrogenase activity was barely detectable and certainly not at a high enough level to account for the relatively high growth rate on nitrate. In addition the uptake of ^{15}N labelled KNO_3 from the medium indicated nitrogen fixation plays a very minor role, if any, in fulfilling the nitrogen requirements of CcI3 grown on nitrate. It is possible that nitrogen fixation associated with vesicles may become important if the concentration of nitrate in the medium was limited and nitrate uptake was therefore restricted. The longer time taken for KNO_3 to have an inhibitory effect on nitrogenase compared with NH_4Cl may be a reflection of a slightly less effecient nitrate uptake system than ammonium uptake. It would be interesting to determine if a higher level of nitrogenase activity could be detected once the nitrate concentration in the medium was reduced. In situations where a particular nitrogen source may become limiting, it would be an advantage to possess the ability to fix nitrogen as well as being able to uptake combined nitrogen from the medium. Under certain situations *Rhizobium* obtains nitrogen simultaneously from substrate nitrogen and nitrogen acquired through fixation (Tubb 1976) and a similar situation has been reported for some *Azotobacter* and *Beijerinckia* (Dobriereiner and Day 1975).

CcI3 previously grown on nitrate had a considerably shorter time-lag to the appearance of nitrogenase activity when transferred to derepressing conditions than cultures previously grown on ammonium. This was presumably because a small population of vesicles already existed in nitrate-grown cultures and the time lag was due mainly to the time taken to synthesise nitrogenase within existing vesicles. In NH_4^+ / -N cultures the lag time probably involved formation of new vesicles as well as nitrogenase synthesis. Tisa and Ensign (1987) showed a similar result in *Frankia* strains $EAN1_{pec}$ and $EUN1_f$ which, because they form vesicles when grown on +N medium, derepress nitrogenase more rapidly on -N medium than *Frankia* strain CpI1 and $ACN1^{AG}$ which do not form vesicles on ammonium. These observations suggest a potential advantage in forming vesicles when *Frankia* does not need to fix nitrogen i.e. under conditions where a source of

combined nitrogen is readily available, by allowing a faster response and earlier onset of activity when nitrogenase is derepressed.

Although numerous lines of evidence indicate vesicles are the site of nitrogenase it is possible that, under certain conditions, vesicles may play a more general role in cell metabolism. It is possible, for example, that vesicles formed on nitrate may have a role in nitrate-reduction activity. The effect of oxygen on vesicle development in nitrate-grown Frankia is examined in the following chapter and should show whether these vesicles still have a role, or at least a potential role, in nitrogen fixation and the consequent oxygen protection of nitrogenase.

Clearly this study, together with previous reports (Gauthier *et al.* 1981, Burggraaf and Shipton 1983; Lamont *et al.* 1985; Meesters *et al.* 1985; Tisa and Ensign 1987) which have noted the presence in *Frankia* cultures that show no detectable nitrogenase activity, suggest the link between vesicles and nitrogenase activity is not absolute. These results indicate a differential expression of nitrogen fixation genes and the genes regulating vesicle development. However there is no evidence to suggest a role, other than one involving nitrogen fixation, for vesicles which, under certain conditions, apparently contain no nitrogenase.

CHAPTER FIVE

OXYGEN CONTROL OF VESICLE DEVELOPMENT

5.1 Introduction

Nitrogenase in all nitrogen-fixing bacteria is extremely oxygen sensitive and, as a consequence, the majority of diazotrophs exist in ecological or symbiotic situations of decreased ambient oxygen. Various "oxygen protection strategies" have been described (Gallon 1981) which may be differentiated by whether the organism relies on its immediate environment, either ecological or symbiotic, to provide low oxygen conditions, or whether the organism can express nitrogenase in air and therefore provide intrinsic oxygen control and protection. Most diazotrophs belong to the former group and are described as microaerophiles or anaerobes, being unable to sustain nitrogenase at atmospheric concentrations of oxygen. *Rhizobium*, for example, only expresses nitrogenase in culture at greatly decreased pO_2 (Tjepkema and Evans, 1975; Bergersen *et al.* 1976) and is unable to adapt to varying external pO_2 (Bergersen *et al.* 1976). Instead oxygen protection is provided in symbiosis by the legume host.

The second group of organisms provide their own oxygen protection and includes the heterocystous cyanobacteria and *Frankia*. Both these diazotrophs possess specialized, thick-walled cells that apparently provide oxygen diffusion resistance thus protecting the nitrogenase within. *Frankia* therefore shows active nitrogenase at atmospheric pO_2 (Tjepkema *et al.* 1986) and is able to adapt to below-atmospheric (Murry *et al.* 1985) and above-atmospheric (Parsons *et al.* 1987) oxygen levels.

Both in pure culture (Tjepkema *et al.* 1980; Murry *et al.* 1984*b*) and in symbiosis (Mian and Bond 1978), the development of nitrogenase activity in *Frankia* coincides with the formation of vesicles, the sites of nitrogenase. Vesicles are bounded by a multilaminate outer envelope with *Frankia's* ability to adapt to varying pO_2 is directly related to the

number of lipid-like layers in this envelope (Parsons *et al.* 1987). Vesicles also have a role in oxygen protection in symbiosis though the importance of this appears to vary across the associations formed with a range of taxonomically diverse angiosperms (Tjepkema *et al.* 1986; Silvester *et al.* 1988*a*, *b*).

The aim of this work was to use the method described earlier for growing *Frankia* in continuous, derepressed culture to investigate the role of oxygen in controlling the thickness and thus the diffusion resistance provided by the vesicle envelope. The role of oxygen in regulation of vesicle wall thickness was also studied under growth conditions where nitrogen fixation activity was inhibited. Effects of low oxygen concentrations on nitrogenase activity were also studied.

5.2 Oxygen control of vesicle development

To date, all work on vesicle development has been conducted on *Frankia* grown in batch culture. Under such conditions a cohort of provesicles arises and goes through a series of physical and biochemical changes including thickening of the vesicle wall (Fontaine *et al.* 1984). In batch culture, the onset and rise in nitrogenase activity coincides with the formation of mature vesicles. However *Frankia* is unable to maintain a high rate of nitrogenase activity in batch culture and once peak level is reached, activity then undergoes rapid decline. At this stage vesicles appear damaged and vesicle number and protein level also decrease (Fontaine *et al.* 1984; Murry *et al.* 1984*b*). Consequently it is difficult in *Frankia* to distinguish between effects of external environmental changes and normal endogenous developmental changes. To obtain unequivocal evidence of environmental influences on vesicle development it is desirable to establish steady state growth conditions for *Frankia* and thus separate developmental changes from environmental effects.

Previous attempts to maintain *Frankia* in continuous culture, or to prolong a stage of constant activity have been unsuccessful (Murry *et al.* 1984*b*). The aim of this experiment was to use a continuous culture system to determine whether formation of a thickened vesicle envelope is a "normal" morphological event and whether oxygen has a controlling role in this process.

Continuous cultures of *Frankia* sp. strain HFPCcI3 were established as previously described. Following inoculation the pO_2 of the bottle headspace was adjusted to 5, 21 or 40 kPa (three replicate cultures at each pO2 level) using Tylan mass flow controllers to produce a predetermined sterile gas mixture. Oxygen levels were checked regularly using a Teledyne portable oxygen analyser and the oxygen concentration in the headspace adjusted to maintain the pO_2 within 10 % of the nominal value. Acetylene reduction assays were performed on three replicate subsamples from each culture at intervals throughout the experiment. Care was taken to conduct assays at the growth pO2 of the culture. Protein level was measured and observations of vesicles were also made using these samples.

In CcI3, low numbers of provesicles and hyphae with swollen tips were observed 41 h after removal of nitrogen at all three concentrations of oxygen (5, 21 and 40 kPa). The provesicles and stalk attachments were thin-walled and differed little from the hyphal wall in thickness.

After 72 h, large numbers of provesicles and thin-walled vesicles were present but no acetylene reduction was detected in any culture (Fig. 5.1). At this time, the distribution of vesicles according to apparent wall thickness was similar at each pO_2 (Fig. 5.2) with a mean wall thickness of 0.39 μm.

Nitrogenase activity first appeared after 91 h in cultures growing at 5 kPa O_2 (Fig. 5.1). At this time, vesicles had a mean wall thickness of 0.41 μm (Table. 5.1). In cultures incubated at 5 kPa O_2, the distribution of vesicles, according to apparent wall thickness,

changed little throughout the remainder of the experiment (Fig. 5.2) although nitrogenase activity increased and then became constant after 160 h (Fig. 5.1).

In contrast, vesicles grown at 21 or 40 kPa O_2 continued to form thicker walls and stalks beyond 91 h. At 119 h, vesicles were thicker-walled than those at 5 kPa O_2 (Fig.5.2). However, while cultures at 21 and 40 kPa O_2 vesicles had similar distributions according to wall thickness measurements (Fig. 5.2), only in cultures at 21 kPa O_2 was there detectable nitrogenase activity after 119 h (Fig. 5.1). Vesicle wall thickness and nitrogenase activity in 21 kPa O_2 cultures continued to increase until 184 h after removal of nitrogen and then became constant. Nitrogenase activity was not measurable at 40 kPa O_2 until 170 h (Fig. 5.1) when the mean wall thickness had reached 0.55 μm (Table 5.1).

At the conclusion of the experiment, nitrogenase activity was stable at 120 to 140 nmol C_2H_4. mg protein^{-1}. h^{-1} at all three pO_2 values (Fig. 5.1) and vesicles had considerably thicker walls and stalks at higher pO_2 (Fig. 5.2).

Table 5.1 Apparent vesicle wall thickness in *Frankia* sp. strain HHPCcI3 at different concentrations of oxygen. Measurements were made at the onset of nitrogenase activity and again 232 h after removal of nitrogen from the medium. Results show mean and (standard deviation; sample size).

	Apparent vesicle wall thickness (μm)	
kPa O_2	at appearance of nitrogenase activity	after 232 h
5	0.41 (0.02; 405)	0.42 (0.03; 415)
21	0.48 (0.04; 378)	0.54 (0.04; 399)
40	0.55 (0.03; 461)	0.61 (0.03; 387)

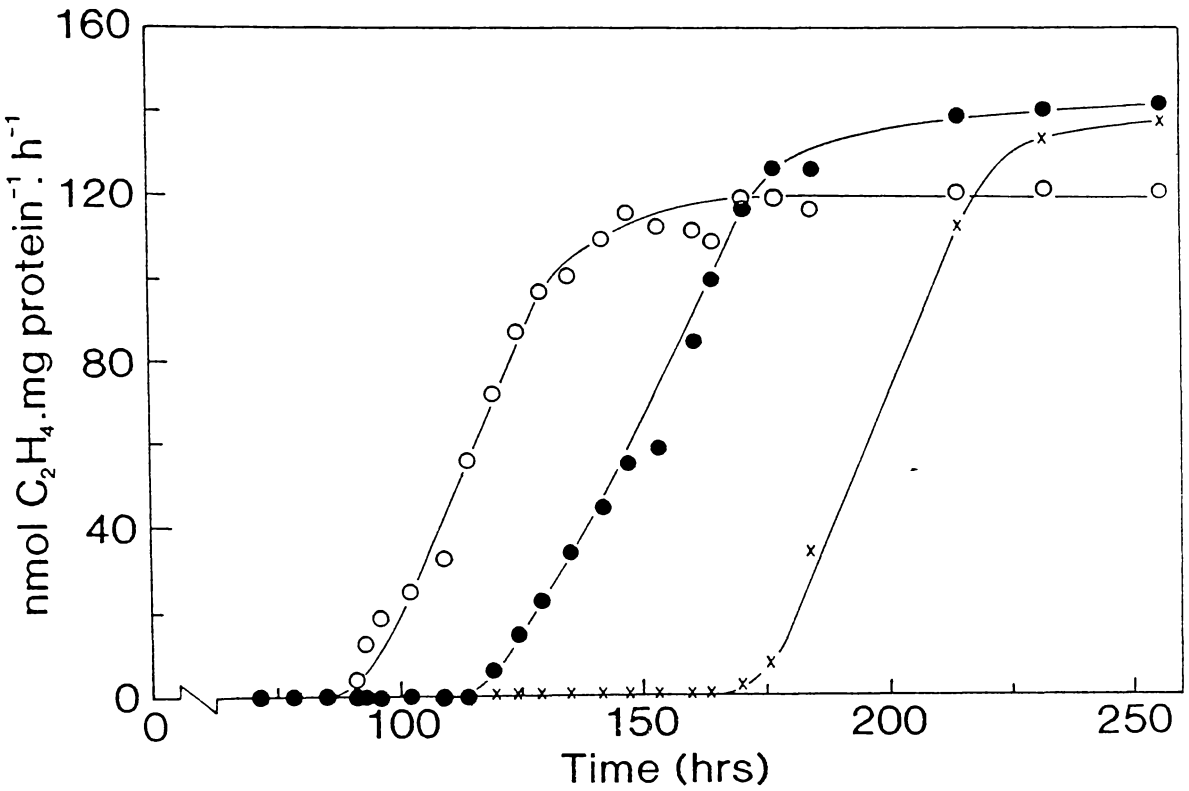


Figure 5.1 Effect of oxygen on development of nitrogenase activity. Continuous cultures of *Frankia* sp. strain HFPCcI3 were grown at 5 kPa O_2 (—o—), 21 kPa O_2 (—●—) and 40 kPa O_2 (—×—).

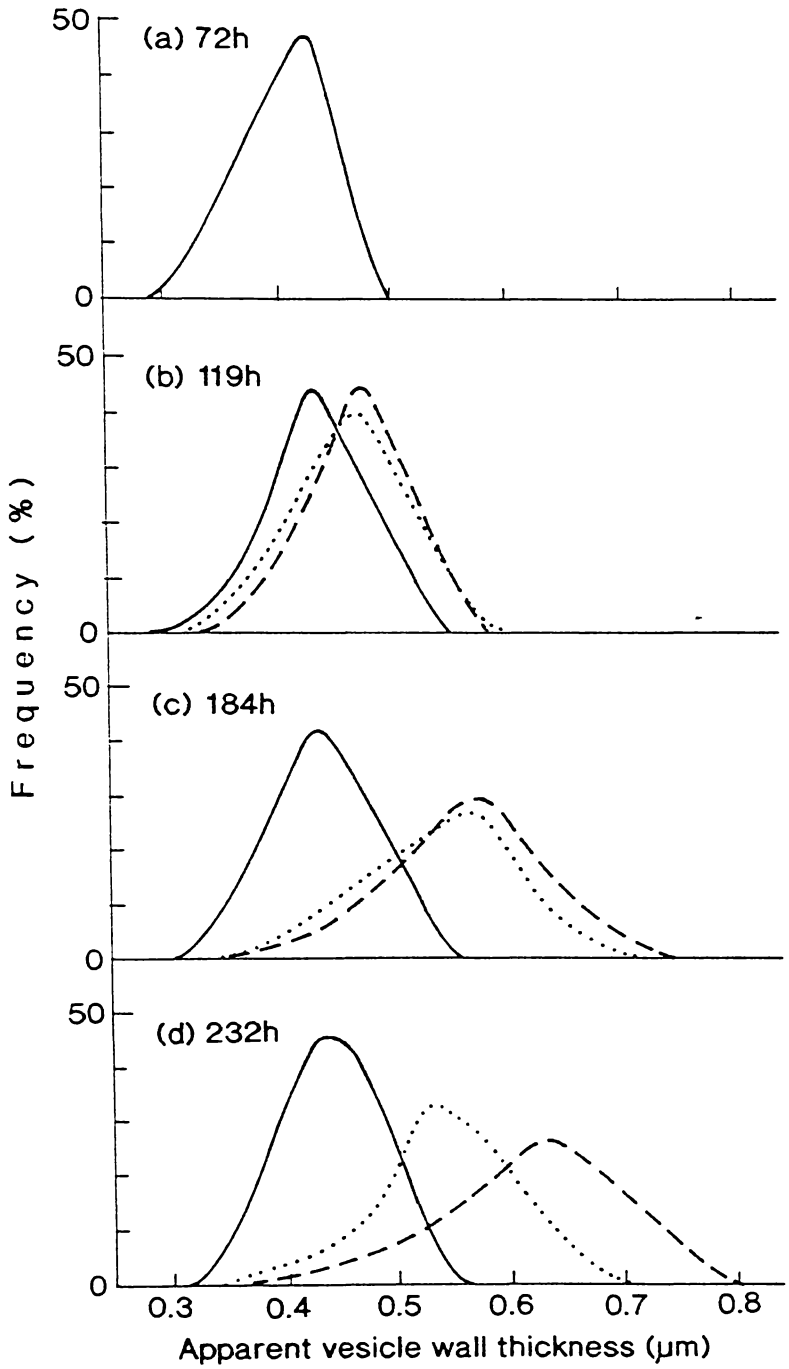


Figure 5.2 Development of thickened vesicle walls in *Frankia* sp. strain HFPCcI3 grown at 5 kPa O_2 (———), 21 kPa O_2 (·····) and 40 kPa O_2 (– – – –) following removal of nitrogen from the medium at time zero.

5.3 Nitrogenase activity and vesicle development in Ar : O_2

Since nitrogenase and the presence of vesicles in *Frankia* cultures are usually synonymous, it is possible that the products of nitrogen fixation have a role in regulation of vesicle development and, in particular, vesicle envelope thickness. If *Frankia* is grown under conditions where gaseous nitrogen is not available as a substrate for nitrogenase then the effects of oxygen on vesicle ontogeny can be studied independent of the influence of products of nitrogen fixation. Thus, in this experiment, nitrogenase activity and vesicle wall thickness was measured in *Frankia* grown at different pO2 levels with argon, rather than nitrogen, as the balance gas. It was hypothesised that if oxygen concentration regulates vesicle envelope thickness then a difference in vesicle wall thickness should still be observed but if products of nitrogen fixation somehow regulate envelope thickness then no difference in wall thickness would be measured at different pO_2 levels.

Continuous cultures were established at 5, 21 and 40 kPa O_2 as described in the previous experiment but with argon rather than nitrogen as the balance gas. Three cultures were also set up at 21 kPa O_2 in nitrogen to function as a control. Possible leakage of nitrogen into Ar : O_2 cultures was tested for throughout the experiment by running samples of the headspace gas on a TCD GC. Vesicle counts from Ar : O_2 and N_2 : O_2 cultures at 21 kPa O_2 were made at intervals throughout the experiment while measurements of protein level, nitrogenase activity and vesicle wall thickness were conducted after 240 h. Acetylene reduction assays were conducted at the growth pO_2 of the culture in an Ar : O_2 atmosphere.

Growth rate (protein level) in Ar : O_2 was low and after 240 h cultures had only a slightly higher protein level than was measured immediately after inoculation (Table 5.2). By comparison, protein level in the control cultures after 240 h was almost double. Despite the difference in growth rate the specific rate of nitrogenase activity was approximately the same in all three argon treatments and the control (Table 5.2). Vesicle number in Ar : O_2

cultures was however considerably greater than in N_2 : O_2 cultures and after 240 h was almost double the control counts (Fig. 5.3). Vesicle number in the control treatment reached a steady state maximum after 180 h but the number of vesicles in the Ar : O_2 cultures continued to increase throughout the experiment.

The main aim of this experiment however was to determine whether *Frankia* vesicles formed in an Ar : O_2 atmosphere still showed a response to oxygen concentration. The results from measurement of vesicle wall thickness are given in Table 5.2. Vesicles formed at 5 kPa O_2 were noticeably thinner-walled than those formed at either 21 or 40 kPa O_2 and the measurements are similar to those given for vesicles formed at different oxygen concentrations in N_2 : O_2 (section 5.2).

Table 5.2 Growth, nitrogenase activity and apparent vesicle wall thickness of *Frankia* sp. strain HFPCcI3 grown at different oxygen concentrations in Ar : O_2. Results are also given for control cultures grown at 21 kPa O_2 in N_2 : O_2. Measurements were made after 240 h. Values give mean and (standard deviation; sample size).

Treatment	Protein level [a.] (μg protein. ml^{-1})	Nitrogenase activity (nmol C_2H_4. mg protein^{-1}. h^{-1})	Vesicle wall thickness (μm)
Ar : O_2 5 kPa O_2	23.87 (0.41; 9)	122.93 (5.37; 9)	0.46 (0.03; 466)
Ar : O_2 21 kPa O_2	24.19 (0.32; 9)	134.09 (6.97; 9)	0.52 (0.02; 431)
Ar : O_2 40 kPa O_2	23.25 (0.47; 9)	129.95 (5.15; 9)	0.60 (0.03; 379)
N_2 : O_2 21 kPa O_2	38.29 (0.51; 9)	141.27 (6.54; 9)	0.54 (0.04; 399)[b.]

a. Initial protein level = 20 μg protein. ml^{-1}

b. Result from section 5.2

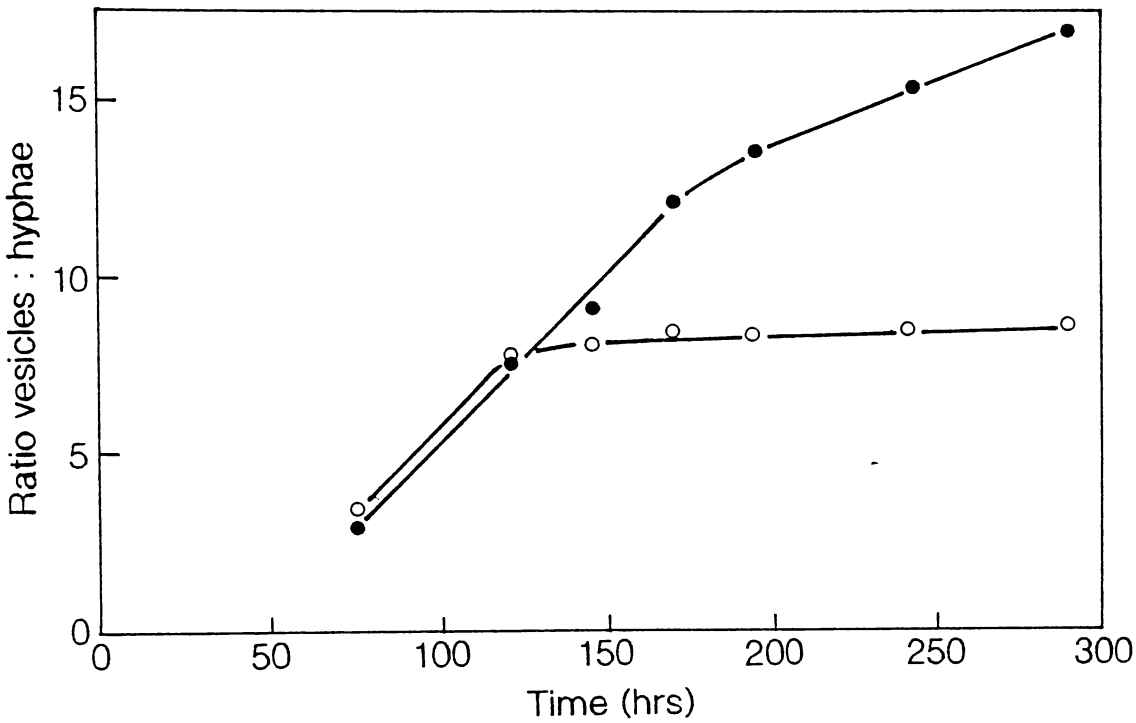


Figure 5.3 Number of vesicles in cultures of *Frankia* sp. strain HFPCcI3 grown in Ar : O_2 (—●—) or N_2 : O_2 (—○—) at 21 kPa O_2. Vesicle number was determined as the ratio of vesicles : unit hyphae (see section 2.1.7).

5.4 Effect of oxygen on nitrate-grown vesicles

The extent of vesicle envelope thickening in derepressed continuous cultures is regulated by the growth pO_2 (section 5.2). Since vesicle structure and vesicle induction in CcI3 grown on nitrate is similar to that on -N medium but acetylene reduction activity is undetectable, an experiment was designed to examine the effect of growth pO_2 level on vesicle wall thickness in nitrate-grown *Frankia*. The aim of this experiment therefore was to investigate the role of oxygen in vesicle development under conditions where nitrogen fixation activity could not be detected.

Continuous cultures of CcI3 were established on BAP medium containing KNO_3 (5 mM) at 5, 21 and 40 kPa O_2 and the experiment conducted as described above (section 5.2).

Thin-walled provesicles were first observed 48 h after inoculation at all three oxygen concentrations and mature vesicles were present in the cultures by 76 h. At this time the distribution of vesicles according to apparent wall thickness was similar at each pO_2 level, with a mean wall thickness of 0.40 μm (Fig. 5.4). This measurement is approximately the same as for vesicles on -N medium at a similar time (section 5.2).

Throughout the remainder of the experiment, vesicles growing at 5 kPa O_2 showed only a slight increase in wall thickness to reach a maximum of 0.42 μm after 240 h. In contrast vesicles at 21 and 40 kPa O_2 formed progressively thicker walls. After 240 h the mean vesicle wall thickness at 21 kPa O_2 was 0.51 μm compared with 0.56 μm at 40 kPa O_2. Comparison with measurements of apparent wall thickness from vesicles on -N medium show that nitrate-grown vesicles at equivalent oxygen concentrations are slightly thinner walled. It should be noted however that the low number of vesicles present in nitrate-grown cultures meant the sample numbers for vesicle wall measurements were comparatively low.

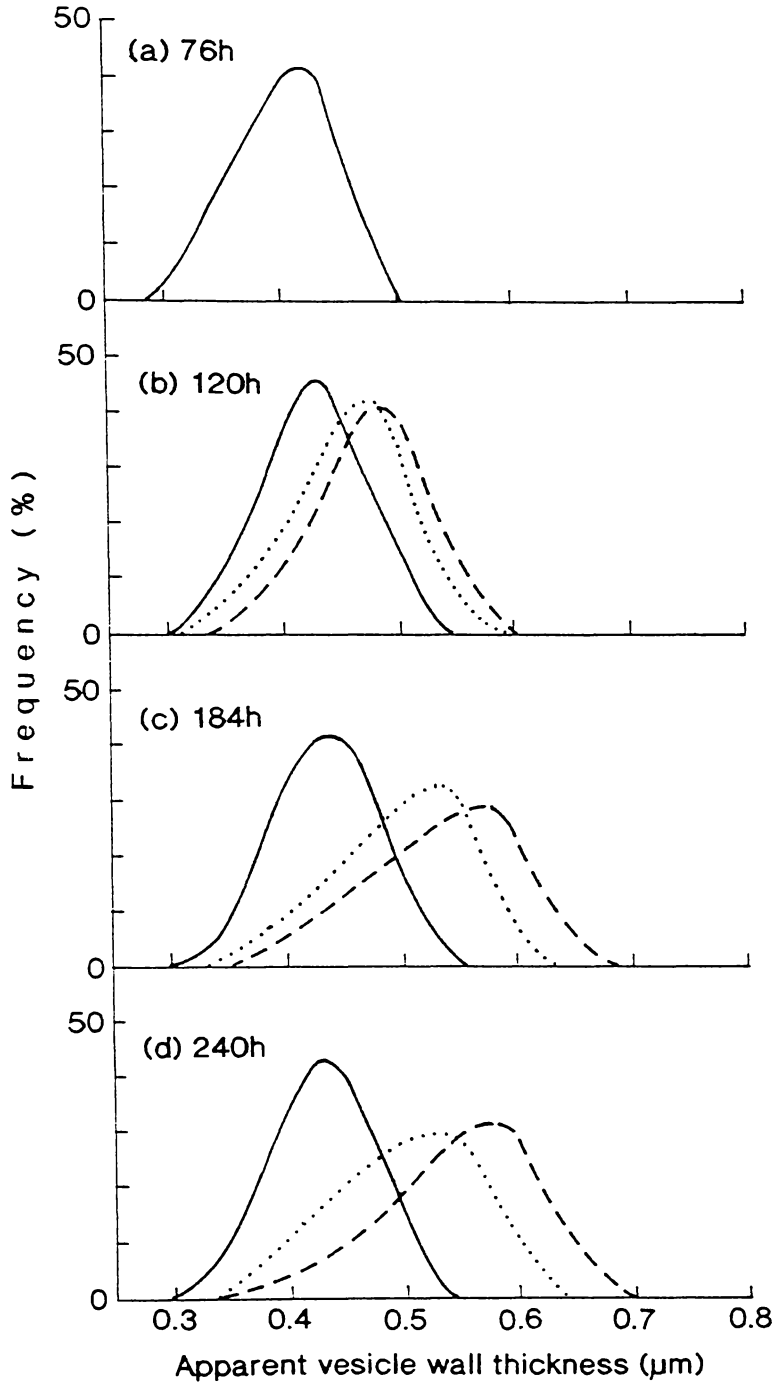


Figure 5.4 Development of thickened vesicle walls in *Frankia* sp. strain HFPCcI3 grown on nitrate-containing medium at 5 kPa O_2 (———), 21 kPa O_2 (----------) and 40 kPa O_2 (– – – –).

Although the effect of oxygen concentration on vesicle wall thickness in *Frankia* grown on nitrate-containing medium was clear, no acetylene reduction activity was detected at any pO_2 during the course of the experiment.

5.5 Effect of oxygen shock on nitrogenase and vesicles

If a thickened vesicle envelope is an absolute requirement for the expression of nitrogenase at high pO_2, then the recovery of nitrogenase after an oxygen shock should coincide with the formation of new, thicker-walled vesicles and/or the production of thicker walls on existing vesicles. To test this hypothesis, continuous cultures of *Frankia* sp. strain ArI3 were grown at 2 kPa O_2 for 210 h and then treated as follows (numbers refer to kPa O_2)

A: control: grown at 2, regassed with 2 and maintained at 2 (2:2:2)

B: oxygen shock 1: grown at 2, shocked with 21 for 30 minutes and then returned to 2 (2:21:2)

C: oxygen shock 2: grown at 2, shocked with 21 for 30 minutes and maintained at 21 (2:21:21).

Exposure to 21 kPa O_2 destroyed all nitrogenase activity but cultures returned to low pO_2 (treatment B) soon recovered enzyme activity and within 30 h had reached the same level as the control (treatment A) (Fig. 5). Cultures remaining at 21 kPa O_2 (treatment C) showed a 36 hour lag before activity returned although the final activity rate, after 97 h at 21 kPa O_2, was six times that in the control (Fig. 5.5).

Measurements of the apparent vesicle wall thickness indicated prolonged exposure to increased pO_2 was accompanied by thickening of the vesicle wall and stalk. Vesicles exposed to 21 kPa O_2 for 97 h (treatment C) had a mean apparent wall thickness of 0.58 μm compared to 0.43 μm at 2 kPa O_2 (treatments A and B) and appeared considerably brighter and thicker than the walls of vesicles at low pO_2 when viewed under dark-field

microscopy (Fig. 5.6). In addition, 83 % of vesicles at 21 kPa O_2 had formed a thickened stalk compared to only 7 % at 2 kPa O_2.

Thickening of the vesicle walls and stalks following oxygen shock occurred gradually (Fig. 5.7) and coincided with the recovery of nitrogenase activity (Fig. 5.5). Initial recovery of activity at 257 h occurred once apparent vesicle wall thickness had reached 0.50 μm while maximum activity coincided with attainment of mean wall thickness of 0.58 μm at 308 h.

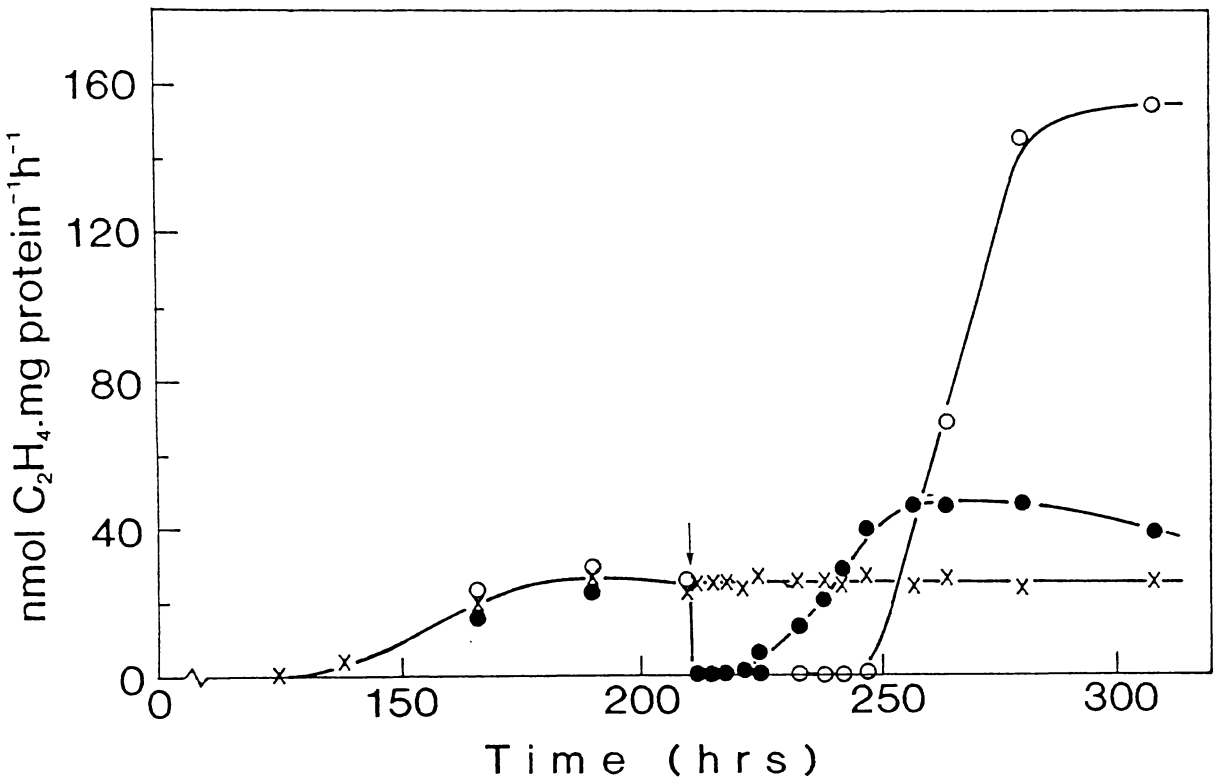


Figure 5.5 Time course of nitrogenase recovery in *Frankia* sp. strain HFPArI3 following oxygen shock at 210 h after removal of combined nitrogen from the medium (↓). Treatment A (control) cultures (—x—) were maintained at 2 kPa O_2. Remaining cultures were exposed to 21 kPa O_2 and either returned 2 kPa O_2 (treatment B; —●—) or maintained at 21 kPa O_2 (treatment C;—o—).

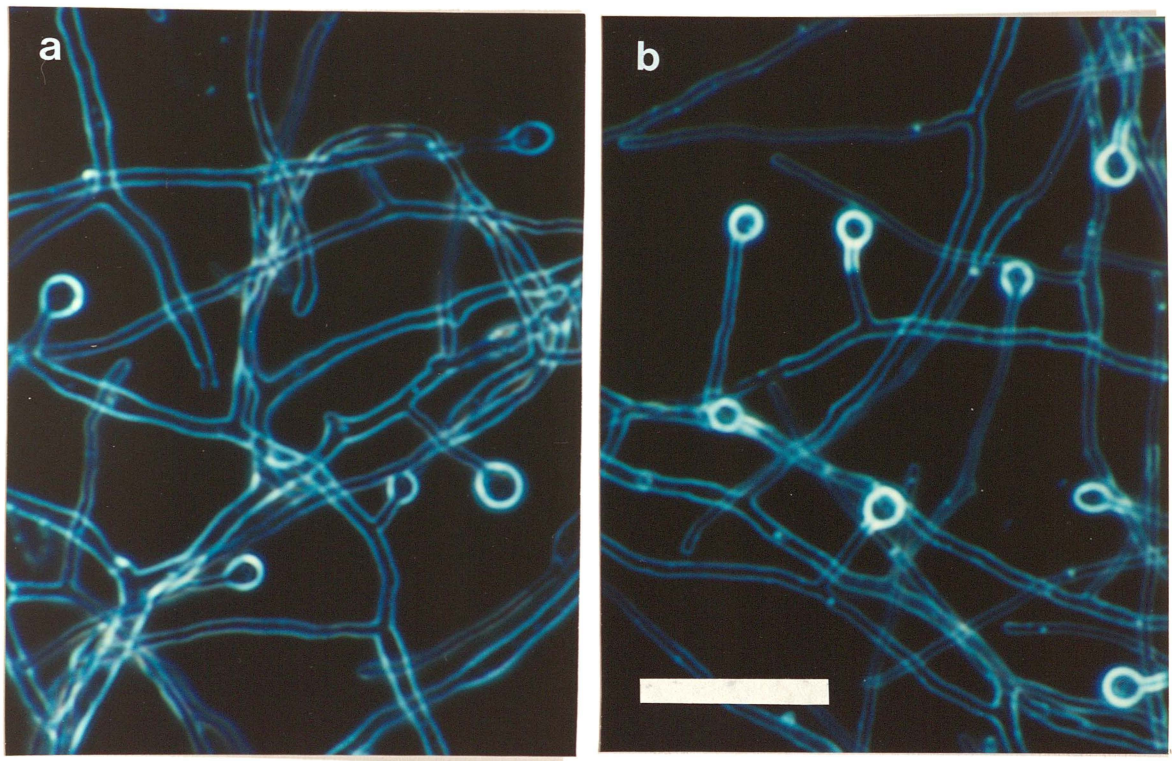


Figure 5.6 Dark field photographs of mature vesicles of *Frankia* sp. strain HFPArI3 grown to steady state conditions (at 308 h, see Fig. 5.5). (a) 2 kPa O_2, (b) 21 kPa O_2. Scale bar = 10μm.

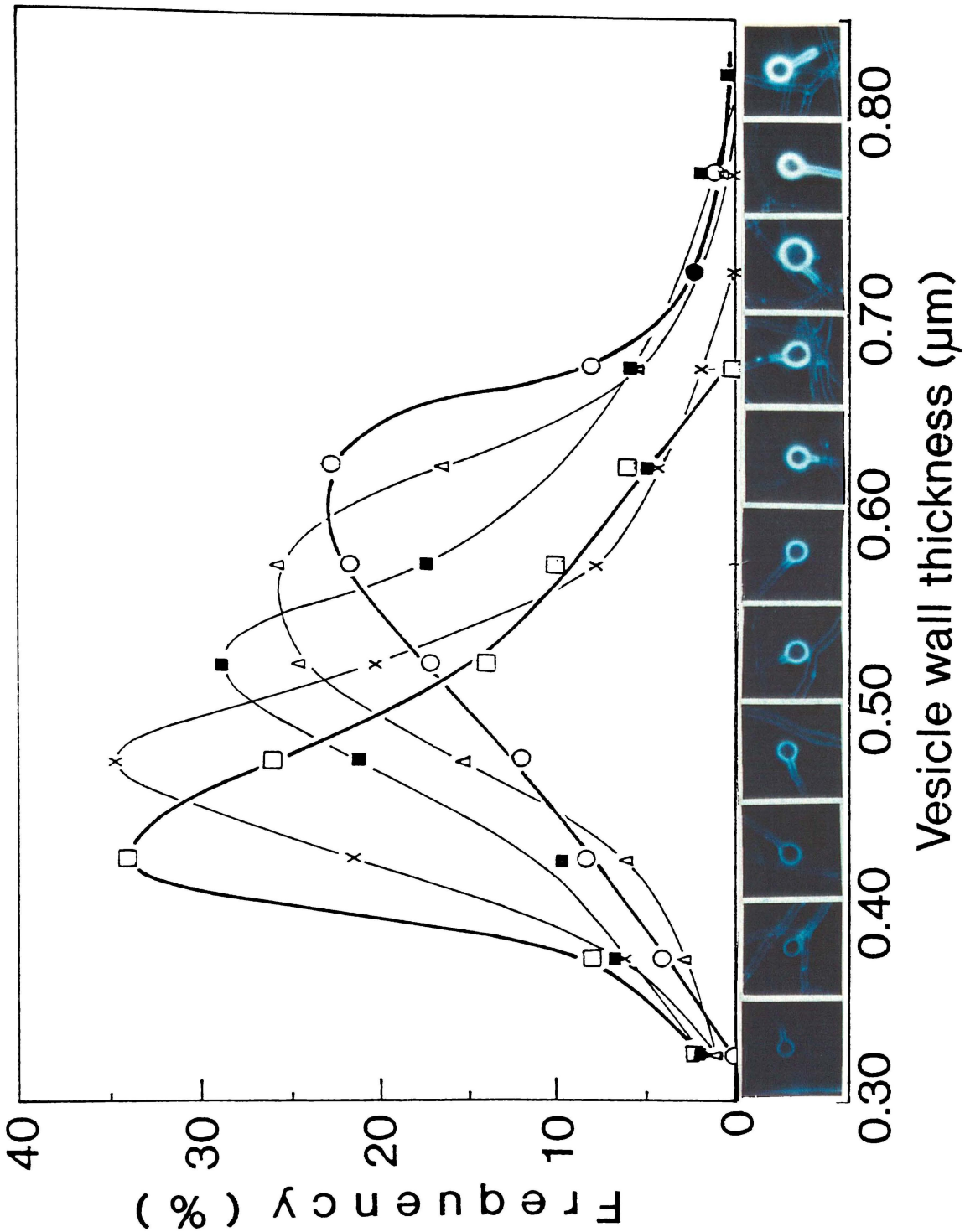


Figure 5.7 Distribution of vesicles according to their apparent wall thickness before (—□— , 165 h) and after oxygen shock (—×— , 233 h), (—■—, 264 h), (—△— , 280 h), (—○—, 308 h). Cultures were transferred from 2 kPa O_2 to 21 kPa O_2, 210 h after removal of combined nitrogen. Dark field photographs of selected vesicles of *Frankia* sp. strain HFPArI3 illustrate the range of wall thicknesses observed corresponding to the measurements shown on the graph.

5.6 Nitrogenase activity at low pO_2

Too little oxygen, like too much oxygen, can also limit nitrogenase activity. Based on the results of previous experiments modification of vesicle envelope thickness clearly allows *Frankia* to adapt to varying oxygen concentration. Thus at low pO_2 the vesicle envelope is thin and provides little resistance to oxygen diffusion so facilitating an adequate oxygen supply at the site of nitrogenase. An extreme example of this adaptation was shown by Murry *et al.* (1985) who reported nitrogenase activity in *Frankia* sp. strain HFPCcI3 but no formation of vesicles at very low pO_2 (0.3 kPa O_2). Thus at very low oxygen concentrations some *Frankia* strains may localise nitrogenase in the hyphae since the thinner hyphal walls offer lower resistance to oxygen diffusion than vesicle walls. Despite the ability of *Frankia* to modify the vesicle envelope, nitrogenase activity at 2 kPa O_2 in the previous experiment (section 5.5) was particularly low suggesting cultures were still oxygen limited to some extent.

The aim of this experiment was to examine nitrogenase activity of *Frankia* at low oxygen concentrations under stirred and static conditions to confirm the apparently poor response of *Frankia* to low pO_2 noted in the previous experiment and also to repeat the work of Murry *et al.* (1985) outlined above.

Continuous cultures CcI3 were established and the bottle headspace adjusted to give four replicates each at oxygen concentrations of 0.5, 1, 2 and 5 kPa O_2. Two replicates at each pO_2 level were placed on a rotary shaker (90 rpm) and the remaining two replicates incubated under static conditions. Although the dissolved pO_2 in the medium was not measured the oxygen concentration of the headspace gas was checked regularly and oxygen concentration adjusted to maintain pO_2 close to the nominal value. It was assumed that the dissolved pO_2 in stirred cultures was close to the headspace pO_2 but in static cultures the dissolved pO_2 would be lower than the nominal value since mixing was limited. Measurement of nitrogenase activity in each culture was conducted in triplicate at

intervals throughout the experiment. At the conclusion of the experiment (320 h) cultures were examined for the presence of vesicles.

The maximum rate of nitrogenase activity measured in each treatment and the time at which the maximum steady state rate was reached are given in table 5.3. No activity was measured in static cultures at 0.5 and 1 kPa O_2 and no vesicles were observed after 320 h. Nitrogenase activity in the presence of vesicles was however observed in all other cultures although the level and timing of the maximum rate varied. Stirred cultures at 5 kPa O_2 had the highest acetylene reduction rate which occurred 196 h after inoculation. Stirred cultures grown at 2 kPa O_2 also had maximum activity at 196 h but the rate was considerably lower than at 5 kPa O_2. In other cultures the maximum rate of activity decreased and the time taken to reach this maximum rate was longer as growth pO_2 decreased. All cultures which showed nitrogenase activity also had vesicles and while visual observation indicated vesicles were thinner-walled at progressively lower oxygen concentrations the differences were not significant.

Table 5.3 Effect of low oxygen concentration on nitrogenase activity in *Frankia* sp. strain HFPCcI3 under stirred and static continuous culture conditions. Results show the maximum rate of nitrogenase activity and the time the maximum was reached. Values for nitrogenase activity show mean and (standard deviation; sample size).

Treatment / growth pO_2	Nitrogenase activity (nmol C_2H_4. mg protein^{-1}. h^{-1})	Time of maximum activity (h)
stirred / 5 kPa O_2	132.72 (8.01; 6)	196
stirred / 2 kPa O_2	38.94 (5.78; 6)	196
stirred / 1 kPa O_2	24.60 (5.01; 6)	214
stirred / 0.5 kPa O_2	14.1 (3.98; 6)	224
static / 5 kPa O_2	31.3 (4.71; 6)	206
static / 2 kPa O_2	11.0 (2.78; 6)	224
static / 1 kPa O_2	0	-
static / 0.5 kPa O_2	0	-

5.7 Discussion

The differentiation of *Frankia* into a filamentous hyphal form with discrete, thick-walled vesicles enables it to fix nitrogen aerobically. Early reports of nitrogenase in *Frankia* showed an optimum pO_2 at near atmospheric levels (Tjepkema *et al.* 1981; Gauthier *et al.* 1981) and, as illustrated here, nitrogenase activity in static culture, or at least at very low pO_2, is severely oxygen limited. Adaptation of nitrogenase activity to oxygen concentrations above and below atmospheric (Murry *et al.* 1985; Parsons *et al.* 1987) is attributed to the diffusion resistance properties of the multilaminate vesicle wall. Parsons *et al.* (1987) demonstrated the relationship between pO_2, dark field brightness of vesicle walls and the number of layers in the vesicle envelope. Results presented here show the

thickening to be a "normal" process during vesicle development but one which is specifically under oxygen control and essential for nitrogenase function.

Timing of vesicle induction appears to be independent of pO_2 since appearance of provesicles and vesicles was simultaneous regardless of oxygen concentration and vesicle walls were initially of similar thickness. However, "normal" endogenous vesicle wall and stalk thickening is subsequently influenced by pO_2. Thickening continues to a certain point which is clearly related to the pO_2 prevailing during growth, in that vesicle envelopes and stalks are thicker at higher pO_2 levels and therefore afford a greater degree of resistance to oxygen diffusion. The role of oxygen in regulation of vesicle envelope thickness was confirmed by the response of vesicles to oxygen concentration in the presence of argon and when grown on nitrate. Thus, even under growth conditions where vesicles apparently have no active role in nitrogen fixation, oxygen is the sole factor regulating vesicle envelope thickness.

The hypothesis that nitrogenase in *Frankia* is a diffusion limited system, in which oxygen supply is limited by the vesicle, is supported by work on the kinetics of oxygen and acetylene uptake by *Frankia* cultures. The K_m for acetylene uptake by most nitrogen-fixing organisms is approximately 0.6 kPa (Hardy *et al.* 1973) however the apparent K_m for *Frankia* is approximately 2 kPa (Murry *et al.* 1984*a*). Murry *et al.* (1984*a*) also showed the acetylene K_m does not vary when V_{max} is altered by changing the pO_2 of the assay. These results are consistent with a diffusion limited system in which the apparent Km is the result of a combined diffusion and enzyme kinetics. It has also been shown the oxygen uptake kinetics of repressed and derepressed *Frankia* are significantly different (Murry *et al.* 1984*a*). In ammonium grown (repressed) cultures which lack vesicles, oxygen uptake shows simple kinetics saturating at 18 μM O_2 (K_m = 1 μM O_2). In derepressed cultures oxygen uptake shows a biphasic curve with two apparent K_m values, one at 1 μM O_2 and the other at 170 μM O_2 (V_{max} = 350 μM O_2). The conclusion from these experiments is that the high acetylene Km and the second high oxygen K_m are associated with the diffusion resistance provided by the vesicle.

Although induction of nitrogenase is triggered by removal of combined nitrogen from the media, the timing of induction of enzyme activity is also indirectly influenced by pO_2. Activity at 21 and 40 kPa O_2 began 28 and 79 h respectively after activity was first measured at 5 kPa O_2. The longer time lag at higher pO_2 levels is apparently a consequence of the time taken to form thicker-walled vesicles and stalks. This suggests a certain minimum wall thickness must be reached in order for nitrogenase to be adequately protected and therefore active at any given concentration of oxygen. This is confirmed by the work of Huss-Danell and Bergman (1990) who showed that immunologically detectable nitrogenase does not appear until some time after apparent vesicle maturity.

Recovery of nitrogenase follows a similar lag period when *Frankia* is exposed to a sudden increase in oxygen concentration. Again the lag is attributable to the time taken for the formation of thicker-walled vesicles and stalks and the thickening of walls of existing vesicles. Interestingly, once nitrogenase activity recovers following transfer from 2 kPa O_2 to 21 kPa O_2, the time taken to reach the level of activity in control cultures maintained at 2 kPa O_2 is much shorter in cultures remaining at 21 kPa O_2 (treatment C, Fig. 5.5) than in cultures returned after oxygen shock to 2 kPa O_2 (treatment B, Fig. 5.5). The slower rate of increase in activity at 2 kPa O_2 may be due to oxygen limitation of nitrogenase and respiration, a phenomenon shown previously by Murry *et al.* (1984*a*). Likewise, the lower level of activity at 2 kPa O_2 is probably due to oxygen limitation.

In this work *Frankia* showed an apparent threshold effect of oxygen on nitrogenase activity at low oxygen concentrations. Activity at 5 kPa O_2 was comparable to that under atmospheric conditions but activity at 2 kPa O_2 was reduced at least three-fold. Although no measurements were made, vesicles at 2 kPa O_2 appeared only slightly thinner-walled than those at 5 kPa O_2. This suggests that, under the growth conditions described here, *Frankia* was physically incapable of forming vesicles thin enough to maintain an adequate oxygen supply at very low external oxygen concentrations i.e. the thin-walled vesicles formed at 5 kPa O_2 were close to the lower limit of vesicle wall thickness. Low levels of nitrogenase activity under oxygen-limited conditions would also result in a slower growth

rate and cell metabolism. This in turn may have contributed to the longer time taken to reach peak nitrogenase activity observed in cultures grown at very low pO_2.

The observation of Murry *et al.* (1985) suggests that under conditions of severe oxygen-limitation *Frankia* does not form vesicles and nitrogenase activity is therefore located in the hyphae which are thinner-walled and therefore offer less diffusion resistance. However no such parallel situation was observed here even in static cultures where the dissolved pO_2 would have been particularly low. This may have been due to the differences in growth conditions since Murry *et al.* (1985) grew their CcI3 cultures at 33 ^{o}C with gas-sparging.

The products of nitrogen fixation clearly have no role in regulating vesicle envelope thickness as illustrated by the response of *Frankia* to Ar : O_2 atmospheres. However nitrogen fixation products probably do have a role in regulating the number of vesicles formed since in Ar : O_2 the vesicle number continued to increase throughout the experiment. Inhibition of nitrogenase activity due to the presence of argon and consequently the absence of nitrogenous compounds resulting from fixation may have been interpreted by the *Frankia* as a need for more nitrogenase to be synthesised and thus more vesicles to be formed. A similar feedback mechanism involving the products of nitrogen fixation has been described for regulation of nitrogenase synthesis (Gauthier 1983).

An absence of nitrogenous compounds produced during nitrogen fixation in *Frankia* grown in Ar : O_2 would be expected to result in inhibition of growth since precursor compounds for protein synthesis would not be present. However a low level of growth was observed in cultures in Ar : O_2. This may have been because the inoculum was not washed during transfer from ammonia-grown cultures and some residual nitrogenous compounds may have been available to support vesicle formation and an increase in protein level.

Other factors, in addition to the diffusion resistance provided by the vesicle may also contribute to protection of nitrogenase from oxygen inactivation in *Frankia*. Lopez *et al.* (1986) showed that utilisation of carbon substrates by *Frankia* was related to the pO_2 at which they were assayed. Cells utilising an organic acid such as propionate had maximum nitrogenase activity at 15 to 20 kPa O_2. However cells able to respire glucose or trehalose supported lower nitrogenase activity at an optimum of less than 10 kPa O_2. One conclusion drawn from this work is that the higher rate of respiration associated with utilisation of the organic acid provides a lower internal pO_2 and thus greater oxygen protection at higher external oxygen concentrations.

Many nitrogen-fixing organisms have high levels of enzymes, such as superoxide dismutase and catalase, responsible for scavenging potentially harmful oxygen-derived species. Puppo *et al.* (1989) for example reported high superoxide dismutase activity in purified *Frankia* vesicles. The involvement of oxygen scavenging enzymes in the protection of nitrogenase is further supported by the presence of high levels of catalase activity in *Frankia* gown on nitrogen-free media and in purified vesicle preparations. It would be interesting to determine whether oxygen concentration has an effect on the concentrations or activity of such enzymes in *Frankia.*

Although other factors such as cell respiration and the presence of enzymes involved in scavenging oxygen-derived species may contribute to oxygen protection in *Frankia,* it is most likely the vesicle which provides most protection for nitrogenase and allows *Frankia* to adapt to both a range of ambient growth pO_2 levels and to changes in pO_2. Some of the structural and biochemical properties of the vesicle which contribute to this oxygen diffusion resistance are discussed in the following chapter.

CHAPTER SIX

BACTERIOHOPANETETROL IN *FRANKIA*

6.1 Introduction

The close relationship between nitrogenase activity and the formation of vesicles in *Frankia* has been discussed previously. Direct assay after vesicle isolation (Noridge and Benson 1986) and immunogold labelling using antibodies against nitrogenase (Meesters 1987; Huss-Danell and Bergman 1990) confirm the vesicle as the site of nitrogenase both in pure culture and in most symbioses. *Frankia* vesicles have a cell envelope, or capsule, that completely surrounds the vesicle and subtending stalk. In freeze-fracture and freeze-substituted preparations the envelope appears as repeated lipid monolayers each about 3.5 to 4 nm thick (Torrey and Callaham 1982, Lancelle *et al.* 1985). Results from the previous chapter indicate the multilaminate vesicle envelope may function as a barrier to oxygen diffusion and the wall thickness is regulated by pO_2. Freeze fracture views of vesicles grown at different pO_2 (Parsons *et al.* 1987) showed an average of 17 lipid layers in the outer walls of vesicles at 4 kPa O_2 compared with 40 lipid layers in vesicles grown at 40 kPa O_2.

Initial studies of the lipid composition of *Frankia* vesicles (Tunlid *et al.* 1989) showed they contain a significantly higher amount and different composition of fatty acids than hyphal cells recovered from *Frankia* grown on ammonium. In particular, vesicles showed a higher proportion of fatty acids in the neutral and glycolipid fractions but lower amounts of polar lipids than vegetative cells. More recent work (Berry *et al.* 1991) detected an unusual class of lipids with amphiphilic properties associated with *Frankia* vesicles. This lipid was identified as bacteriohopanetetrol ($C_{35}H_{62}O_4$) and belongs to the hopanoid class of lipids. HPLC and mass spectrometry studies (Berry *et al.* 1991; Berry

pers. comm.) on isolated vesicles indicated the vesicle envelope consists mainly of bacteriohopanetetrol and a derivative, bacteriohopanetetrol phenylacetate monoester ($C_{43}H_{62}O_5$), in approximately equal amounts. Bacteriohopanetetrol has also been identified in extracts of vegetative cells but the phenylacetate monoester appears to be specific to the vesicle (Berry pers. comm). Both these hopanoids have also been identified in extracts of nodule tissue from *Alnus* and *Ceanothus* (Berry *et al.* 1991; Berry pers.comm).

Hopanoids are pentacyclic triterpenoids found widely distributed among bacteria and some plants and, like their animal equivalents, the sterols, are derived from squalene. Bacterial hopanoids are generally distinguishable from plant hopanoids by the presence of an extended tail (Prince 1987) and are found in cell membranes where they have an important role in membrane stability (Schmidt *et al.* 1986; Hermans *et al.* 1991). Flesch and Rohmer (1987) showed that, in those bacteria which possess hopanoids, hopanoid synthesis is essential to survival under all growth conditions. It is possible therefore that hopanoids such as bacteriohopanetetrol in *Frankia* also function in stabilising membranes and may be involved in altering vesicle envelope permeability.

The aim of this work was to confirm the role of the vesicle envelope as a diffusion barrier by conducting a quantitative analysis of hopanoids present in *Frankia* grown at a range of pO_2 levels. It was anticipated the presence of thickened vesicle walls in vesicles at high pO_2 would be correlated with an increase in hopanoid content. The study also involved identification of the major hopanoids present in lipid extracts from *Frankia* grown under repressed and derepressed conditions and from nodules of several actinorhizal species.

6.3 Lipid extraction

Lipids were extracted from *Frankia* sp. strain HFPCcI3 and nodules of *Alnus glutinosa*, *Casuarina cunninghamiana* and *Coriaria arborea* using the method described by Berry *et al.* (1991). Prior to lipid extraction *Frankia* cells, which had been grown in continuous culture, and washed nodules were stored at -70 °C. *Frankia* cells (2.0 g fwt) were harvested, centrifuged, re-suspended in hexane : isopropanol (3 : 2) and thoroughly homogenised in this solvent mixture using a Kontes microtissue grinder. Nodules (5.0 g fwt) were ground first in a mortar and pestle in liquid nitrogen, suspended in hexane : isopropanol and then homogenised. Lipid extracts suspended in solvent were then centrifuged and the supernatant filtered using a sintered glass Buchner filter to remove cell debri. The remaining residue was washed three times by re-suspending in hexane : isopropanol for approximately two minutes and then re-filtering. The lipid extract was then partitioned against 0.5 M Na_2SO_4 (anhydrous salt) to remove any water. Finally the extract was dried under a stream of nitrogen and the pellet re-suspended in chloroform : methanol (1 :1).

Immediately prior to analysis a 0.1 ml sample of the extract was removed, vacuum dried and re-suspended in 0.1 mls acetic anhydride : pyridine (1 :1) in order to acetylate the extract. Acetylation allowed for detection of the tetra-acetate of bacteriohopanetetrol. Fatty acids were derivatised by the addition of an ethereal solution of diazomethane (0.05 ml per sample) prepared by gently heating a two-phase mixture of aqueous potassium hydroxide and N-nitrosomethylurea in ether.

6.3 GC/MS analysis

GC/MS analyses were conducted on a 12 m HP-1 methyl silicone gum capillary column installed in a HP 5980 GC interfaced to a HP 5970(B) mass selective detector (MSD). Initial oven temperature was held at 150 °C for 0.5 minutes and then increased at 10 °C / minute to 325 °C, where it was held for 30 minutes. Typically 3 µl of the derivatised lipid extract were injected into the GC/MS system using a Grob injection time of 36 seconds at a head pressure of 10 psi. The MSD was operated in scan mode between masses 70 to 720. Peaks were integrated using the standard HP GC/MS software package and identified on the basis of characteristic ionisation mass spectra. Measurement of peak size was determined against an internal n-alkane standard added to the derivatised extract. It was intended to use a C_{18} internal standard (Octadecane, 1 mg. ml-1) to quantify early eluting lipids and a C_{28} standard (n-Octacosane, 1mg. ml^{-1}) for quantifying the C_{30} hopanes and C_{35} hopane alcohols. However it was found that in many of the extracts, squalene ($C_{30}H_{50}$) was present and partly or completely obscured the C_{28} standard peak. Consequently all components were quantified relative to the C_{18} internal standard. A response factor of 0.636 was calculated from a mixture of the C_{18} internal standard and an authenticated bacteriohopanetetrol tetra-acetate standard isolated from *Frankia* sp. strain HFPArI3 provided by Alison Berry, University of California, Davis, California. This response factor was used to quantify the tetra-acetate peaks while a response factor of one was used for other hopanoid peaks.

6.4 Major lipids present in *Frankia* and nodule extracts

GC/MS analysis of derivatised lipid extracts from *Frankia* cultures all gave similar chromatograms. Figure 6.1 shows a typical chromatogram obtained from *Frankia* grown under derepressed conditions.

Early eluting lipids were identified as saturated and unsaturated fatty acids (Fig. 6.1: FA) with a characteristic fragmentation ion at m/z 74. Major fatty acid peaks included (in order of elution): palmitic acid (anti-isomer) (C16:0 ai.), palmitoleic acid (C16:1, cis-9), palmitic acid (C16:0), margaroleic acid (C17:1), linoleic acid (C18:2 cis-9,12), oleic acid (C18:1 cis-9) and stearic acid (C18:0). Also eluted in this region was the C18 internal standard, octadecane (Fig. 6.1: S) which was detected between the margaroleic and linoleic acid peaks.

Many of the lipid extracts also had a major peak at approximately six minutes (Fig. 6.1: ?). This compound is an unknown with major fragmentation ions at m/z 135, 312, 202 and 354. A second unknown lipid (Fig. 6.1: ??) was eluted after approximately 17 minutes and had major fragmentation ions at m/z 178 and 329 with molecular weight 515. Neither of these unknown compounds was a hopanoid.

A large peak eluting at approximately eleven minutes and present in all samples was identified as squalene (Fig. 6.1: SQ). Squalene ($C_{30}H_{50}$, mwt 410) is the uncyclised precursor of hopanoids and also their animal equivalents, the sterols.

Most hopanoids (Fig.6.1: H) appeared on the chromatograms after eleven to 17 minutes though it should be noted that not all peaks detected in this time frame were hopane lipids. Hopanoids are relatively easy to identify using the major fragmentation ion at m/z 191 as a marker. This distinctive ion is formed as a result of cleavage of the basic hopane ring

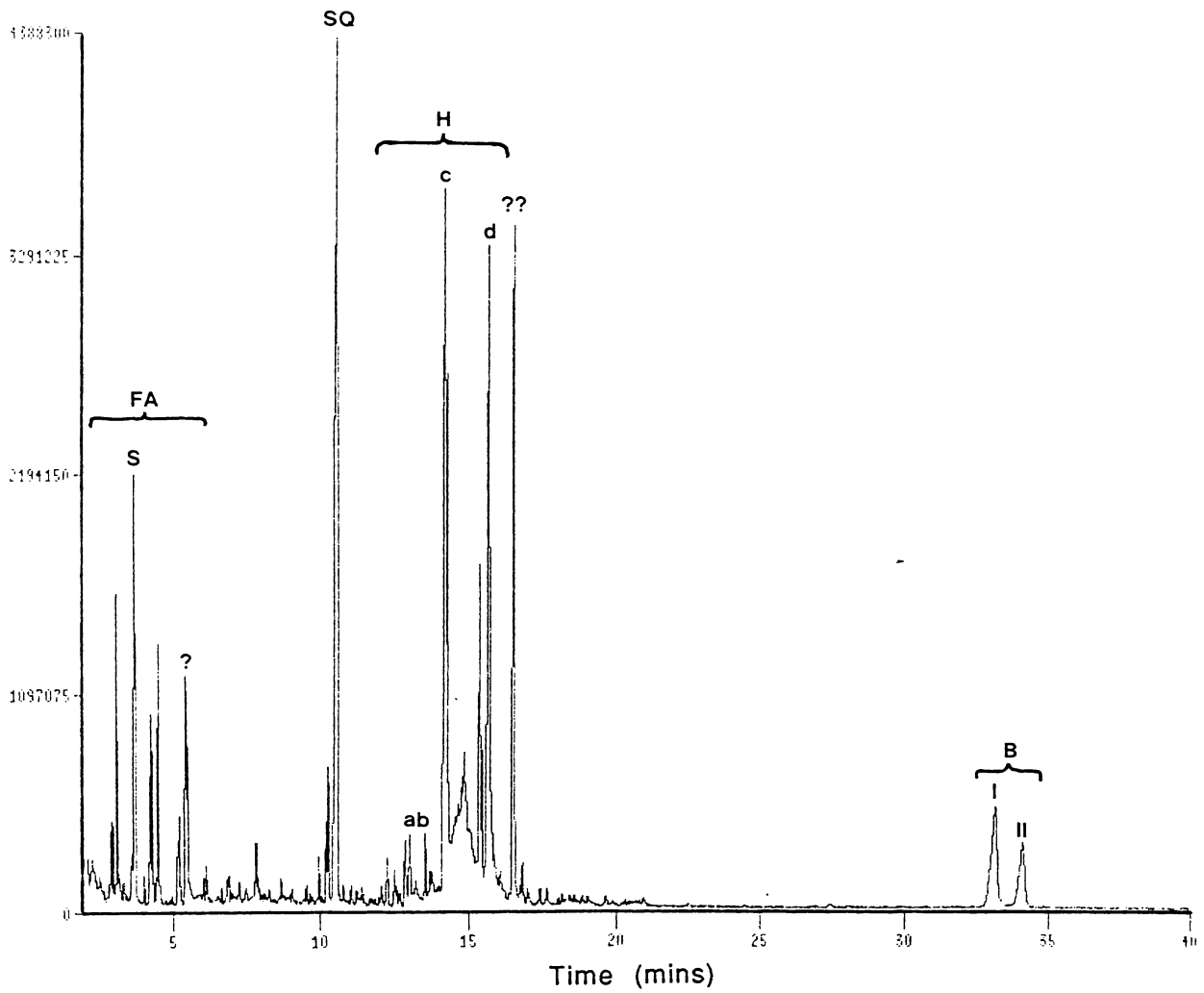


Figure 6.1 Example of GC/MS chromatogram typically obtained from lipid extracts of *Frankia* cells and actinorhizal nodules. Major peaks were identified as fatty acids (FA), C_{18} internal standard (S), unknown (?), squalene (SQ), hopanoids (H) including hopene I (a), hopene II (b), diplotene (c) and diploterol (d), unknown (??), and bacteriohopanetetrol tetra-acetatae (B) including the 22R (I) and 22S (II) stereoisomers.

structure. A number of hopanoids and their isomers were detected in the lipid extracts and the four major peaks identified on the basis of their mass spectra were:

a. hopadiene isomer I (hopene I)

b. hopadiene isomer II (hopene II)

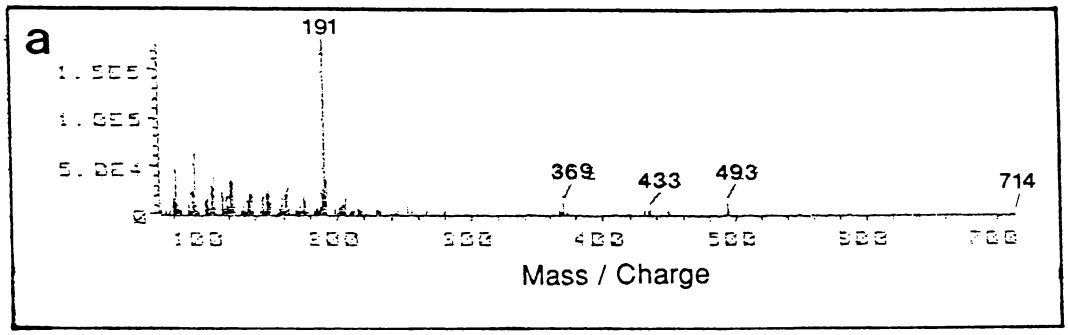
c. hop-22(29)-ene (diplotene)

d. hop-22-ol (diploterol).

A fifth hopanoid, bacteriohopanetetrol tetra-acetate (Fig. 6.1: B), took approximately 30 to 35 minutes to elute due to its relatively high molecular weight (mwt 714). All samples contained two bacteriohopanetetrol tetra-acetate peaks ascribed to the 22R (Fig. 6.1:I) and 22S (Fig, 6.1:II) isomers respectively. Although not obvious in figure 6.1, other isomers were often detected in extracts as earlier eluting pairs of peaks. Both bacteriohopanetetrol tetra-acetate isomers give the same mass spectra (Fig. 6.2a). A scheme for the major fragmentation ions, at m/z 191, 369, 433 and 493, is shown in figure 6.2b. These fragmentation ions were present in all samples but only one extract, from *Alnus* nodules, contained the molecular ion (714) since this is usually present in only small amounts.

Derivatised lipid extracts from nodules gave chromatograms similar to extracts from *Frankia* and contained a range of fatty acids and hopanoids including bacteriohopanetetrol tetra-acetate. Nodule extracts did however contain lupanes which appeared as peaks in the same time frame as the hopanoids. Lupanes are also pentacyclic triterpenoid lipids and, like hopanoids, are characterised by a major fragmentation ion at m/z 191. However lupanes are a constituent of terrestrial higher plants while hopanoids are largely restricted to bacteria.

It is worth noting that the bacteriohopanetetrol phenylacetate monoester, described by Berry *et al.* (1991) was not detected in any of the lipid extracts in this study since its comparatively high molecular weight (793) was beyond the detection capabilities of this system. In addition, since bacteriohopanetetrol phenylacetate monoester is specific to vesicles (Berry pers. comm.), and vesicles comprise only a small proportion of *Frankia*



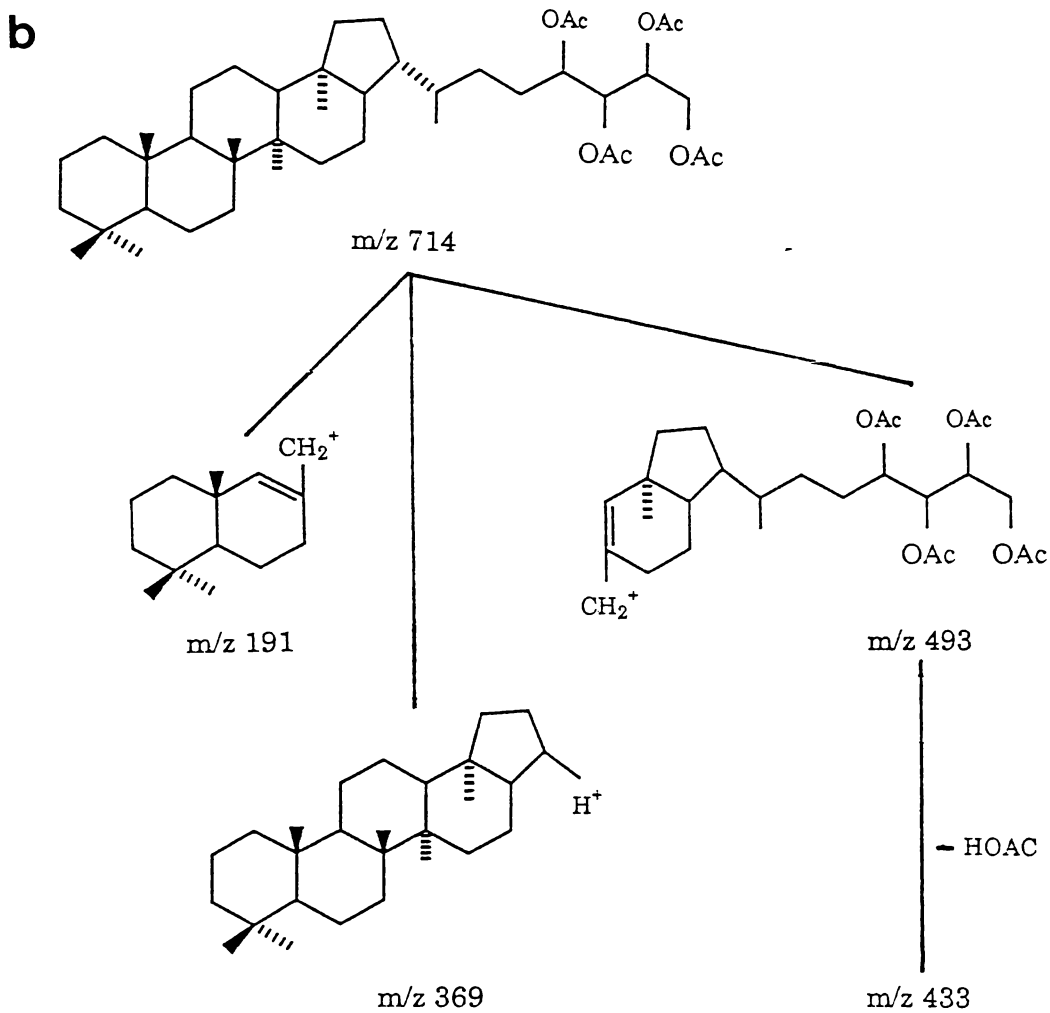


Figure 6.2 Mass spectrum obtained for bacteriohopanetetrol (Fig. 6.2a) showing major fragmentation ions at m/z 191, 369, 433 and 493 and a scheme for the origin of these ions (Fig. 6.2b).

cells grown under derepressed conditions, the concentration of this compound may have been too low for it to be detected. Berry and coworkers investigated hopanoids present in preparations of isolated vesicles rather than using exractions from both vesicles and hyphae.

Measurement of the hopanoid content in lipid extracts from *Frankia* was based on quantification of six major hopane compound peaks: hopene I, hopene II, diplotene, diploterol, bacteriohopanetetrol tetra-acetate I (22R isomer) and bacteriohopanetetrol tetra-acetate II (22S isomer). In extracts from nodules, quantification did not include either hopene I or hopene II since these lipids were not present in significant amounts.

6.5 Hopanoid content of nodules

Nodules of all three actinorhizal species, *Alnus glutinosa*, *Casuarina cunninghamiana* and *Coriaria arborea*, contained a range of hopanoid lipids although visual assessment of chromatograms indicated *Coriaria* nodules contained only a small concentration of hopanoids in terms of all lipids detected. *Alnus* and *Casuarina* nodules however showed larger proportions of hopanoids. Many of the hopane compounds present had the typical fragmentation ion at m/z 191 but had additional side chains attached to the basic ring structure.

Table 6.1 gives the relative amounts of the four major hopanoid compound in each nodule extract. In all three species diplotene was the most abundant hopanoid. Both bacteriohopanetetrol tetra-acetate isomers were present in all three species but only in very low concentrations in *Casuarina* nodules (Fig. 6.3a). In *Alnus* and *Coriaria*, bacteriohopanetetrol tetra-acetate comprised approximately 25 % to 28 % of total hopanoids although *Alnus* also had an earlier eluting pair of isomers (Fig. 6.3b). Bacteriohopanetetrol tetra-acetate in *Alnus* nodule extracts also differs from that in

Casuarina and *Coriaria* nodules in that the 22R isomer was less abundant than the 22S isomer although the significance of this is unclear.

Table 6.1 Relative hopanoid contents of *Alnus glutinosa*, *Casuarina cunninghamiana* and *Coriaria arborea* nodules.

	% of total hopanoids		
Hopane compound	*Alnus*	*Casuarina*	*Coriaria*
Diplotene	44.6	69.7	49.2
Diploterol	17.3	29.5	24.9
Bact. tetra-acetate I[a.]	8.9 (3.7)[b.]	0.5	20.3
Bact. tetra-acetate II[a.]	20.7 (4.8)[b.]	0.3	5.6

a. Bact. = bacteriohopanetetrol

b. Figures in brackets refer to the second pair of isomers

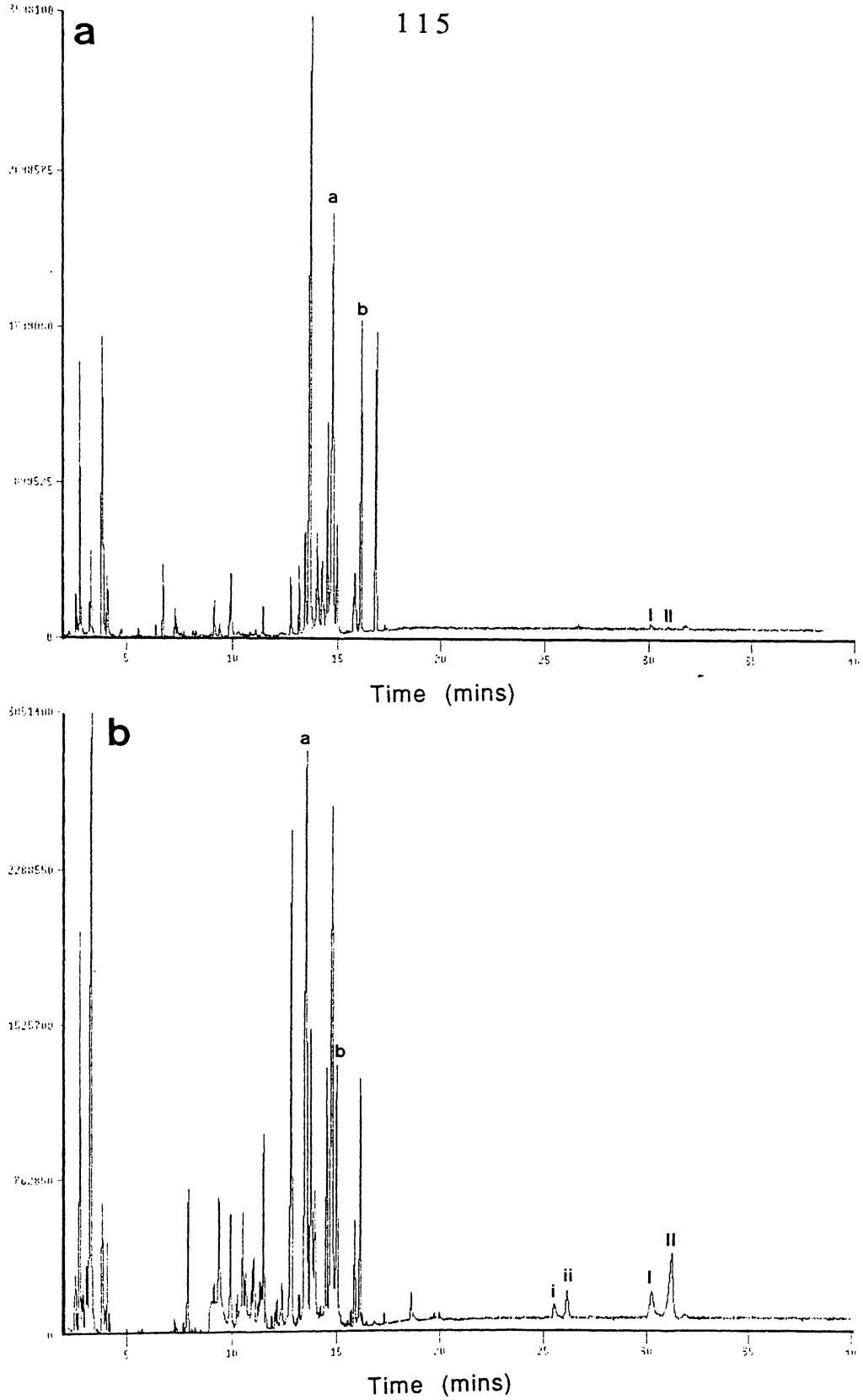


Figure 6.3 GC/MS chromatograms of lipid extracts from (a) *Casuarina cunninghamiana* and (b) *Alnus glutinosa* nodules. The major hopanoid peaks are: diplotene (a), diploterol (b) and the two bacteriohopanetetrol tetra-acetate isomers (I and II). The *Alnus* chromatogram also shows a second pair of tetra-acetate isomers (i and ii).

6.6 Hopanoid content of repressed and derepressed *Frankia*

Analysis of lipid extracts from *Frankia* grown on ammonium (repressed) and on nitrogen-free medium (derepressed) showed similar hopanoid contents (Table 6.2). In both extracts, bacteriohopanetetrol tetra-acetate comprised at least 50 % of the hopanoids, significantly more than in nodules, and in both cases the 22R isomer was present in higher concentrations than the 22S isomer. Of the remaining hopane compounds quantified, diplotene was the most abundant, though in derepressed *Frankia* the diploterol component was also significant.

In terms of absolute amounts of hopane lipids in the extracts, derepressed *Frankia* had a slightly higher hopanoid content than cultures lacking vesicles (Table 6.2).

Table 6.2 Hopanoid contents of repressed (+ N) and derepressed (- N) *Frankia* sp. strain HFPCcI3.

	% of total hopanoids	
Hopane compounds	+ N	- N
Hopene I	3.6	1.8
Hopene II	2.6	1.1
Diplotene	32.3	20.5
Diploterol	10.9	16.3
Bact. tetra-acetate I[a.]	31.9	37.9
Bact. tetra-acetate II[a.]	18.7	22.4
Total hopanoids (mg. g dwt cells^{-1})	1.65	1.83

a. Bact. = bacteriohopanetetrol

6.7 Hopanoid content of *Frankia* grown at a range of oxygen concentrations

The relative amounts of the major hopanes present in *Frankia* grown at 2, 21 and 60 kPa O_2 are given in Table 6.3. Bacteriohopanetetrol tetra-acetate was the major hopane compound present in each treatment, comprising approximately 50 % of the hopane compounds. The 22R isomer was the more abundant in each case although the difference between the two isomers was reduced as the oxygen concentration increased. A second pair of isomers was also present in *Frankia* at all three growth pO_2 levels (Fig 6.4). Increased oxygen concentration resulted in a considerable increase in the proportion of the minor hopane compounds (hopene I and hopene II) present. Changes in the relative amounts of diplotene and diploterol present were also observed with increased oxygen concentration (Table 6.3).

Increased growth pO_2 was accompanied by a slight increase in the absolute amounts of hopanoids present in *Frankia* (Table 6.3).

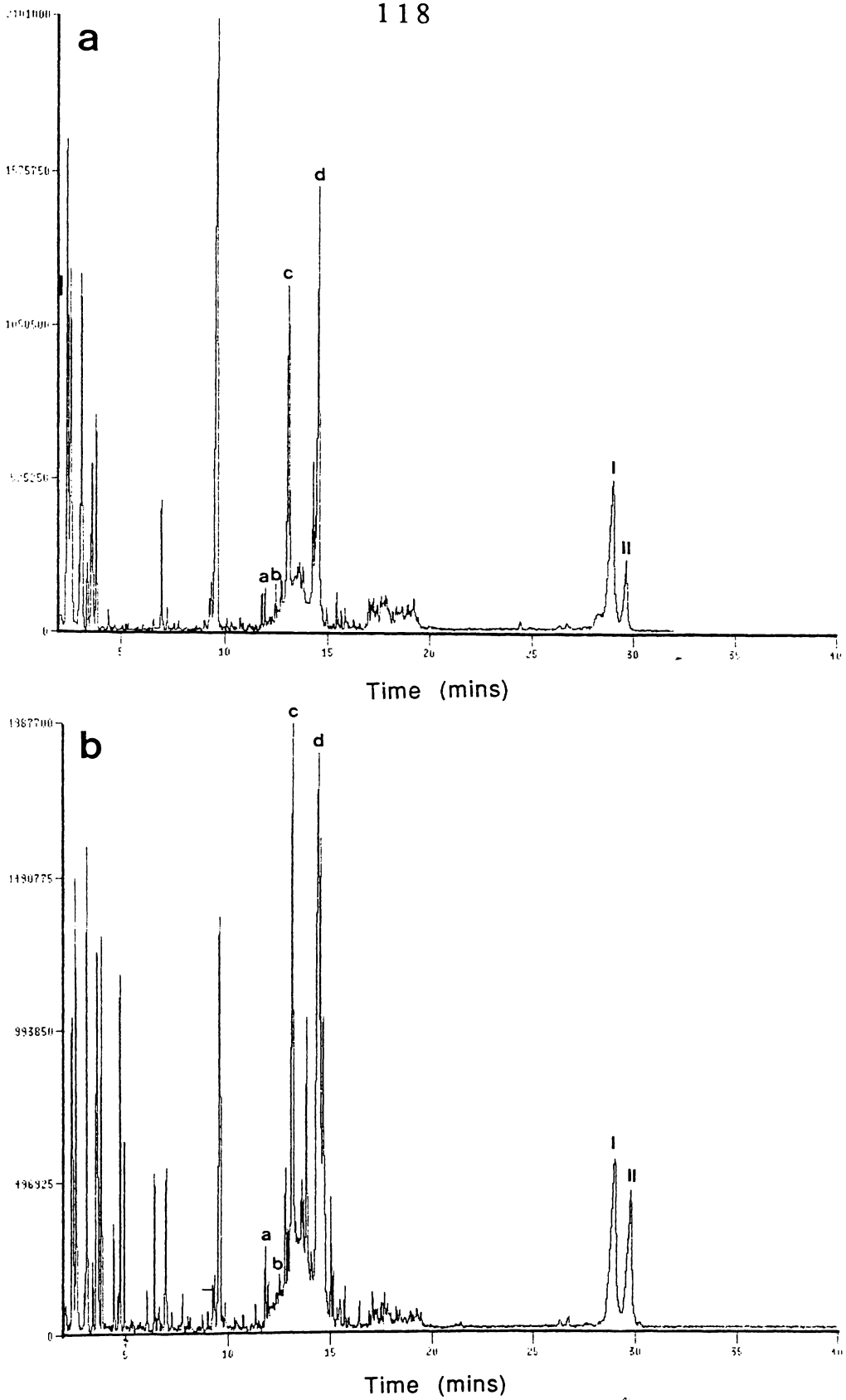


Figure 6.4 GC/MS chromatograms of lipid extracts from *Frankia* sp. strain HFPArI3 grown at 2 kPa O_2 (Fig. 6.4a) and 60 kPa O_2 (Fig. 6.4b). The major hopanoid peaks are: hopene I (a), hopene II (b), diplotene (c), diploterol (d) and the two bacteriohopanetetrol tetra-acetate isomers (I and II). Both chromatograms also show a second pair of earlier-eluting tetra-acetate isomers.

Table 6.3 Hopanoid contents of *Frankia* sp. strain HPFCcI3 grown at three different oxygen concentrations.

	% of total hopanoids		
Hopane compounds	2 kPa O_2	21 kPa O_2	60 kPa O_2
Hopene I	1.3	2.8	8.7
Hopene II	1.0	2.8	7.4
Diplotene	14.9	19.2	26.9
Diploterol	26.5	18.7	10.6
Bact. tetra-acetate I[a.]	42.4	36.6	25.5
Bact. tetra-acetate II[a.]	14.1	19.9	20.9
Total hopanoids (mg. g dwt cells^{-1})	1.88	1.98	2.18

a. Bact. = bacteriohopanetetrol

6.8 Discussion

Hopanoids are among the most widespread biomolecules and are also a major component of soluble organic matter in sediments (Ourisson *et al.* 1987). Rohmer *et al.* (1984) reported the presence of hopanes in more than half the 100 strains of prokaryotes they tested including cyanobacteria, obligate methylotrophs, purple non-sulphur bacteria and many Gram- positive and Gram-negative chemoheterotrophs. The amount of hopanoids present in *Frankia* was calculated as approximately 1.8 mg. g dwt cells-1 which lies at the upper end of the range of 0.4 to 2 mg. g dwt cells^{-1} given by Rohmer *et al.* (1984) for a number of different bacteria.

Hopanoids are structurally similar to sterols in their molecular dimensions and amphiphilic properties and both arise from the same precursor, squalene. However, cyclisation of squalene during the synthesis of hopanoids is an anaerobic process whereas sterol synthesis requires oxygen (Rohmer *et al.* 1979). It is surprising then that, although hopanoid biosynthesis is independent of oxygen, hopanoids have never been found in strict anaerobes (Ourisson *et al.* 1987).

The simplest prokaryotic hopanoids, the C_{30} compounds diplotene and diploterol, were first found in a few cyanobacteria (Gelpi *et al.* 1970) and have since been shown to be present in all hopanoid-containing prokaryotes. As expected, both these hopanes were detected in *Frankia* lipid extracts where they comprised approximately 38 % of hopanoids. Diplotene and diploterol were also detected in large amounts in the nodule extracts and in most samples diplotene was the more abundant. It is unlikely there is any real significance in the relative amounts of these two C_{30} compounds particularly since Rohmer *et al.* (1984) showed the relative amounts of the two hopanes varies considerably among those bacteria tested and even between closely related species. It is interesting to note however that the relative abundance of diplotene in *Frankia* lipid extracts increased with increasing pO_2 whereas diploterol concentration decreased.

The major hopanes in most bacteria are the C_{35} bacteriohopanepolyols (Rohmer *et al.* 1984) which have the basic C30 skeleton linked at C-30 to a C_5 *n*-alkyl polysubstituted chain. Bacteriohopanetetrol, which was first discovered in *Acetobacter aceti* ssp. *xylinum* (Forster *et al.* 1973), is the most widespread of these hopanes. Berry *et al.* (1991) showed bacteriohopanetetrol is a major constituent of *Frankia* vesicles and results presented here showed an acetylated derivative of this hopane composed approximately 50 % of the hopanoids present in *Frankia*. This figure is consistent with that given for other hopanoid-containing bacteria (Hermans *et al.* 1991).

In all *Frankia* and nodule extracts at least two stereoisomers of bacteriohopanetetrol tetra-acetate were detected and in some of the extracts a second pair of isomers was present. Berry *et al.* (1991) also detected a second smaller bacteriohopanetetrol tetra-acetate peak in their HPLC analysis of lipid extracts from *Frankia* and *Alnus* and *Ceanothus* nodules but they could find no evidence of other stereoisomers (Berry pers. comm). In all lipid extracts in this study, except that from *Alnus* nodules, the 22R isomer was more abundant than the 22S isomer. Very few other hopanoid-containing bacteria contain two bacteriohopanetetrol stereoisomers but those which do, also show a significantly higher concentration of the 22R isomer (Rohmer *et al.* 1984). Two stereoisomers were also detected following degradation of oil shale keragen which is a sediment with microorganism origins (Mycke *et al.* 1987). However any significance of the presence or absence of two bacteriohopanetetrol stereoisomers is unknown.

Numerous studies have investigated the role of hopanoids in providing membrane stability. Bacteriohopanetetrol was shown to induce organisation of microfibrils from cellulose micells in *Acetobacter aceti* ssp. *xylinum* (Haigh *et al.* 1973). Since then, increases in hopanoid content of a range of bacteria have been reported in response to increased temperature (Poralla *et al.* 1984; Schmidt *et al.* 1986; Hermans *et al.* 1991) and ethanol concentration (Bringer *et al.* 1985; Schmidt *et al.* 1986; Hermans *et al.* 1991) i.e. in conditions where more membrane stabilisers would be necessary. Berry *et al.* (1991) therefore proposed a role for bacteriohopanetetrol in altering cell and, in particular, vesicle permeability in *Frankia*.

Initial studies of vesicle composition in *Frankia* compared the lipid contents of vesicles and hyphae. Tunlid *et al.* (1989) found a range of fatty acids, including those detected in this study, in both vesicles and hyphae although vesicles had considerably higher amounts of neutral and glycolipid fatty acids but lower amounts of polar fatty acids than hyphal preparations. In addition they detected several long-chain compounds with molecular ions at m/z 408 and 410 at high concentrations in vesicles. Although

unidentified at the time, these compounds probably correspond to the hopenes (mwt 408) and diplotene and diploterol (mwt 410) found in this study. Berry *et al.* (1991) looked more specifically at the bacteriohopanetetrol content of *Frankia* cells grown under derepressed conditions i.e. with vesicles, and those grown on ammonium but they could find no significant differences between the two. In this study however the overall hopanoid content and bacteriohopanetetrol tetra-acetate concentration of derepressed *Frankia* was slightly higher than in repressed cultures lacking vesicles although it should be emphasised this data is based on single lipid extractions. An increase in the absolute hopanoid content was also measured in response to increased growth pO_2 suggesting oxygen does have a regulatory influence on the amount of hopanoid synthesised and agreeing with observations that vesicle walls are thicker at higher oxygen concentrations. More significant differences in hopanoid content may have been measured if lipid extractions were made from isolated vesicle preparations, since oxygen only has an effect on vesicle wall thickness and not on the hyphal wall. However, vesicles make up only a small proportion of cells in derepressed cultures and differences in the hopanoid content of vesicles in response to oxygen may well be masked to a large extent. The effects of oxygen on hopanoid content may also become clearer if comparisons are made based on bacteriohopanetetrol phenylacetate monoester content since this hopanoid is vesicle specific.

Major differences in the bacteriohopanetetrol tetra-acetate content of nodules were also observed. *Alnus* and *Coriaria* contained similar amounts of the tetra-acetate but only very low concentrations were detected in *Casuarina* nodules. This may be because *Frankia* in *Casuarina* lacks vesicles whereas vesicles are present in *Alnus* and *Coriaria* nodules. However the difference observed in the bacteriohopanetetrol content of nodules was considerably greater than the difference between repressed and derepressed *Frankia* where vesicle presence or absence is also the major distinguishing feature.

The importance of hopanes to hoipanoid-containing bacteria was illustrated by Flesch and Rohmer (1987). They showed hopanoid-containing bacteria were unable to survive in the presence of inhibitors of the squalene cyclisation reactions since the resulting synthesis of hopanes was also inhibited. A similar technique could be used to confirm the importance of hopanoids in *Frankia* since squalene cyclisation inhibitors would most probably affect vesicle development and therefore have an indirect influence on the response on nitrogenase activity in *Frankia* to oxygen.

A parallel exists between *Frankia* vesicles and the envelopes surrounding cyanobacterial heterocysts. The inner laminated layers of the heterocyst envelope are composed of glycolipid with long chain (C_{26} to C_{28}) hydroxy fatty acids and polyhdroxy alcohols linked by ether or ester bonds to hexoses (Lambein and Wolk 1973). Mutant studies on *Anabaena vulgaris* have indicated these lipids are essential for protecting cyanobacterial nitrogenase from oxygen (Haury and Wolk 1978). However the bacteriohopanetetrol phenylacetate monoester, which occurs in *Frankia* vesicle envelopes, is not present in cyanobacteria (Berry pers. comm.). Thus while there may be structural and functional similarities between the vesicle envelope and the heterocyst envelope these structures appear to have evolved along different metabolic pathways.

CHAPTER SEVEN

ACETYLENE-INDUCED DECLINE IN *CORIARIA*

7.1 Introduction

The acetylene reduction assay is widely used as a measure of nitrogenase activity in both legume and actinorhizal symbioses (section 2.3.1). However work by Minchin *et al.* (1983) showed that when nodulated roots of many legume species were exposed to acetylene, ethylene production declined rapidly before reaching a new steady state. This decrease in nitrogenase activity has been termed the acetylene-induced decline.

Minchin *et al.* (1983) also showed that respiratory CO_2 production linked to nitrogenase activity, decreased rapidly following exposure to acetylene before reaching a new equilibrium level. Normally a reduction in respiration rate would be expected to result in an increase in pO_2 concentration within the nodule and subsequent inactivation of nitrogenase. However such inactivation was not observed and both respiration and nitrogenase activity returned to their pre-exposure level when acetylene was removed (Minchin *et al.* 1983). This observation provides the basis for the hypothesis that acetylene exposure in legumes induces an increase in nodule diffusion resistance and subsequent oxygen-limitation of nitrogenase resulting in a decline in nitrogenase activity. Further work on the oxygen dependence of acetylene-induced declines (Witty *et al.* 1983; Hunt *et al.* 1987) has provided support for this theory.

Actinorhizal plants also show a decline in nitrogenase activity in response to acetylene exposure. Large acetylene-induced declines occur in *Alnus*, *Myrica* (Tjepkema *et al.* 1988*b*; Silvester and Winship 1990) and *Coriaria* (Silvester and Harris 1989) while *Casuarina*, *Gymnostoma* (Silvester and Winship 1990) and *Datisca* (Tjepkema *et al.*

1988*b*)generally show smaller declines. However most actinorhizal species differ from legumes in that nitrogenase activity is recovered following the decline to a rate that is 79 % to 98 % of the pre-decline maximum. A similar response occurs when actinorhizal plants are exposed to stepwise increases in pO_2 i.e. Oxygen-induced transients, and consequently the recoverable acetylene-induced declines observed in some actinorhizal species, are probably more accurately termed acetylene-induced transients (Silvester and Winship 1990). Such recoverable acetylene-induced declines have been documented for *Casuarina*, *Myrica* and *Alnus*. *Datisca* however shows only a 63 % recovery of maximum nitrogenase activity which is followed by a further decline (Tjepkema *et al.* 1988*b*)while *Coriaria* shows a non-recoverable decline similar to many legume species (Silvester and Harris 1989).

Unlike legumes, actinorhizal species have had little research work on the effect of acetylene on respiration. In *Myrica* however, introduction of acetylene had only a small effect on CO_2 production (Tjepkema *et al.* 1988 which contrasts to the effect of acetylene on respiratory activity in legumes. The effect of acetylene on nitrogenase and respiratory activity in most actinorhizal plants studied (Tjepkema *et al.* 1988*b*;Silvester and Winship 1990) suggests a change in nodule diffusion resistance is not a satisfactory explanation for acetylene-induced declines and the pattern of response is quite different from that in legumes.

However, shutting off of nitrogen fixation so that ammonia production is inhibited and electrons are diverted from nitrogen reduction to acetylene reduction is common to both legumes and actinorhizal species. Replacement of nitrogen in the gas stream with argon causes a similar decline in legumes (Minchin *et al.* 1983) i.e. argon-induced decline, which has been attributed to the same mechanisms as acetylene-induced declines. Consequently, most recent investigation of such declines in legumes (Hunt *et al.* 1987; King and Layzell 1991) has been on argon-induced declines where H_2 evolution is used as a measure of nitrogenase activity.

The aim of this work was to investigate acetylene-induced decline in *Coriaria* and, to a lesser extent, *Casuarina* using an approach similar to that previously used for legumes in an effort to determine the mechanisms involved in acetylene-induced declines in these two actinorhizal species. Preliminary experiments with *Coriaria* have shown acetylene-induced declines are superficially similar to those in legumes (Harris 1989) while *Casuarina's* response to acetylene appears more typical of other actinorhizal species (Tjepkema *et al.* 1988*b*; Silvester and Winship 1990).

7.2 Acetylene-induced decline in *Coriaria* and *Casuarina*

The effect of acetylene exposure on nitrogenase and respiratory activity in intact *Coriaria arborea* and *Casuarina cunninghamiana* plants was investigated using a continuous flow system (section 2.3.4). Four month old plants were equilibrated in a cuvette under 21 kPa O_2. After two minutes, 10 kPa acetylene was added to the gas stream. Samples were taken from the outflow gas every 30 seconds for GC analysis and the remainder of the outflow gas passed to the IRGA.

When nodulated *Coriaria* plants were exposed to 10 kPa acetylene, the rate of ethylene production increased rapidly during an initial mixing period to reach a peak value (maximum rate) after approximately three minutes (Fig. 7.1). Activity then decreased rapidly over the following five minutes before attaining a new equilibrium rate approximately ten minutes after acetylene exposure. The peak level of ethylene production was never recovered and activity was maintained at an equilibrium rate of about 61 % of the initial peak rate (Table 7.1).

Respiratory CO_2 production of nodulated *Coriaria* plants also declined in response to acetylene (Fig. 7.1). However acetylene had no effect on the root respiration of unnodulated *Coriaria* plants (Fig. 7.2). It should be noted that the nodulated plant in

figure 7.1 showed a slight rise in root respiration during the first three minutes of acetylene exposure that was not a typical response for the majority of plants tested. Usually CO_2 evolution was steady until the decline began approximately three minutes after acetylene was added to the gas stream. Like nitrogenase activity, the initial maximum rate of respiration was not recovered and a new steady rate was reached around ten minutes after acetylene exposure. All *Coriaria* plants assayed showed a decline in nitrogenase activity consistently larger than the accompanying acetylene-induced decline in nitrogenase-linked respiration.

Nitrogenase activity of nodulated *Casuarina* plants was also affected by addition of 10 kPa acetylene to the gas stream. Maximum activity was reached approximately four minutes after acetylene addition (Fig. 7.3), one minute later than for *Coriaria* (Table 7.1). Nitrogenase activity then declined rapidly until it was approximately 72 % of the pre-decline rate (minimum rate). However, unlike *Coriaria*, *Casuarina* plants showed a gradual recovery of nitrogenase activity so that 22 minutes after acetylene addition approximately 97 % of the initial level of activity had been recovered (equilibrium rate) (Table 7.1).

A further difference in the responses of *Casuarina* and *Coriaria* to 10 kPa acetylene was in respiratory activity. Unlike *Coriaria*, *Casuarina* root respiration was largely unaffected by acetylene exposure (Fig. 7.3).

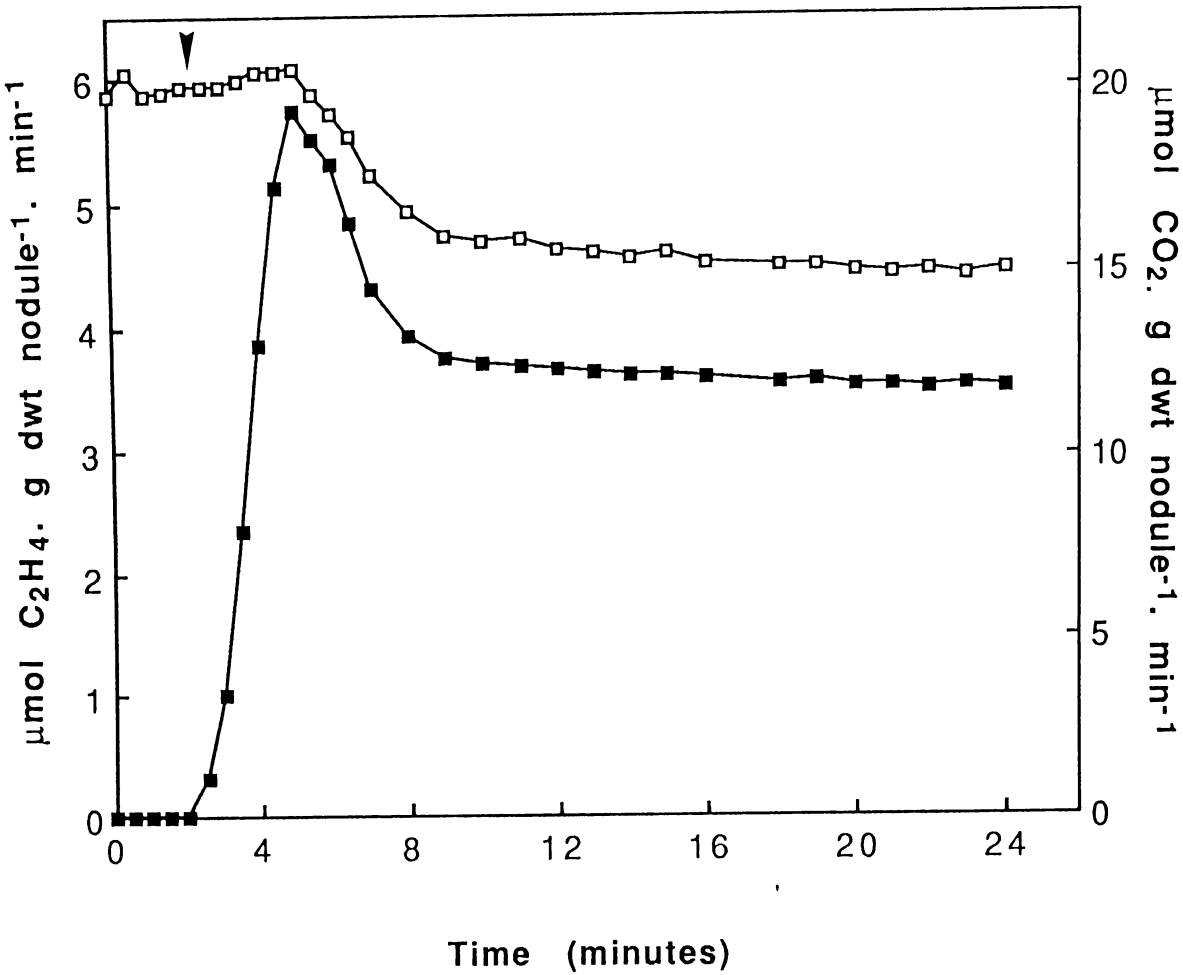


Figure 7.1 Effect of acetylene on nitrogenase activity (—■—) and nodule respiration (—□—) in *Coriaria arborea*. Plants were assayed at 21 kPa O_2 and 10 kPa acetylene was added after two minutes (▼).

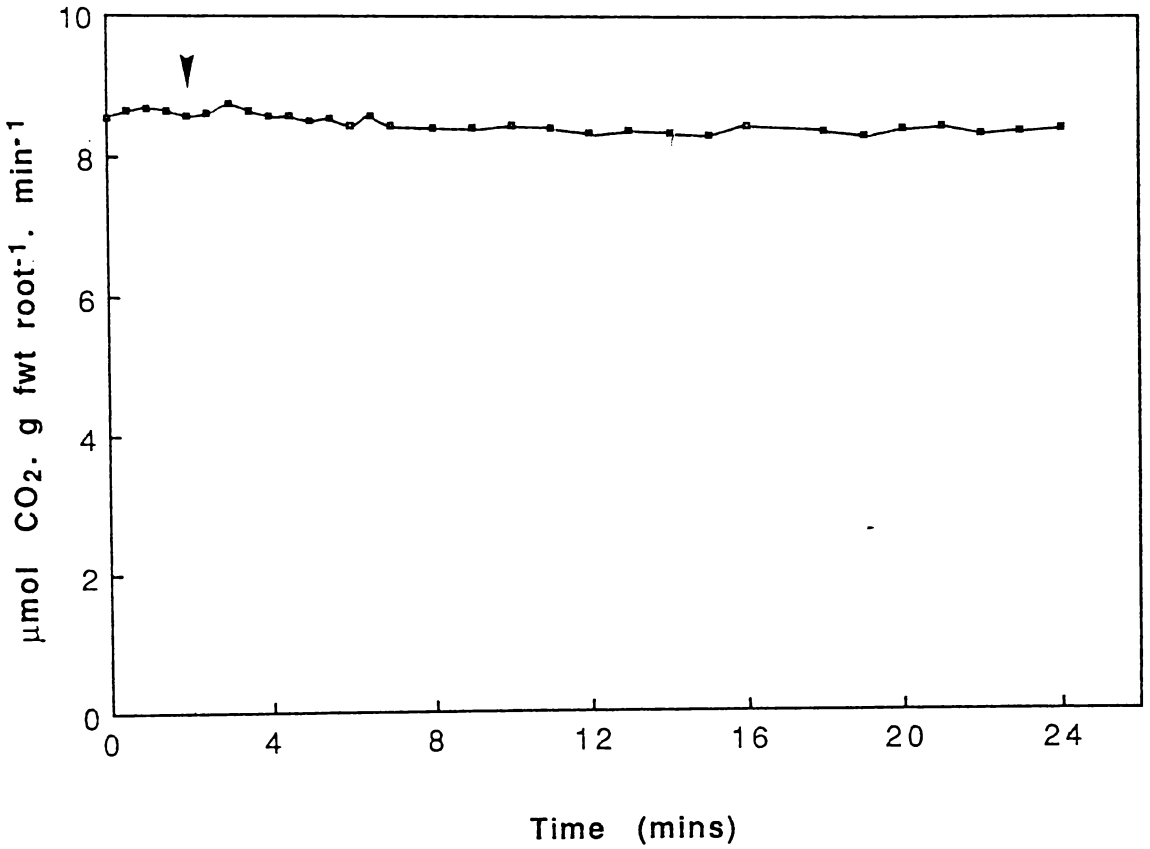


Figure 7.2 Effect of acetylene on root respiration of an unnodulated *Coriaria arborea* plant. The plant was assayed at 21 kPa O_2 and 10 kPa acetylene added after two minutes (▼).

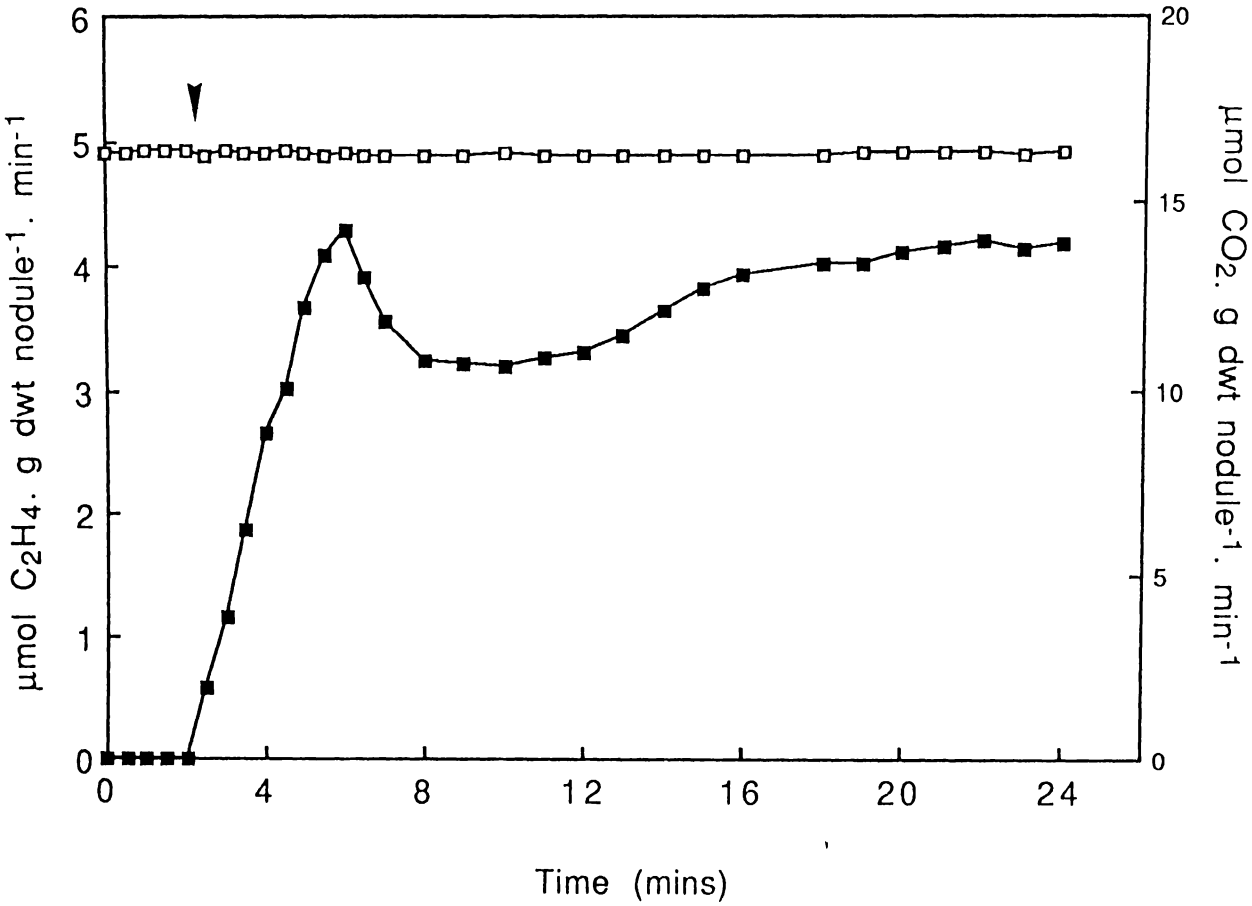


Figure 7.3 Effect of acetylene on nitrogenase activity (—■—) and nodule respiration (—□—) in *Casuarina cunninghamiana*. Plants were assayed at 21 kPa O_2 and 10 kPa acetylene was added after two minutes (▼).

Table 7.1 Acetylene-induced decline in *Coriaria arborea* and *Casuarina cunninghamiana*. Results show mean (standard deviation).

	Coriaria arborea		*Casuarina cunninghamiana*	
	Nitrogenase activity	Nodule respiration	Nitrogenase activity	Nodule respiration
Time to maximum (minutes)	2.98 (0.65)	2.92 (0.72)	3.95 (0.75)	N.A
Maximum rate	5.28 (0.72)[a.]	22.14 (3.96)[b.]	4.31 (0.50)[a.]	16.19 (1.29)[b.]
Minimum rate	N.A	N.A	3.11 (0.54)[a.]	16.16 (1.27)[b.]
Equilibrium rate	3.06 (0.67)[a.]	15.66 (3.37)[b.]	4.20 (0.40)[a.]	16.16 (1.27)[b.]
% minimum rate / maximum rate	N.A	N.A	72.20 (6.71)	99.87 (0.32)
% equilibrium rate / maximum rate	61.25 (7.41)	70.73 (5.72)	97.37 (3.05)	99.87 (0.32)
% decline	38.75 (7.41)	29.27 (5.72)	27.80 (6.71)	0.13 (0.32)
Number plants assayed	5		4	

[a.] μmol C_2H_4. g dwt nodule^{-1}. min^{-1}
[b.] μmol CO_2. g dwt nodule^{-1}. min^{-1}

7.3 Effect of argon on *Coriaria* respiration

As stated above, substitution of nitrogen in the gas stream for argon produces a decline in nitrogenase and respiratory activity in legumes like that induced by acetylene addition. The objective of the following experiment was to determine whether argon causes a similar decline in *Coriaria* nodule activity. However the impossibility of using hydrogen evolution as a measure of nitrogenase activity in actinorhizal plants meant that only the effect of argon on *Coriaria* root respiration could be measured.

A *Coriaria* plant was established in the continuous flow system and exposed to a gas stream containing N_2 : O_2 (79 : 21). After several minutes equilibration argon (Ar : O_2, 79 : 21) was introduced into the system and the effect on *Coriaria* root respiration measured.

Argon caused an immediate, non-recoverable decline in root respiration similar to the decline caused by acetylene exposure (Fig. 7.4). Within eight minutes CO_2 evolution had reached a new steady state level at approximately 84 % of the pre-decline rate. A decline of this magnitude is comparable to the decrease in *Coriaria* root respiration caused by acetylene exposure (section7.2).

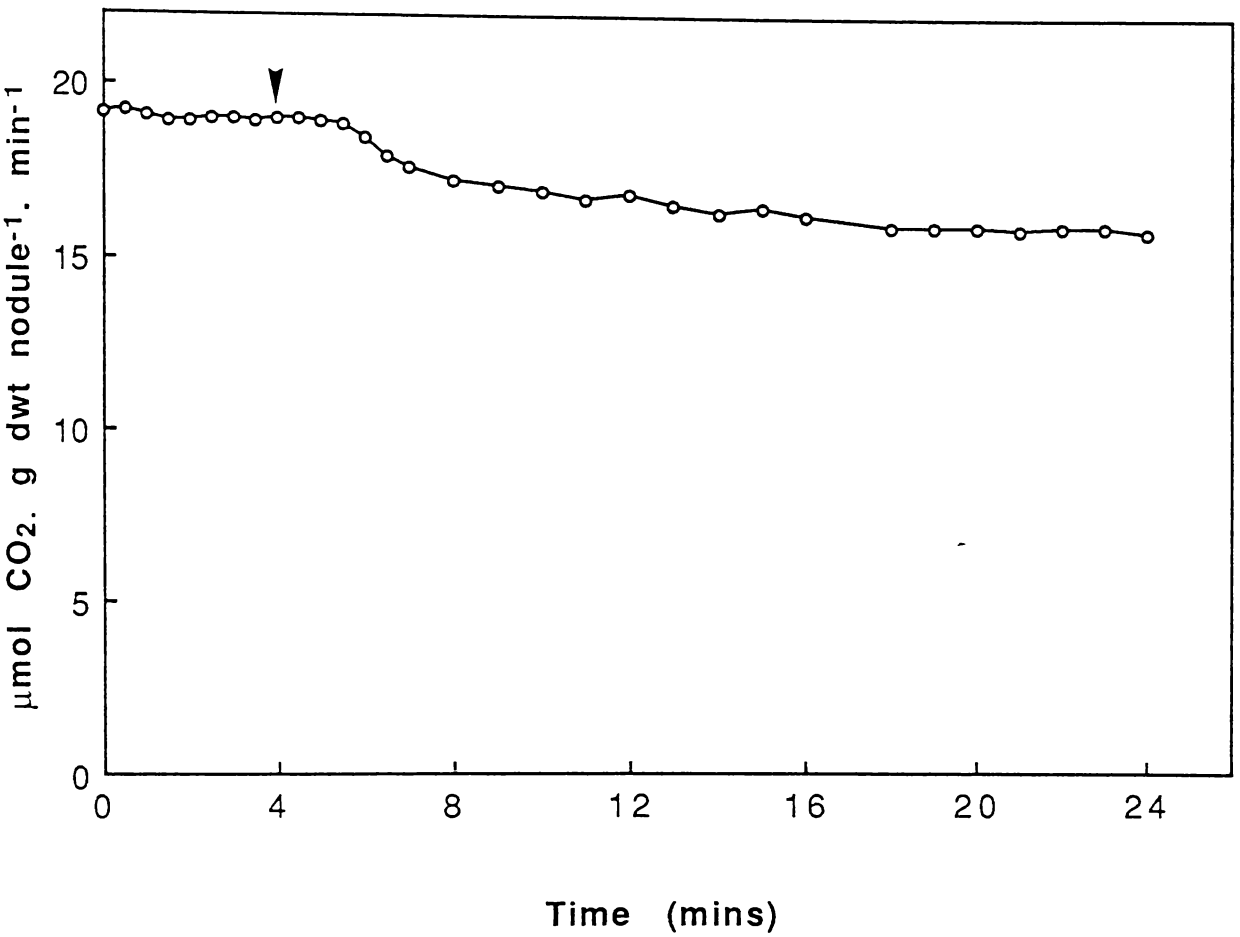


Figure 7.4 Effect of argon on nodule respiration in *Coriaria arborea*. Plants were initially assayed in N_2 : O_2 (79 : 21) and the inflow gas changed to Ar : O_2 (79 : 21) after four minutes (▼).

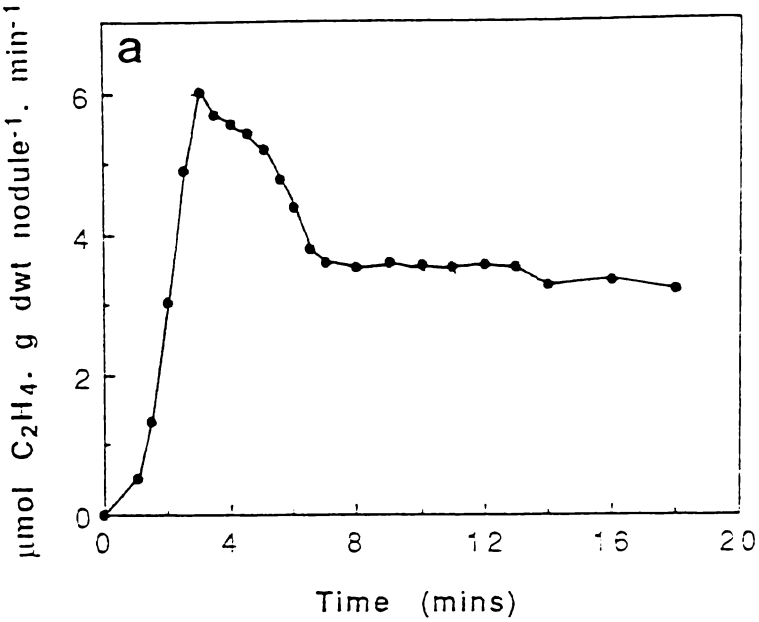
7.4 Effect of plant age on acetylene-induced decline in *Coriaria*

Most *Coriaria* plants used during the acetylene-induced decline work were three to four months old and had been nodulated in habitat soil before transfer, firstly to sand and then to aeroponics. All plants of this age showed a non-recoverable acetylene-induced decline in both nitrogenase activity and root respiration (section 7.2). However, several nine-month-old *Coriaria* plants assayed showed very little evidence of acetylene-induced decline. It was therefore decided to investigate the effect of plant age on the timing and size of the acetylene-induced decline in nitrogenase activity.

Replicate *Coriaria* seedlings were nodulated in habitat soil and transplanted to sand once nodulated. Nodulated seedlings were held in sand until transfer to aeroponics four weeks prior to use. Plants ranged in age from three to twelve months.

The size of the acetylene-induced decline in nitrogenase activity decreased as plant age increased (Fig. 7.5). Three- and four-month-old plants showed non-recoverable declines measuring approximately 46 % and 39 % respectively (Table 7.2). The mean acetylene-induced decline was significantly smaller in six-month-old plants while nine- and twelve-month-old plants had a barely detectable decline (Fig. 7.5).

The maximum rate of nitrogenase activity and the time taken to reach this pre-decline peak also changed with plant age. Maximum nitrogenase activity rate was approximately the same in three, four and six month old plants and occurred about 3.15 minutes after acetylene was added to the gas stream (Table 7.2). Older plants however had a noticeablely lower peak rate and, for twelve month old plants in particular, the time taken to reach the predecline peak was considerably longer than in younger plants (Table 7.2).


a
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8
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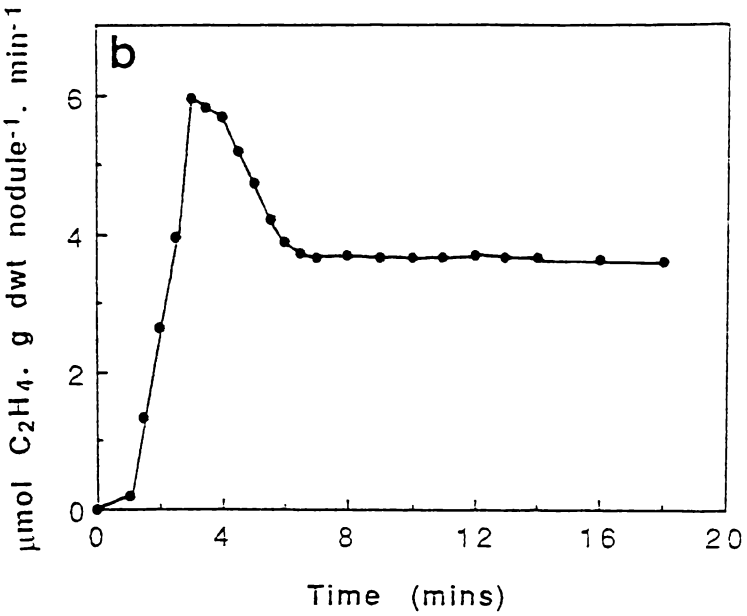
Time (mins)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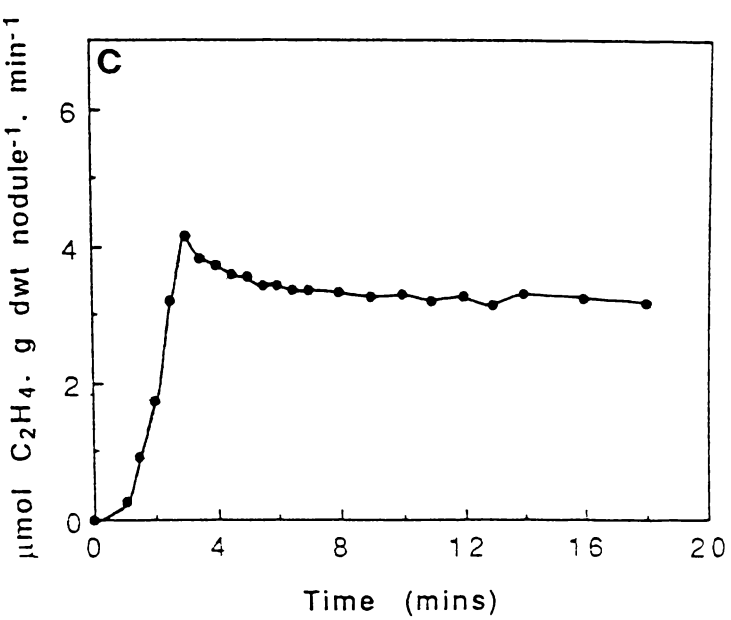
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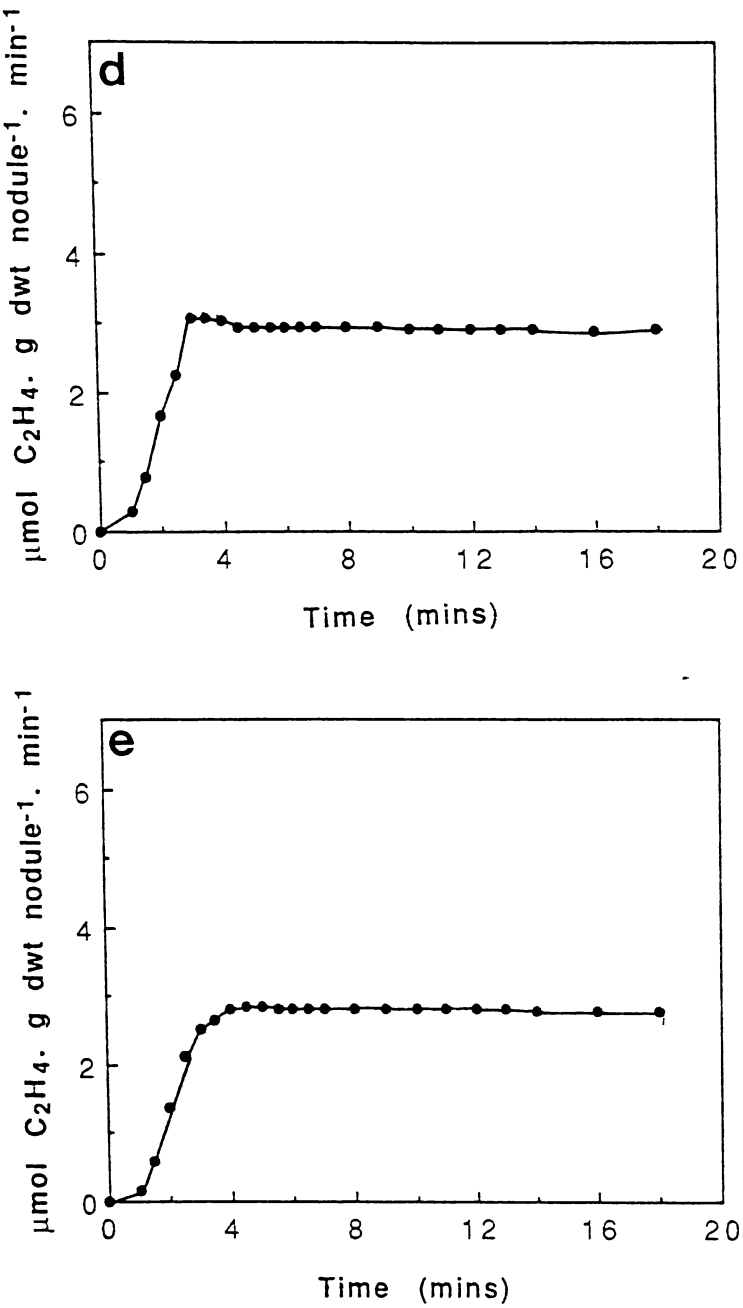

Figure 7.5 Effect of plant age on acetylene-induced decline in *Coriaria arborea*. Plants were assayed at (a) three months; (b) four months; (c) six months; (d) nine months and (e) twelve months. Assays were conducted at 21 kPa O_2 and 10 kPa acetylene was added at 0 minutes.

Table 7.2 Effect of plant age on the acetylene-induced decline in *Coriaria arborea*.

Results show mean (standard deviation).

Plant age (months)	3	4	6	9	12
Maximum rate (μmol C_2H_4. g dwt nodule^{-1}. min^{-1})	6.05 (0.31)	5.81 (0.51)	4.30 (0.20)	3.11 (0.40)	2.88 (0.50)
Equilibrium rate (μmol C_2H_4. g dwt nodule^{-1}. min^{-1})	3.62 (0.44)	3.56 (0.22)	3.24 (0.40)	2.91 (0.48)	2.83 (0.27)
% equilibrium rate / maximum rate	54.85 (2.09)	61.27 (2.75)	75.27 (2.65)	93.63 (1.29)	98.38 (1.25)
% decline	46.15 (3.58)	38.73 (2.75)	24.73 (2.65)	6.43 (1.33)	1.62 (1.25)
Time to peak activity (minutes)	3.09 (0.11)	3.14 (0.20)	3.27 (0.36)	3.52 (0.17)	4.30 (0.71)
Number plants assayed	4	3	4	5	5

7.5 Effect of oxygen on acetylene-induced decline in *Coriaria*

If an increase in nodule diffusion resistance to oxygen and resultant oxygen limitation of nitrogenase and respiratory activity is the mechanism responsible for the decline in activity upon acetylene exposure, oxygen concentration would be expected to have some effect on the acetylene-induced decline. Thus an increase in external oxygen concentration would be expected to relieve the decline to some extent. Such a pO_2 response has been shown in the acetylene-induced declines in some legume species (Witty *et al.* 1983). Alternatively a decline in nitrogenase activity may be a result of increased internal pO_2 induced by acetylene exposure. If this is so then a decrease in external pO_2 would serve to alleviate the acetylene-induced decline. Tjepkema and Schwintzer (1991) showed decreasing the pO_2 during the acetylene-induced decline, reduced the effect of acetylene on nitrogenase activity in *Myrica gale*. They (Tjepkema and Schwintzer 1990) also found the acetylene-induced decline was smaller if *Myrica* plants were assayed at a lower pO_2 level. A similar mechanism may therefore be associated with acetylene-induced declines in other actinorhizal species.

The aim of this work was to investigate the effect of prior adaptation to different pO_2 concentrations on acetylene-induced declines in *Coriaria arborea.*

Three to four month old plants were assayed using a continuous flow system. Plants were initially exposed to an O_2 : N_2 gas mixture (21 kPa O_2). Acetylene (10 kPa) was added to the gas stream after a suitable equilibration period and ethylene production measured over the ensuing 20 minutes before acetylene was removed from the gas stream. Care was taken to maintain a constant pO_2 during acetylene exposure. Plants were then allowed approximately 40 minutes to recover from the effects of acetylene before the assay was repeated. Following duplicate measurements of the acetylene-induced decline at 21 kPa O_2, the oxygen concentration in the gas stream was changed to either 12 kPa or 35 kPa. Plants were allowed one hour for adaptation to the new pO_2

level before acetylene was again added and duplicate measurements of the acetylene-induced decline made as described above. Some plants were also assayed in reverse i.e. first under either low (12 kPa) or high (35 kPa) pO_2, and then again when returned to atmospheric (21 kPa) pO_2 in order to overcome effects of any inherent changes in activity during the course of the experiments.

Coriaria plants assayed at 21 kPa O_2 showed a typical non-recoverable decline in nitrogenase activity measuring approximately 36 %(Fig. 7.6b). However when plants were previously adapted to and assayed at a higher pO_2 level (35 kPa O_2) the acetylene-induced decline was significantly smaller (Fig. 7.6c) and measured about 17 % (Table 7.3). At this higher pO_2 the equilibrium activity rate was approximately the same as at 21 kPa O_2 but the peak activity rate was considerably lower (Table 7.3). In contrast, *Coriaria* plants previously adapted to and assayed at 12 kPa O_2 had a mean acetylene-induced decline slightly larger than at 21 kPa O_2 (Fig. 7.6a). Furthermore the equilibrium rate, as well as the peak nitrogenase activity rate, was reduced with the decrease in pO_2 (Table 7.3).

Table 7.3 Effect of oxygen on the acetylene-induced decline in nitrogenase activity *Coriaria arborea*. Results show mean (standard deviation).

pO_2 (kPa O_2)	12	21	35
Maximum rate μmol C_2H_4. g dwt nodule^{-1}. min^{-1}	3.99 (0.51)	5.00 (0.47)	3.72 (0.49)
Equilibrium rate μmol C_2H_4. g dwt nodule^{-1}. min^{-1}	2.46 (0.43)	3.20 (0.38)	3.07 (0.51)
% equilibrium rate / maximum rate	61.63 (3.04)	64.02 (3.55)	82.55 (2.78)
% decline	38.37 (3.04)	35.98 (3.55)	17.45 (2.78)
Number plants assayed	5	4	6

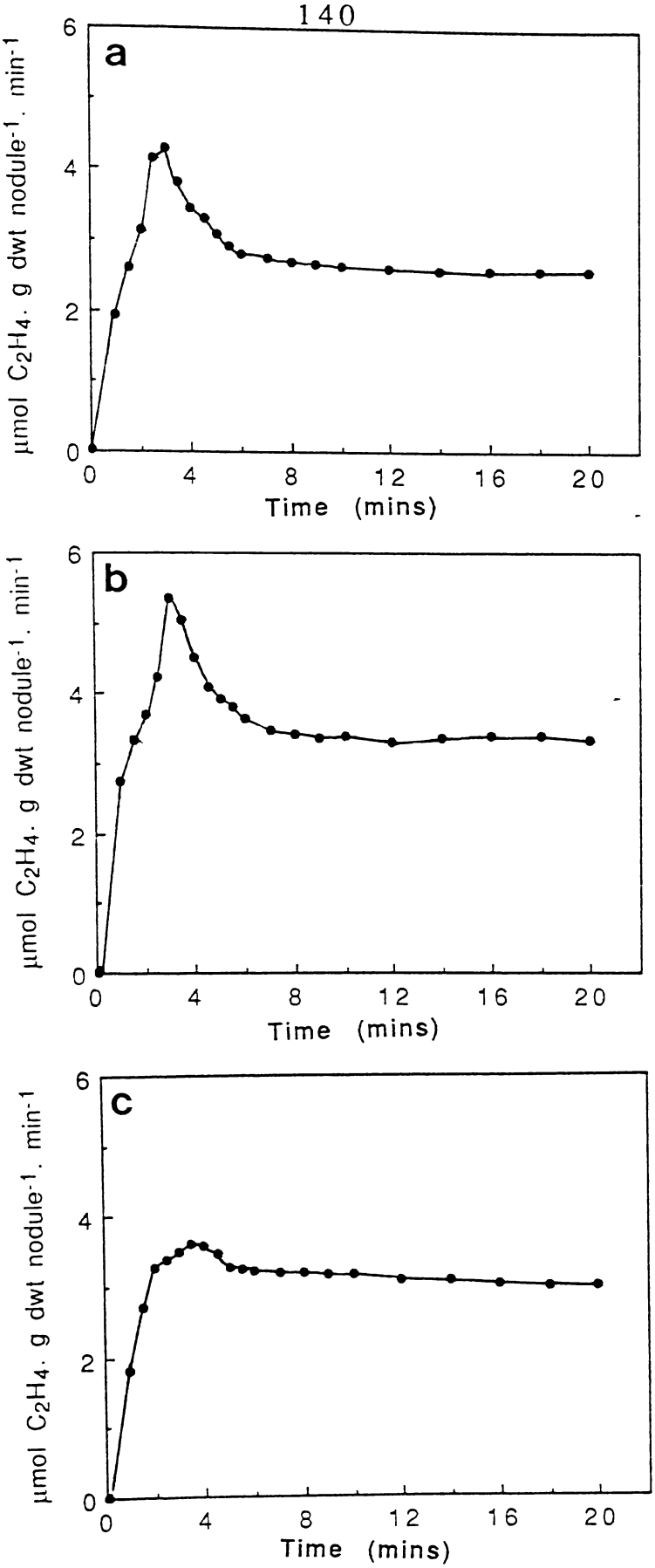

Figure 7.6 Effect of acetylene on nitrogenase activity of *Coriaria arborea* plants previously adapted to (a) 12 kPa O_2; (b) 21 kPa O_2 or (c) 35 kPa O_2. Acetylene (10 kPa O_2) was added at 0 minutes.

7.6 Argon induced changes in nodule diffusion resistance

Since acetylene- and argon-induced declines in legumes both probably involve a change in nodule diffusion resistance, most recent work on this phenomena (Hunt *et al.* 1987; Hunt *et al.* 1989; King and Layzell 1991) has looked at the response to argon rather than acetylene. One advantage of studying argon-induced declines is that any changes in nodule diffusion resistance can then be measured using the lag-phase method (Weisz and Sinclair 1987) which is an assay involving the use of acetylene.

Earlier (section 7.3) it was demonstrated *Coriaria* shows an argon-induced decline in root respiration similar to the decline caused by acetylene. The aim of this experiment was to determine whether nodule diffusion resistance was affected by argon exposure using the lag-phase method.

No direct measure of nodule diffusion resistance is available although several techniques for indirectly determining resistance have been reported. Sheehy *et al.* (1983) used a steady-state one dimensional diffusion equation to convert CO_2 evolution rate in white clover to a measure of nodule diffusion resistance. Denison *et al.* (1983) and Winship and Tjepkema (1983) both developed a technique for estimating nodule permeability using Michaelis-Menten kinetics to model ethylene production based on data from acetylene reduction assays. However both these methods made assumptions which Weisz and Sinclair (1987) later showed to be incorrect. Most estimates of nodule diffusion resistance are now made using the lag-phase method proposed by Weisz and Sinclair (1987).

The lag-phase method measures the lag-time to steady state ethylene production in intact nodules and expresses this as the time constant (T) which can be used as a measure of nodule diffusion resistance. The method assumes a general model in which nodules are spherical with most resistance to gas diffusion occurring outside the infected tissue.

Inside the diffusion barrier, a system of intercellular air spaces permeates the nodule, and therefore gas diffusion in the nodule interior is rapid compared with the restricted diffusion across the external barrier. Concentrations of acetylene and ethylene are assumed to be uniform and in equilibrium with the inner surface of the diffusion barrier. Weisz and Sinclair (1987) tested the validity of the lag-phase method using simulated and experimental data and showed it to be independent of the biochemical characteristics of nitrogenase. This independence from Vmax and Km allows use of the lag-phase method for estimating nodule diffusion resistance even if Vmax or Km change over the course of the experiment or as part of the experimental treatment.

Although the lag-phase method was developed for legume nodules, similar assumptions can be made for *Coriaria* nodules and, as well as having been used successfully in several legume studies (Weisz and Sinclair 1987; King and Layzell 1991), the technique was also used to show changes in *Coriaria* nodule diffusion resistance in response to pO_2 (Silvester and Harris 1989).

Coriaria plants were assayed in a modified continuous flow system and initially subjected to a gas stream containing N_2 : O_2 (79 : 21). Acetylene (10 kPa) was added to the flow gas at five minutes and samples taken every five seconds (section) for a 90 second period. Acetylene was then removed from the inflow gas to prevent occurrence of an acetylene-induced decline. This procedure was repeated several times in N_2 : O_2 before the inflow gas was changed to Ar : O_2 (79 : 21) and the procedure again repeated.

During the assay period, acetylene diffuses into the nodule and ethylene diffuses out. The time taken to reach steady-state ethylene production is termed the lag-time and provides a measure of the diffusion resistance of the system. This lag is made up of five components: (1) the time required for the chamber to reach final acetylene concentration, (2) the lag required for acetylene diffusion into the nodule, (3) the time taken for ethylene to reach its final concentration within the nodule, (4) the time required for ethylene to

diffuse out of the nodule, (5) the time required for ethylene concentration in the cuvette to reach steady-state. It is essential in this method that the time taken for the cuvette to reach final acetylene concentration and the time required for ethylene to reach steady-state are negligible compared to the lag-time of the nodule. To minimise these effects a small cuvette volume (10 mls) and a high flow rate (140 ml. min^{-1}) were used giving a cuvette flushing time of 4.2 seconds.

Figure 7.7 shows the increase in nitrogenase activity following addition of acetylene to the system for a plant assayed under N_2 : O_2 and then under Ar : O_2. Only the curves for the assays at 40 minutes under N_2 : O_2 and after two minutes and 50 minutes in Ar : O_2 are shown although the plant was assayed three times in nitrogen and five times after argon exposure (Table 7.4). When assayed in N_2 : O_2 plants took approximately 30 seconds to reach steady-state ethylene production. This was a much shorter time than the three minute period to maximum activity measured earlier (section 7.2). However this difference is not a real phenomenon but is due to the much higher flow rate used during the lag-phase method.

Nitrogenase activity was considerably lower following addition of argon averaging 74 % of the rate of activity under N_2 : O_2 (Table 7.4). Exposure to argon also resulted in a marked increase in nodule diffusion resistance. Plants assayed in N_2 : O_2 took approximately 30 seconds to reach steady-state ethylene production which equates to a time constant (half the time taken to reach a maximum rate of ethylene production) of 14.8 seconds. By comparison, in Ar : O_2 the time constant averaged 20.8 seconds representing a 40 % increase in apparent nodule diffusion resistance. This increase in diffusion resistance was particularly rapid, since the time constant was significantly higher after only two minutes in argon.

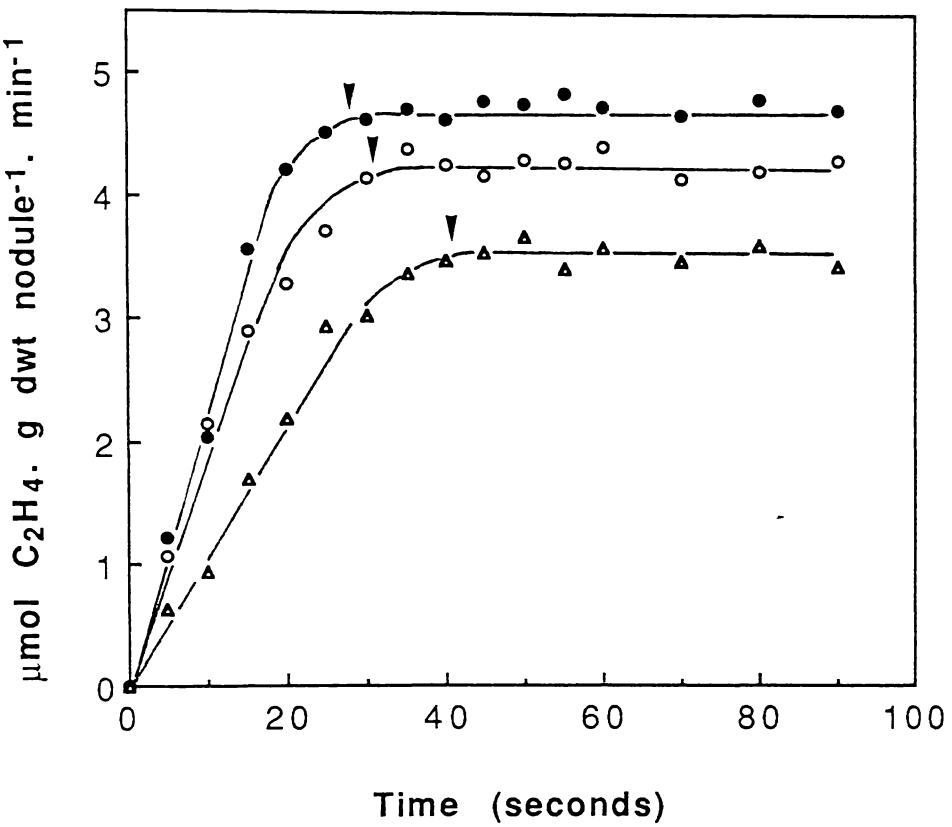

Figure 7.7 Time taken for ethylene production to rise to equilibrium in *Coriaria arborea* assayed after 40 minutes in N_2 : O_2 (—●—) and then after 2 minutes in Ar : O_2 (—○—) and 50 minutes in Ar : O_2 (—△—). Arrows indicate the time taken for ethylene production to reach equilibrium. Note the plant was exposed to acetylene for 90 second periods only and was not subject to acetylene-induced decline.

Table 7.4 Time constant (half-time to reach equilibrium ethylene production) of *Coriaria arborea* root nodules exposed to acetylene for 90 second intervals. Three results in N_2 : O_2 (79 : 21) and five results in Ar : O_2 (79 : 21). Time is the interval after the first acetylene exposure in each treatment.

Gas stream	Time (mins)	Time constant (s)	Nitrogenase activity μmol C_2H_4. g dwt nodule^{-1}. min^{-1}
N_2 : O_2	5	14.8	5.35
	20	15.2	4.93
	40	14.5	4.73
Ar : O_2	2	16.8	4.26
	30	19.7	3.60
	50	21.5	3.48
	75	20.9	3.54
	100	21.1	3.57

7.7 Discussion

Coriaria and *Casuarina* both showed a decline in nitrogenase activity within minutes of addition of acetylene to the gas stream. However the effect of acetylene on associated respiratory activity in each species is sufficiently different to suggest different physiological mechanisms and structures are responsible for the decline in each case. Superficially *Coriaria* shows a decline very similar to that of legumes (Minchin *et al.* 1983; Witty *et al.* 1983) while *Casuarina* behaves more like other actinorhizal genera such as *Alnus* and *Myrica* (Tjepkema *et al.* 1988*b*).

Coriaria, like a number of legumes including soybean (*Glycine max* L.), lucerne (*Medicago sativa*), lupin (*Lupinus albus*), red clover (*Trifolium pratense*) and white clover (*Trifolium repens*) (Minchin *et al.* 1983), shows a non-recoverable decline in both

nitrogenase activity and root respiration in response to 10 kPa acetylene. The initial rise in ethylene production immediately after acetylene addition, is not accompanied by any change in root respiration, in contrast to the subsequent decline. Minchin *et al.* (1983) found that this initial increase was shown by all legume symbioses assayed in a flow-through system in response to acetylene whether or not they showed a subsequent decline. Thus this initial rise in ethylene production, as observed for both *Coriaria* and *Casuarina*, is due to the equilibration period, as acetylene concentration in the cuvette reaches 10 kPa rather than a rise in nitrogenase activity.

In *Coriaria* the acetylene-induced declines in nitrogenase and respiratory activity occur simultaneously. The fact that 10 kPa acetylene had no effect on root respiration in non-nodulated *Coriaria* indicates the effect of acetylene is specifically on nodule respiration i.e. nitrogenase-linked respiration, rather than total root respiration. However the decrease in nitrogenase-linked (nodule) CO_2 evolution was consistently smaller than the drop in nitrogenase activity. Although the extent of the decline varied between plants, decrease in nitrogenase-linked respiration was usually about 70 % that of ethylene production. Similar figures have been presented for white clover in response to 10 kPa acetylene (Minchin *et al.* 1983). King and Layzell (1991) investigated the effect of argon (Ar : O_2, 80 : 20) on nitrogenase activity (hydrogen evolution) and root respiration in soybean. They found, following the decline, hydrogen evolution reached a new steady state value which was on average 30 % of the original rate while CO_2 evolution approached a new steady state which was on average 77 % of the pre-decline rate (note that this was for total root respiration rather than nitrogenase-linked respiration). This marked difference in response to acetylene between nitrogenase and respiratory activity may be explained by the existence of at least two separate mechanisms working cooperatively to produce the decline in activity. King and Layzell (1991) have suggested such a scheme based on their work with argon-induced declines in soybean (see later in Discussion).

It may be significant that, although the shape of the decline in *Coriaria* is similar to legumes, the time scales involved in each case are different. *Coriaria* for example undergoes an immediate decline upon acetylene addition and attains a new equilibrium level of nitrogenase activity and respiration within 20 minutes as assayed under the above experimental conditions (section). In legumes the response appeared slightly slower when plants were assayed under similar conditions and a comparable gas flow rate (Minchin *et al.* 1983; Witty *et al.* 1983). Often legumes show a brief period of stable activity prior to the decline which is not evident in either *Coriaria* or *Casuarina* as assayed here. Tjepkema *et al.* (1988*b*)also showed the time to maximum rate following acetylene addition varied considerably among the five actinorhizal genera studied. They considered the difference in speed of acetylene diffusion into the nodule and ethylene diffusion out did not explain the differences in the nodules' response to acetylene. Obviously care should be taken when comparing results from different studies but the different time scale of response may be a reflection of relative nodule permeability. Here for example, *Coriaria* reached its maximum rate of nitrogenase activity approximately one minute before *Casuarina* when assayed under identical conditions suggesting perhaps a greater nodule permeability in *Coriaria*. Indeed other measurements of nodule permeability such as oxygen microelectrode probes and India ink studies (Tjepkema and Yocum 1974; Tjepkema 1979; Tjepkema *et al.* 1988*b*; Silvester and Harris 1989) indicate *Coriaria* nodules are more aerated than *Casuarina* nodules. Actinorhizal nodules are also generally considered more permeable to gas diffusion than their legume counterparts (Tjepkema 1979, 1984).

Recent work has shown a number of factors, including plant age and development, nitrate feeding and pO_2, may affect the dynamics of the acetylene- or argon-induced declines in both legumes (Minchin *et al.* 1983; Witty *et al.* 1983; King and Layzell 1991)and actinorhizal species (Tjepkema *et al.* 1988*b*; Tjepkema and Schwintzer 1991). Plant age, for example, had a marked effect on the size of acetylene-induced decline in *Coriaria*. As plants aged the extent of decline decreased so that in 12 month old plants a decline in

nitrogenase activity was barely detectable. In addition, the period of time taken to reach the maximum rate of nitrogenase activity was longer in older plants and older plants also had a lower equilibrium activity rate. A similar decrease in the size of the acetylene-induced decline has also been reported for *Casuarina* (Tjepkema *et al.* 1988*b*), soybean and lucerne (Minchin *et al.* 1983). The effect of plant age on the timing and magnitude of the acetylene-induced decline in *Coriaria* can be largely explained by changes in nodule structure over time. As nodules age the amount of woody, inactive tissue increases. Thus the proportion of active tissue within nodules decreases which accounts for the lower specific rates of nitrogenase activity measured in older plants. Similarly an increase in the amount of woody, inactive tissue in nodules would increase the length of the pathway for gas diffusion from the nodule exterior to the site of nitrogenase. Such an increase would result in greater nodule diffusion resistance which may account for the longer time taken to reach maximum ethylene production rate. Increased diffusion resistance would also minimise the effect of added acetylene on nodule gas permeability, resulting in smaller acetylene-induced declines. Hartwig *et al.* (1987) reported an apparent increase in nodule diffusion resistance of red clover with age, in their study of the effects of defoliation on nitrogenase activity. They suggested young, rapidly growing plants require a large supply of nitrogen and they showed that, in contrast to older clover plants, where the absolute demand for nitrogen is reduced, nodule diffusion resistance in young plants is significantly lower. If nodule permeability does in fact decrease with age, acetylene would be expected to cause little further increase in nodule diffusion resistance and so have little effect on nitrogenase activity of an older nodule with an already high diffusion resistance. Thus the response of nitrogenase activity to various influences, such as acetylene exposure, may be affected by changes in the sinks for nitrogen with plant age and consequently changes in nodule diffusion resistance with age.

In *Coriaria*, nitrogenase and respiratory activity can be recovered following an acetylene-induced decline to a rate close to the original maximum if acetylene is removed from the gas stream (Silvester and Harris 1989). This result was confirmed in section 7.5 where

plants were removed from acetylene for 40 minutes following 20 minutes acetylene exposure and each time nitrogenase activity was recovered to a rate comparable to the original level. Similarly Minchin *et al.* (1983) showed that legume nitrogenase was not permanently impaired by acetylene, since removal of acetylene allowed activity to return to the pre-exposure value within two hours and the decline pattern was repeated upon re-exposure to acetylene. The occurrence of the acetylene-induced decline without permanent damage to nitrogenase has been explained by an increase in the nodules' resistance to gaseous diffusion in response to acetylene exposure and so provides the basis for the variable diffusion mechanism commonly used to account for acetylene-induced declines in legume nodules.

The role of changes in nodule diffusion resistance in regulating acetylene-induced declines in legume nodules has been substantiated by several related observations. Not all legumes show a decline under acetylene but those that do e.g. pea, white clover and lucerne, are distinguished by their tolerance to stepwise increases in pO_2 up to a high oxygen concentration (Witty *et al.* 1983). Hunt *et al.* (1987) provided evidence that such adaptation to increases in pO_2 is due to a variable diffusion barrier operating within nodules. *Coriaria* too is noted for its ability to adapt rapidly to oxygen levels above and below atmospheric and to show a broad pO_2 optimum for nitrogenase activity (20 to 40 kPa O_2) (Silvester and Harris 1989) in contrast to most other actinorhizal plants studied (Silvester *et al.* 1988*a*, *b*; Silvester and Winship 1990). Lag-phase measurements indicated *Coriaria* could rapidly adjust its nodule diffusion resistance in response to changes in pO_2 above or below atmospheric (Silvester and Harris 1989). Since the ability to change nodule diffusion resistance in response to acetylene or argon exposure is common in plants also capable of changing nodule permeability in response to oxygen concentration, the two processes may be regulated by the same mechanisms.

The close association of oxygen control within legume nodules and regulation of the acetylene-induced decline is supported by the effect of oxygen concentration on the size of

the acetylene-induced decline. Witty *et al.* (1983) showed the magnitude of the decline in respiration following acetylene exposure in white clover plants was decreased in plants assayed at high pO_2 concentrations. *Coriaria* plants previously adapted to high (35 kPa O_2) oxygen concentrations also had a smaller decline in nitrogenase activity following acetylene addition than those previously exposed to atmospheric (21 kPa O_2) or below atmospheric (12 kPa O_2) pO_2. This may have been because the nodule diffusion resistance of those plants at 35 kPa O_2 had already increased in response to the above atmospheric pO_2 and so addition of acetylene had limited effect on increasing diffusion resistance further. Consequently the acetylene-induced decline at high pO_2 was half the size of the decline at lower pO_2.

Recently, King and Layzell (1991) showed that 93% of original H_2 evolution and 99% of CO_2 evolution could be recovered in soybean nodules if oxygen concentration was increased in a linear ramp during the argon-induced decline. This suggested the increase in pO_2 relieved an oxygen limitation within the nodule which could only have been caused by a prior increase in diffusion resistance. In fact, measurements of leghaemoglobin oxygenation showed the internal pO_2 had declined to 56% of its initial value after 60 minutes of argon exposure (Ar : O_2, 80 : 20) and that nodule permeability (measured using the lag phase method) had decreased to 26 % of its original level.

Measurement of *Coriaria* nodule diffusion resistance, using the lag-phase method, showed a similar decrease in gas permeability when nodules were exposed to argon (Ar : O_2, 79 : 21). Under N_2 : O_2 (79 :21) the time constant was approximately 71 % of that when nodules were exposed to argon indicating an increase in diffusion resistance in response to argon.

Acetylene-induced declines in most actinorhizal plants studied e.g. *Casuarina* and *Myrica*, appear not to involve any change in nodule diffusion resistance. This theory is based on the observation that acetylene causes no change in the root respiration rate of

Casuarina (Tjepkema *et al.* 1988*b*) or *Myrica* (Tjepkema and Schwintzer 1991). The effect of oxygen on the acetylene-induced decline in *Myrica* (Tjepkema and Schwintzer 1990, 1991) also supports the existence of an alternative mechanism for acetylene-induced declines to that proposed for legumes. Whereas in legumes increased pO_2 alleviates the decline (Witty *et al.* 1983), Tjepkema and Schwintzer (1990) showed prior adaptation to low pO_2 e.g. 5 kPa O_2, resulted in a smaller acetylene-induced decline in *Myrica*. Similarly, lowering the oxygen concentration during the decline helped alleviate the effect of acetylene on *Myrica* nitrogenase activity (Tjepkema and Schwintzer 1991). Tjepkema and Schwintzer (1990) hypothesise nitrogenase turns over more rapidly when acetylene is being reduced than when nitrogen is being reduced. The introduction of acetylene may therefore lead to a depletion in the pool of reductant used by nitrogenase. However, reducing the pO_2 to a level where nitrogenase is strongly oxygen-limited means acetylene reduction is more likely to be limited by the rate of ATP formation i.e. respiratory activity, than by the rate of reductant formation. Thus nitrogenase activity is no longer restricted by competition for the pool of reductant and, as a consequence, the acetylene-induced decline is smaller. Alternatively introduction of acetylene, and thus the higher rate of turn over of nitrogenase, may cause increased competition between nitrogenase and respiratory processes for the pool of metabolites. As a result, oxygen uptake at the site of nitrogenase may decrease causing a rise in internal pO_2 and resultant inhibition of nitrogenase. Lowering the oxygen concentration would therefore relieve this inhibition to some extent, resulting in a smaller acetylene-induced decline.

Despite considerable research into acetylene-induced declines in legumes and actinorhizal species, the actual physical processes and morphological structures involved remain unclear. Experimental evidence strongly suggests that acetylene- and argon-induced declines in legumes are due mainly to an increase in nodule diffusion resistance. The limited experimental evidence presented here suggests acetylene-induced declines in *Coriaria* are due to a similar phenomena. However, *Coriaria* and legume nodules are structurally very different and therefore the precise mechanisms leading to a change in

diffusion resistance are most probably different. Hunt *et al.* (1987) suggest changes in legume nodule permeability are regulated by osmotic changes within the nodule, most probably in the region of the inner cortex where a permanent diffusion boundary exists. In *Coriaria* nodules no such diffusion boundary exists exterior to the infected zone (Silvester and Harris 1989) although gas diffusion may be restricted in the region between the internal periderm and endodermis. This zone may provide a suitable site where osmotic changes e.g. the amount of water in intercellular spaces, could regulate gas diffusion.

Work by King and Layzell (1991) on soybean has indicated the mechanisms regulating argon-induced declines in this legume at least, are more complex than simply a change in nodule diffusion resistance. They found the decline in hydrogen evolution following argon addition occurred as two distinct phases. The initial sharp decline in hydrogen evolution was not accompanied by any equivalent decrease in CO_2 evolution nor internal oxygen concentration, as would be expected if nodule diffusion resistance rapidly increased. Instead, CO_2 evolution and internal pO_2 underwent a more gradual decline. King and Layzell (1991) suggested increased nodule diffusion resistance, and thus a reduced internal oxygen concentration, could account for the long-term decline in nitrogenase activity but not the short-term response. Rather, this short-term decline may be due to some biochemical mechanism similar to the conformational protection of nitrogenase reported for Azotobacter in response to oxygen (Dalton and Postgate 1969) and proposed to account for oxygen transients in actinorhizal species and *Frankia* (Silvester and Winship 1990). The means by which exposure to acetylene, argon or increased pO_2 could cause such reversible inhibition is still not clear although it has been suggested the effect may arise from perturbation of metabolite pools e.g. ATP or reductant pools, within the nodule (Hunt *et al.* 1989). Whether the acetylene-induced decline in *Coriaria* can be separated into short- and long-term responses due to a combination of physical and biochemical mechanisms, as proposed for soybean, remains undetermined.

It should be noted that *Frankia* cultures show no acetylene-induced decline although they do show oxygen transients in response to stepwise increases in pO_2 (Silvester and Winship 1990). This would appear to suggest the host-plant has some role in the regulation of acetylene-induced declines and that the mechanisms causing acetylene-induced declines or transients in actinorhizal species are perhaps different from those processes involved in oxygen transients.

CHAPTER EIGHT

DEFOLIATION EFFECTS AND DIFFUSION PATHWAY IN *CORIARIA* NODULES

8.1 Introduction

Nitrogen fixation, whether by *Frankia* in actinorhizal nodules or by *Rhizobium* in legume nodules, is a highly energy demanding process requiring at least 4 ATP / 2 e- transferred to nitrogen. Legume and actinorhizal species therefore face a similar dilemma in having to maintain a high oxygen flux to support nitrogenase activity but at the same time maintaining a low oxygen concentration at the site of nitrogenase to prevent inactivation of the enzyme. Despite being faced with a similar problem, the structures and mechanisms providing oxygen diffusion in legume and actinorhizal species appear very different. Vacuum infiltration of nodules with India ink (Tjepkema and Yocum 1974; Tjepkema 1979; Tjepkema *et al.* 1988*a*; Silvester and Harris 1989; Zeng *et al.* 1989), oxygen microelectrode probes (Tjepkema 1979, 1983) and indirect measurements of diffusion resistance (Weisz and Sinclair 1987; Silvester and Harris 1989) indicate actinorhizal nodules are generally more aerated than their legume counterparts. In legumes much of the diffusion resistance is attributed to a layer of cells in the nodule cortex which lack air spaces, the cortical diffusion barrier. Among actinorhizal species, nodule structure is more varied and the site of diffusion resistance less well defined. In the absence of a cortical barrier and in view of apparent ventilation via lenticels or breathing roots, it is postulated that the major limitation to oxygen diffusion resides in the host cytoplasm, host cell walls or in *Frankia* vesicles.

This chapter covers a series of experiments conducted, using a variety of techniques, with the intention of gaining more information on the energy costs of nitrogen fixation, diffusion resistance and the effect of defoliation on nodule activity in *Coriaria*.

8.2 Respiratory cost of nitrogen fixation in *Coriaria*

In this study measurements of total root respiration were made. However most environmental parameters affecting respiration eg. oxygen concentration, acetylene exposure and defoliation, only affect the nodule component of total root respiration i.e. nitrogenase-linked respiration. In some situations it is desirable to determine only the response of nitrogenase-linked respiration to environmental changes and therefore it was necessary to calculate the proportion of total root CO_2 evolution due to nodule activity.

CO_2 evolution was measured at 21 kPa O_2 in nodulated, intact plants which had been grown aeroponically for 3 months. After 30 minutes plants were removed from the cuvette and nodules carefully detached from the subtending roots. The plants, minus their nodules, were then replaced in the cuvette and further measures of respiratory activity made over the following 60 minutes. The procedure was repeated on six replicate plants.

Removal of nodules caused an immediate drop in CO_2 evolution rate of 71 % which was equivalent to nodule respiration. Nodule respiration provides for basal metabolic processes, such as growth and cell maintenance, but the bulk of nodule respiration is nitrogenase-linked activity.and nitrogenase activity (Tjepkema and Winship 1980). If values for the maximum rates of nitrogenase activity and nitrogenase-linked respiration from section 7.2 are used, a figure for the cost of nitrogenase activity in terms of the ratio CO_2 evolved : nitrogen fixed can be determined. For example, the maximum rate of nitrogenase-linked CO_2 evolution was 22.14 μmol CO_2. g dwt nodule^{-1}. min^{-1} while maximum nitrogenase activity was measured at 5.28 μmol C_2H_4. g dwt nodule^{-1}. min^{-1} thus giving an energy cost ratio of 4.19 : 1.

8.3 Effect of defoliation on *Coriaria* nodule activity

Nitrogenase activity in root nodules is dependent on photosynthesis in the host plant. Photosynthates produced in the leaves are respired in the root nodules to provide the energy and metabolites for growth and maintenance of plant and bacterial cells, for nitrogenase activity and for ammonia assimilation. Thus changes in the supply of photosynthate to nodules will affect nitrogenase activity. Several legume species, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), lucerne (*Medicago sativa*) and soybean (*Glycine max*) showed a severe decrease in nitrogenase activity following defoliation or darkness (Lindstrom 1984; Minchin *et al.* 1985; Hartwig *et al.* 1987; Walsh *et al.* 1987) presumably due to nodules being deprived of their external carbohydrate supply. Hartwig *et al.* (1987) also provided evidence that the decrease in nitrogenase activity of white clover following defoliation was accompanied by an increase in nodule diffusion resistance and that this, rather than limitation of photosynthate supply, was the main cause of the decline in nodule activity. Huss-Danell and Sellstedt (1985) measured a decrease in nitrogenase activity of *Alnus* incana following defoliation and darkness which they attributed to inhibition of photosynthesis. However they provided no evidence for any changes in nodule diffusion resistance.

The aim of this experiment was to determine whether defoliation had any effect on nitrogenase and respiratory activity in *Coriaria*. Lag-phase measurements were also conducted to investigate whether defoliation caused a change in nodule permeability particularly since *Coriaria* has already been shown to increase nodule diffusion resistance in response to increases in oxygen concentration (Silvester and Harris 1989) and argon exposure (section 7.6).

Four replicate *Coriaria* plants were established in a continuous flow system and subjected to 21 kPa O_2 with nitrogen as the balance gas. Ten kPa acetylene was added to the gas stream after four minutes. After 30 minutes two treated plants were defoliated while

control plants were left intact and the resultant effect on nitrogenase and respiratory activity measured over the ensuing five hours.

In a second experiment four replicate plants were set up in a continuous flow system modified for lag-phase measurements (section 7.6). Calculation of the time constant was made three times on intact plants and then five times following defoliation. Control plants which were left intact were assayed over the same time period.

Defoliation had little initial effect on nitrogenase and respiratory activity and little change in activity was observed for approximately two hours after shoot removal (Fig. 8.1). However five hours after defoliation nitrogenase activity had declined to approximately 38 % of the equilibrium rate prior to detopping. Respiration also showed a delayed decline of similar proportions (Fig. 8.1). Control plants, which remained intact throughout the experiment, showed only a small, gradual decline in both nitrogenase and respiratory activity.

Lag-phase measurements indicate defoliation also had an effect on nodule diffusion resistance (Fig. 8.2). Measurement of the time constant 30 and 90 minutes after defoliation indicated a slight increase in diffusion resistance but the response was much more significant after 150 minutes (Table 8.1). Five hours after defoliation nodule diffusion resistance had increased by 30 %. By comparison, control plants showed little change in activity or nodule diffusion resistance (Table 8.1).

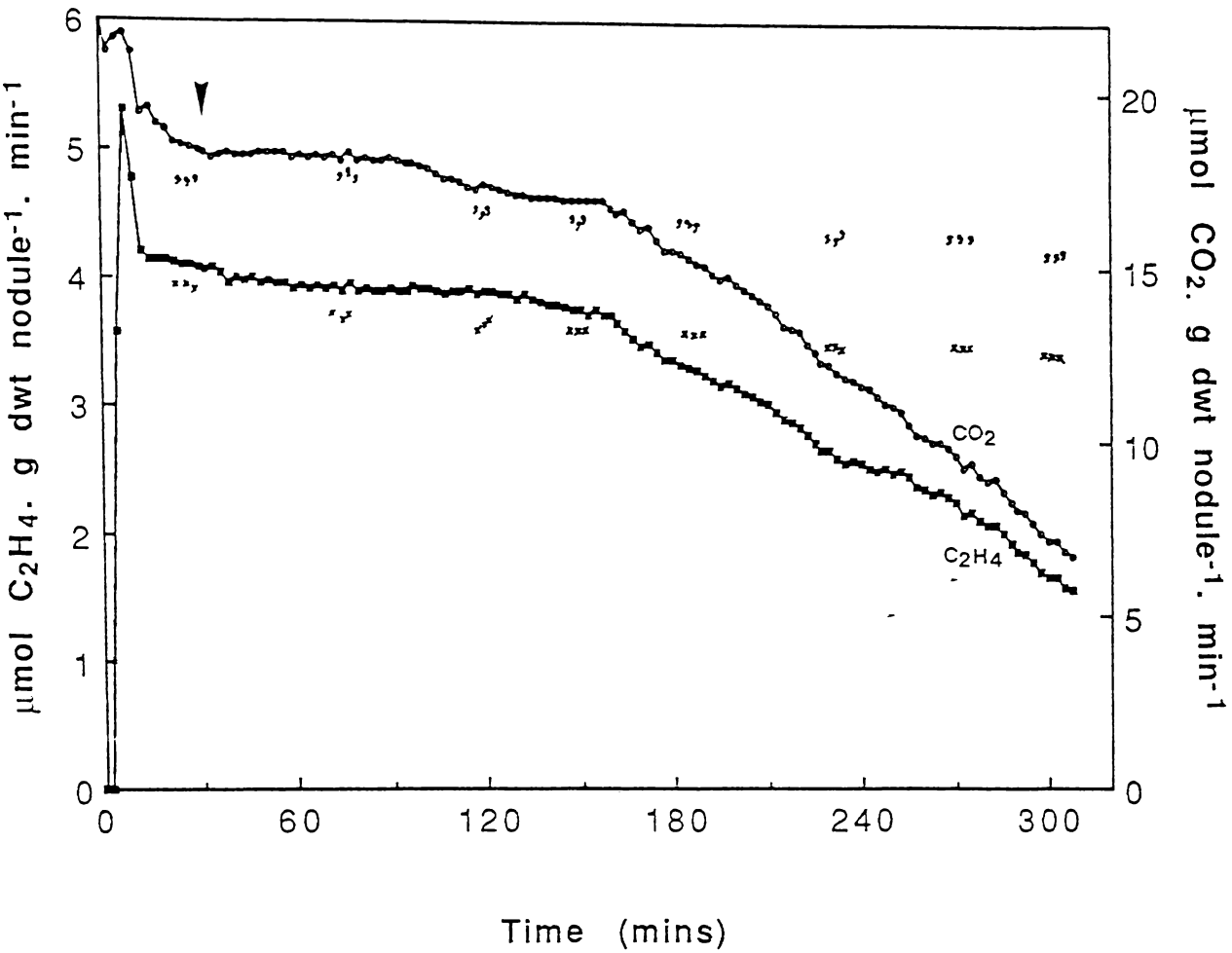

Figure 8.1 Effect of defoliation on nitrogenase activity (—■—) and nodule respiration (—□—) in *Coriaria arborea*. The plant was defoliated after 30 minutes (▼) and only every second data point is shown. Intermittent measurements of nitrogenase (×) and respiratory (›) activity in a control plant, which was not defoliated, are also plotted.

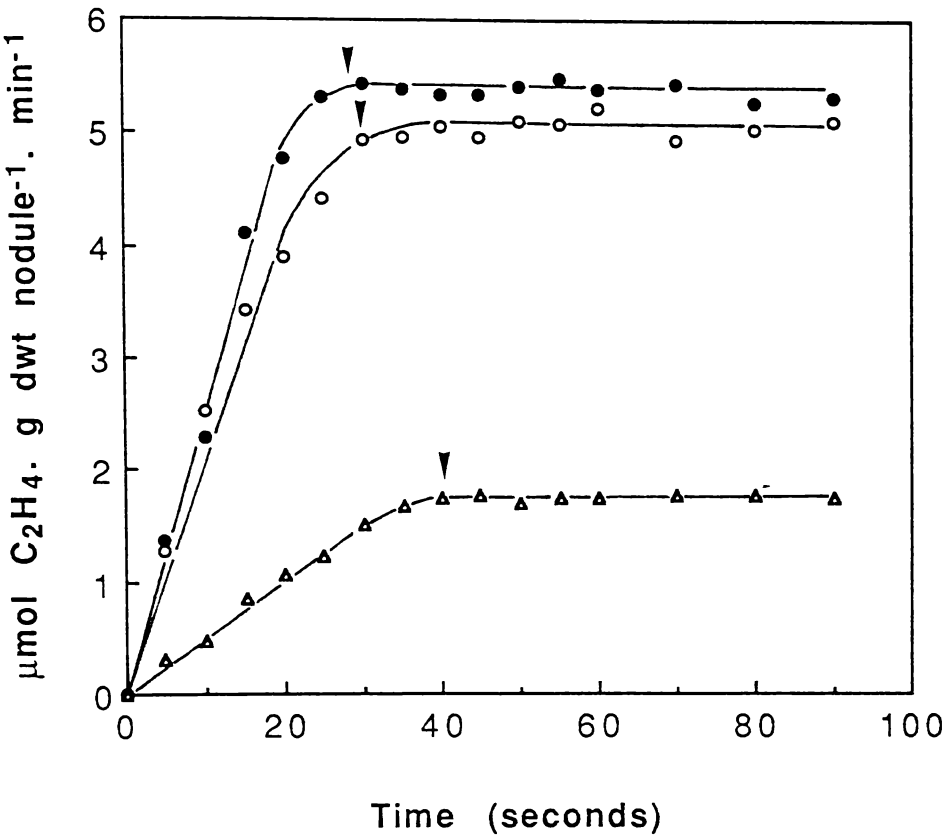

Figure 8.2 Time taken for ethylene production to rise to equilibrium in a *Coriaria arborea* plant before and after defoliation. The plant was exposed to 90 second periods of air / acetylene and therefore was not subject to an acetylene-induced decline. Results show an assay before defoliation at 30 minutes (—●—) and then 90 minutes (—O—) and 300 minutes (—△—) after defoliation. Arrows indicate the time required for ethylene production to reach equilibrium.

Table 8.1 Time constant (half-time to reach an equilibrium rate ofethylene production) in *Coriaria arborea* nodules following defoliation. Three measurements before defoliation and five measurements after defoliation are shown. Data from control plants which were not defoliated is given in italics. Time is the interval after the first acetylene exposure in each treatment.

Treatment	Time (mins)	Time constant (s)		Nitrogenase activity μmol C_2H_4. g dwt nodule^{-1}. min^{-1}	
intact	15	14.4	*14.3*	5.428	*5.298*
	30	14.1	*14.2*	5.376	*5.208*
	45	14.6	*14.5*	5.401	*5.195*
defoliated	30	14.5	*14.6*	5.214	*5.201*
	90	14.7	*14.3*	5.087	*5.193*
	150	15.2	*14.2*	4.643	*5.184*
	210	18.9	*14.3*	3.458	*5.170*
	300	20.4	*14.4*	1.762	*5.162*

8.4 Confirmation of lenticel aeration in *Coriaria* nodules

The structure of a typical *Coriaria* nodule is illustrated in figure 8.3. *Coriaria* nodules are surrounded by a dense, suberised periderm layer which encloses the entire nodule although it is much less extensive in the vicinity of the lenticel. The periderm layer completely surrounds the semi-lunar shaped infected zone; the internal periderm curving inward to leave a small gap between it and the vascular bundle. The lenticel lies on the opposite side of the nodule to the infected tissue and is readily identifiable as an area of white, loosely-packed cells on the nodule surface. Vacuum infiltration of *Coriaria* nodules with India ink (Silvester and Harris 1989) indicated the external periderm and internal periderm are impermeable to gases and aeration is provided via the lenticel.

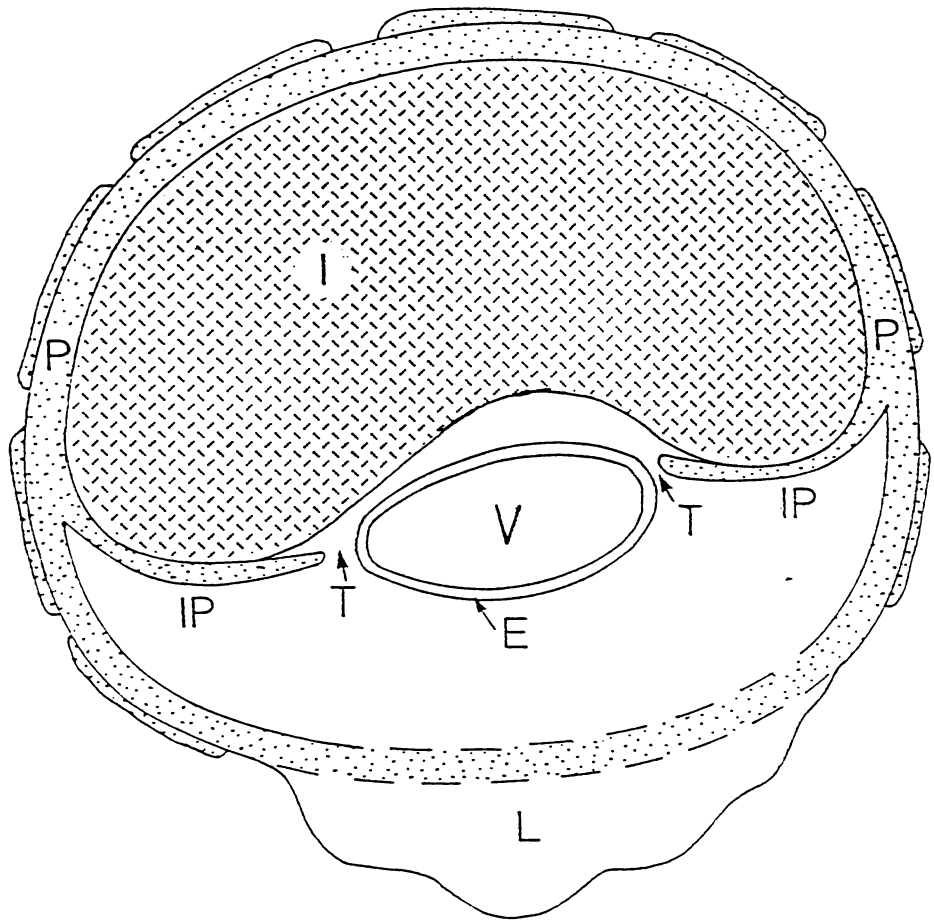

Figure 8.3 Diagramatic representation of a *Coriaria arborea* nodule. The semi-lunar infected zone (I) lies to one side of the nodule and is surrounded by a dense periderm (P). The periderm splits and follows closely under the infected tissue to form the internal periderm (IP) leaving a small gap (T) adjacent to the endodermis(E) of the vascular bundle (V). A prominent lenticel (L) lies opposite the infected tissue (Silvester and Harris 1989).

Intercellular air spaces exist within the infected zone which are continuous with the lenticel via the small gap between the internal periderm and the vascular bundle.

The aim of this experiment was to confirm the role of the lenticel in providing aeration and the effect of restricting gas diffusion via the lenticel on nitrogenase and respiratory activity.

Coriaria plants from sand culture were used rather than aeroponically grown plants since the lenticel region is more clearly defined. In aeroponically grown plants almost the entire nodule surface may become covered with white, loosely-packed cells and the lenticel tissue itself tends to be soft and easily damaged. Plant root systems were gently freed from the sand and plants established in a continuous flow system at 21 kPa O_2. After five minutes equilibration time 10 kPa acetylene was added to the gas stream and nitrogenase and respiratory activity measured automatically. At 45 minutes the plant was removed from the cuvette and grease applied to the lenticel region of each individual nodule lobe under a stereomicroscope, the plant was then replaced in the cuvette and activity measured for a further 45 minutes. The effect of restricting nodule aeration was tested using this procedure on three replicate plants. Two control plants were also treated in the same way except that grease was applied to the opposite side of the nodule from the lenticel i.e. on the external periderm.

Restriction of gas diffusion by application of grease to the external periderm had little effect on nitrogenase and respiratory as illustrated by the control plant shown in figure 8.4. The slight decrease in activity in the control plant was probably due to effects of removal from the cuvette and handling although the effect was not significantly greater than the gradual decline in activity observed following the acetylene-induced decline. However activity of nodules in which the lenticel region was coated with grease declined dramatically following treatment and within 50 minutes nitrogenase and respiratory activity had completely disappeared (Fig. 8.4).

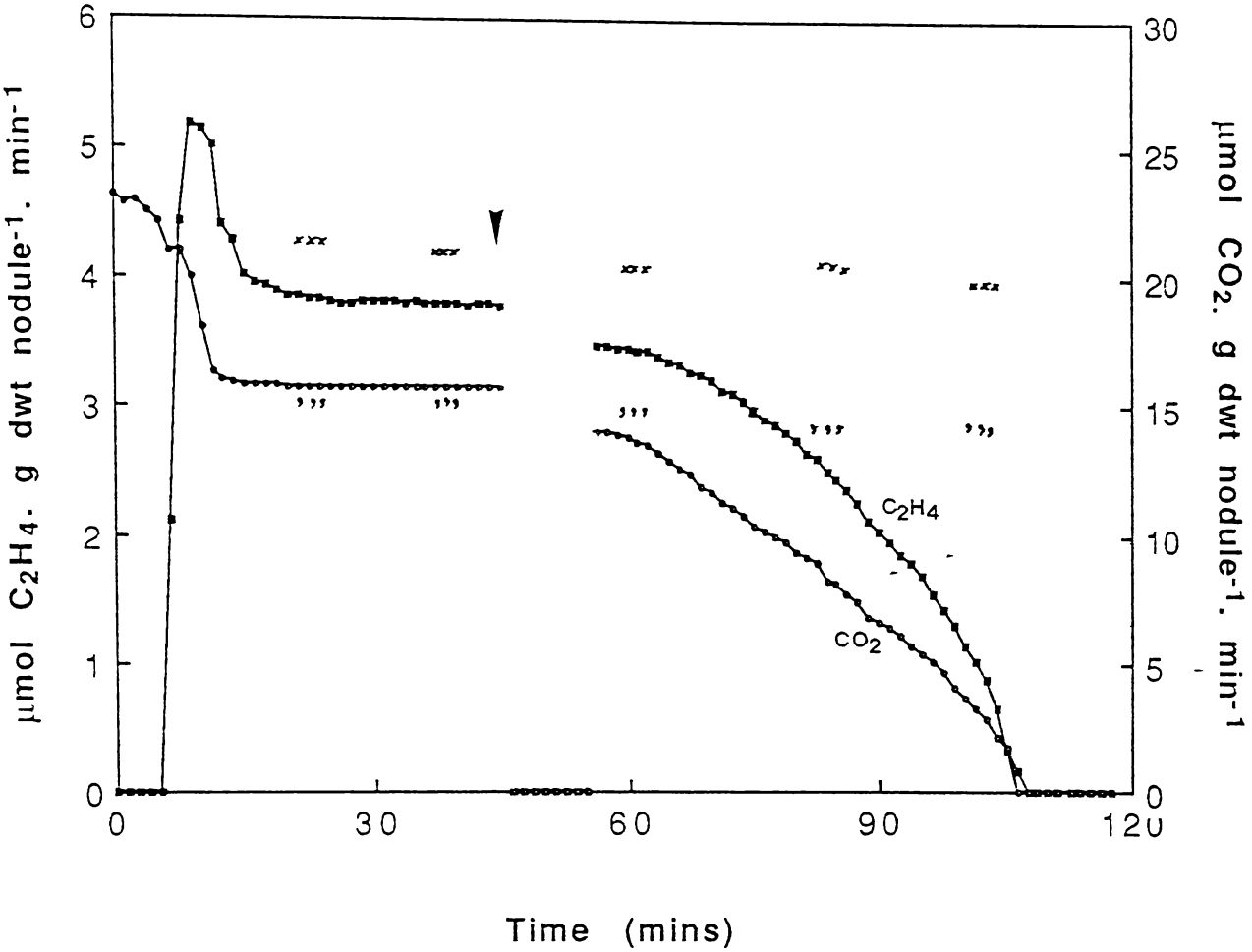

Figure 8.4 Effect of restricting nodule aeration on nitrogenase (—■—) and respiratory (—□—) activity in *Coriaria arborea*. The plant was removed from the cuvette after 45 minutes and lenticels on nodules coated with grease (▼). Measurement of activity, at 21 kPa O_2, was resumed after 55 minutes. Intermittent measurements of nitrogenase (×) and respiratory (❜) activity in a control plant, in which the nodule periderm was coated in grease, are also plotted.

8.5 Nature of diffusion resistance in various nodules

The site and nature of diffusion resistance in legume and actinorhizal nodules is still a source of speculation. All legumes studied so far show evidence of a barrier to gas diffusion in the nodule inner cortex which is completely lacking in air spaces (Tjepkema and Yo- um 1974; Dixon *et al.* 1981). Thus it is unlikely the length of the diffusion pathway in legume nodules could have any major bearing on permeability of the nodule to gases although Dakora and Atkins (1989) suggest that variations in the size, arrangement and distribution of cells in the cortical region could affect the rate of oxygen diffusion across the cortex. As indicated above, the site of diffusion resistance in actinorhizal nodules is more varied and may lie in the host cytoplasm, host cell walls or in *Frankia* vesicles. It is also possible that length and size of the diffusion pathway may contribute to regulation of oxygen diffusion in some species.

The purpose of this work was to determine the nature of diffusion resistance in nodules i.e. whether some component of the diffusion pathway is air-filled and thus resistance is regulated by length and size of this air-filled component or whether resistance is due to a more "solid" barrier such as water-filled intercellular spaces, suberised cell walls or a zone of cells completely lacking intercellular spaces.

The technique used to determine the nature of nodule diffusion resistance was based on a method previously described by Cowan *et al.* (In press) in their study of CO_2 exchange in lichens. The technique relies on the diffusion coefficient for oxygen in helium (0.729 $cm^2. s^{-1}$) i.e. oxygen with helium as the balance gas, being three times greater than the diffusion coefficient of oxygen in nitrogen (0.243 $cm^2. s^{-1}$) (Mason and Marrero 1970). This difference in diffusivity may provide a sudden increase in the oxygen concentration at the site of nitrogenase when the balance gas is changed from nitrogen to helium, which could result in either an increase or decrease in nitrogenase activity depending on whether the pO_2 is above or below the optimum concentration. However such a change in

nitrogenase activity can only occur if all, or at least some component, of the diffusion resistance within the nodule is an air-filled pathway. If all the diffusion resistance is a water-filled pathway or some form of "solid" barrier exists, increasing diffusivity of oxygen by changing the balance gas from nitrogen to helium will have no effect on nitrogenase activity since oxygen diffusion through such a barrier is the same regardless of the balance gas.

It should be noted that helium, like acetylene and argon, causes a decline in nitrogenase (hydrogen evolution) and respiratory activity in legumes (Minchin *et al.* 1983). However, in this experiment, plants were first exposed to acetylene which itself causes a decline in nitrogenase activity, so exposure to helium would have no further effect on nodule activity.

The technique was tested on *Coriaria*, *Casuarina*, *Alnus*, *Myrica* and *Medicago sativa* plants using the continuous flow system. Plants were subjected to alternate O_2 : N_2 and O_2 : He atmospheres, also containing 10 kPa acetylene, at several pO_2 concentrations as indicated on the graphs. Care was taken to ensure oxygen concentrations were equivalent when changing from O_2 : N_2 to the corresponding O_2 : He gas mixture since small changes in oxygen concentration have a significant effect on nitrogenase activity.

Figures 8.5 to 8.9 show the response of *Medicago*, *Casuarina*, *Alnus*, *Myrica* and *Coriaria* to alternating O_2 : N_2 and O_2 : He at several pO_2 concentrations above and below atmospheric. All four actinorhizal species and both legumes responded as expected to the increases in oxygen concentration but only *Myrica* (Fig. 8.8) and *Coriaria* (Fig. 8.9) exhibited a change in nitrogenase activity when the balance gas was changed at a constant pO_2. Throughout the experiment nitrogenase activity in *Coriaria* was less influenced by pO_2 than that of *Myrica* but both these species showed small increases in activity when the balance gas was changed from nitrogen to helium at 10 kPa O_2, presumably because the oxygen limitation at low pO_2 was partially relieved as oxygen diffusivity was

increased. At 21 kPa O_2 *Coriaria* again showed a slight increase in nitrogenase activity following the change to helium but nitrogenase activity in *Myrica* decreased. At 30 kPa O_2 both species exhibited a decrease in activity when the balance gas was changed probably because increased diffusivity of oxygen caused oxygen inhibition of nitrogenase. Following the slight decrease at 30 kPa O_2 : He, *Coriaria* showed a small decline in activity and then recovered to remain relatively stable at a level slightly below the rate of activity at 30 kPa O_2 in nitrogen (Fig. 8.9). In *Myrica* however, activity at 30 kPa O_2 continued to decline following the change to helium (Fig. 8.8). *Coriaria* also exhibited "bounces" in nitrogenase activity immediately following the initial decline and preceding the gradual recovery of activity when oxygen concentration was changed. These "bounces" occurred as small peaks in activity approximately four to six minutes after an increase or decrease in oxygen concentration. A change in the balance gas from nitrogen to helium also resulted in small "bounces" in activity although these were considerably smaller than those associated with a change in pO_2. "Bounces" in nitrogenase activity in *Coriaria* have been recorded previously (Harris 1989; Silvester and Harris 1989) and were attributed to a "hunting effect" as nitrogenase activity is readjusted to internal pO_2. Such an effect probably involves changes in respiration and/or diffusion resistance and conformational changes in nitrogenase.

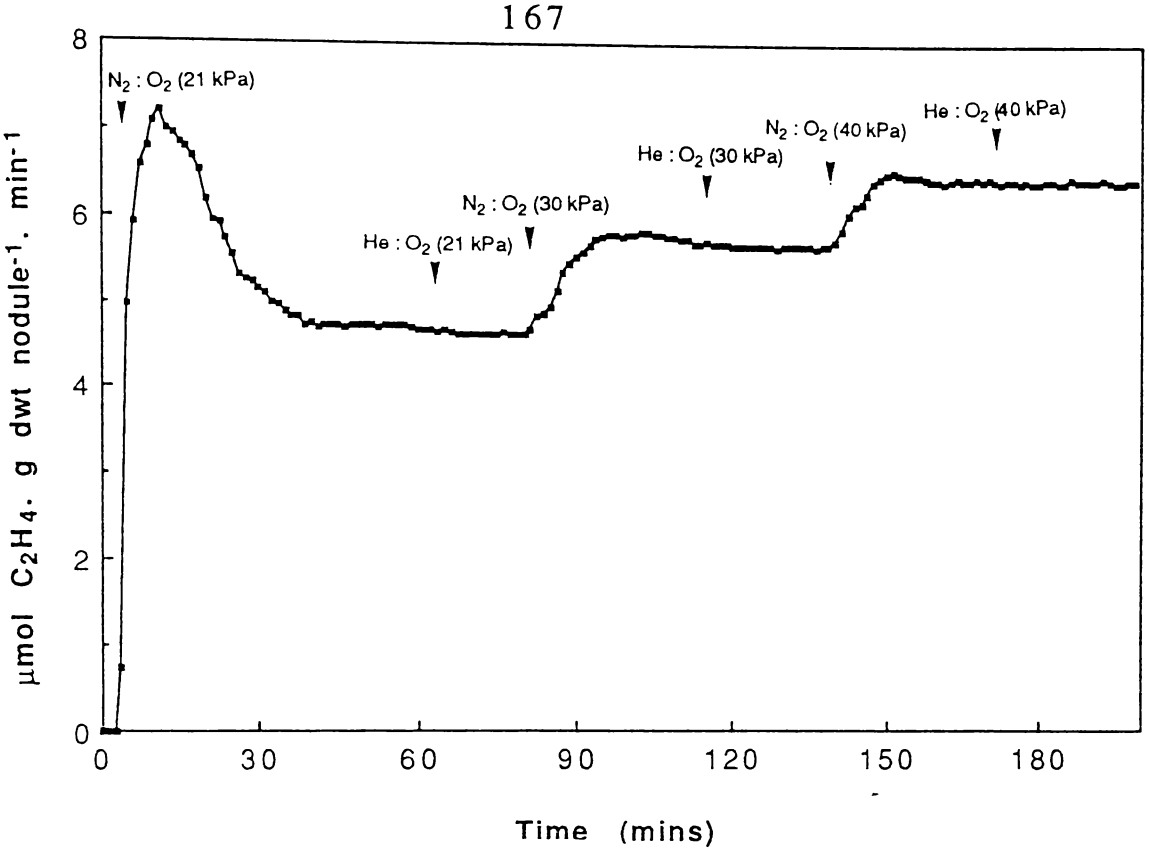

Figure 8.5 Effect of alternating nitrogen and helium in the gas stream on nitrogenase activity in *Medicago sativa*. Gas changes were made as indicated by arrows, over a pO_2 range of 21 kPa O_2 to 40 kPa O_2.

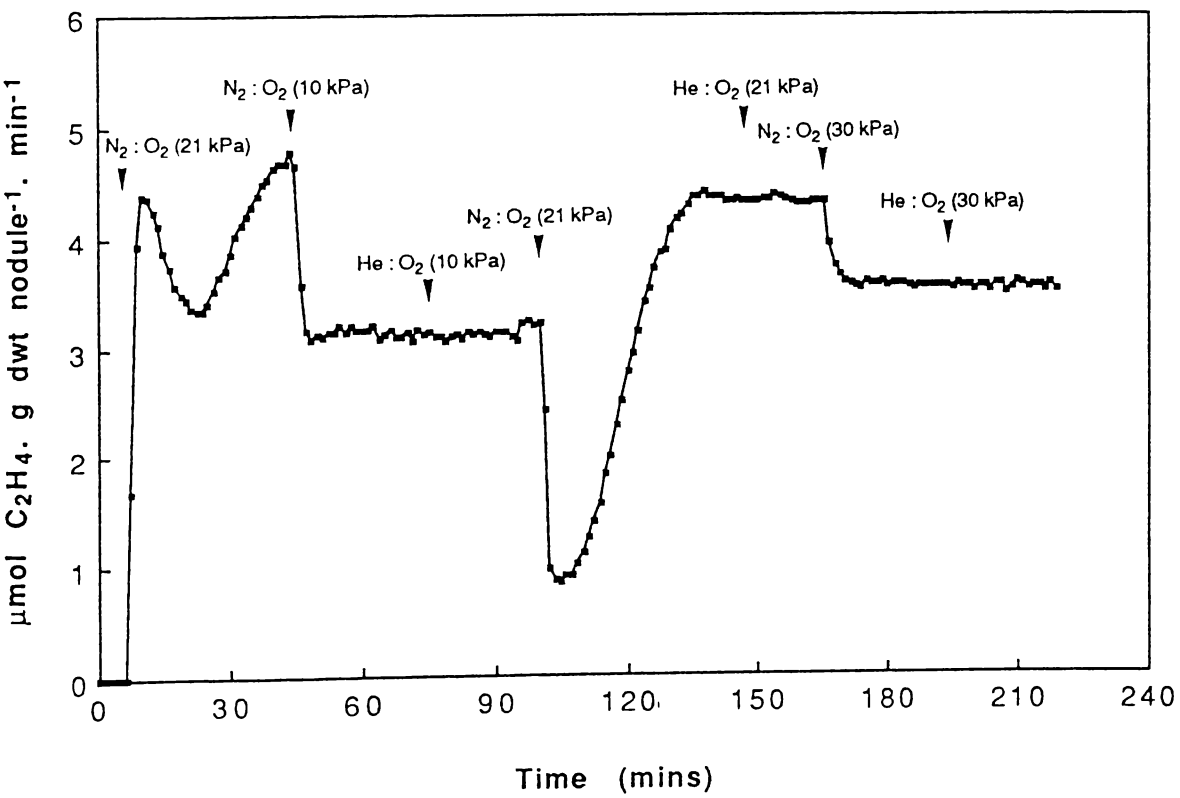

Figure 8.6 Effect of alternating nitrogen and helium in the gas stream on nitrogenase activity in *Casuarina cunninghamiana*. Gas changes were made as indicated by arrows, over a pO_2 range of 10 kPa O_2 to 30 kPa O_2.

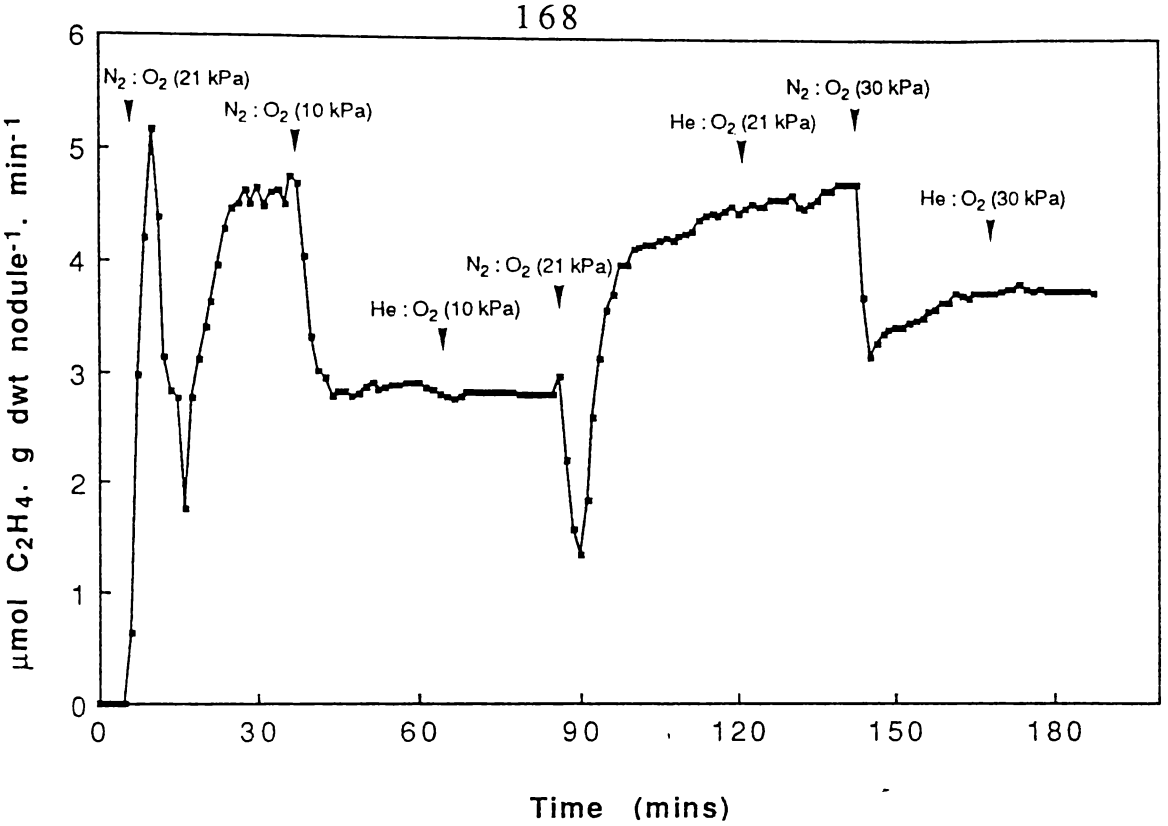

Figure 8.7 Effect of alternating nitrogen and helium in the gas stream on nitrogenase activity in *Alnus glutinosa*. Gas changes were made as indicated by arrows, over a pO_2 range of 10 kPa O_2 to 30 kPa O_2.

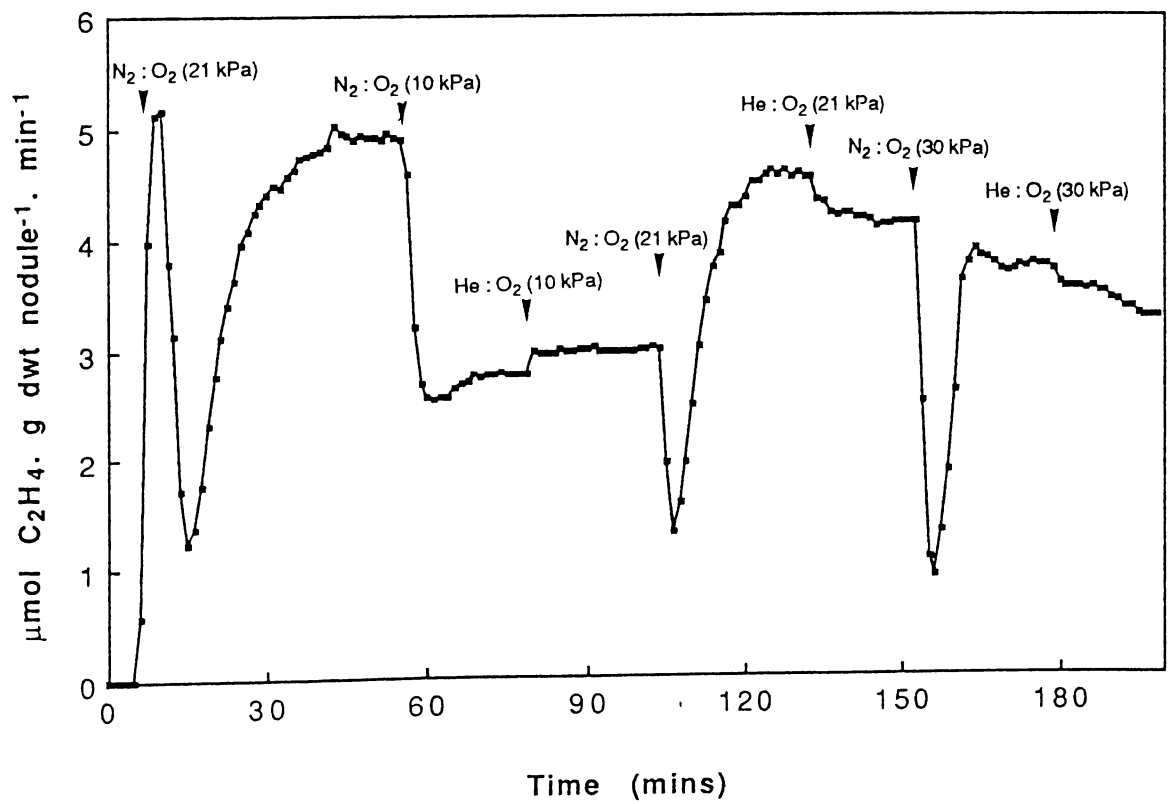

Figure 8.8 Effect of alternating nitrogen and helium in the gas stream on nitrogenase activity in *Myrica gale*. Gas changes were made as indicated by arrows, over a pO_2 range of 10 kPa O_2 to 30 kPa O_2.

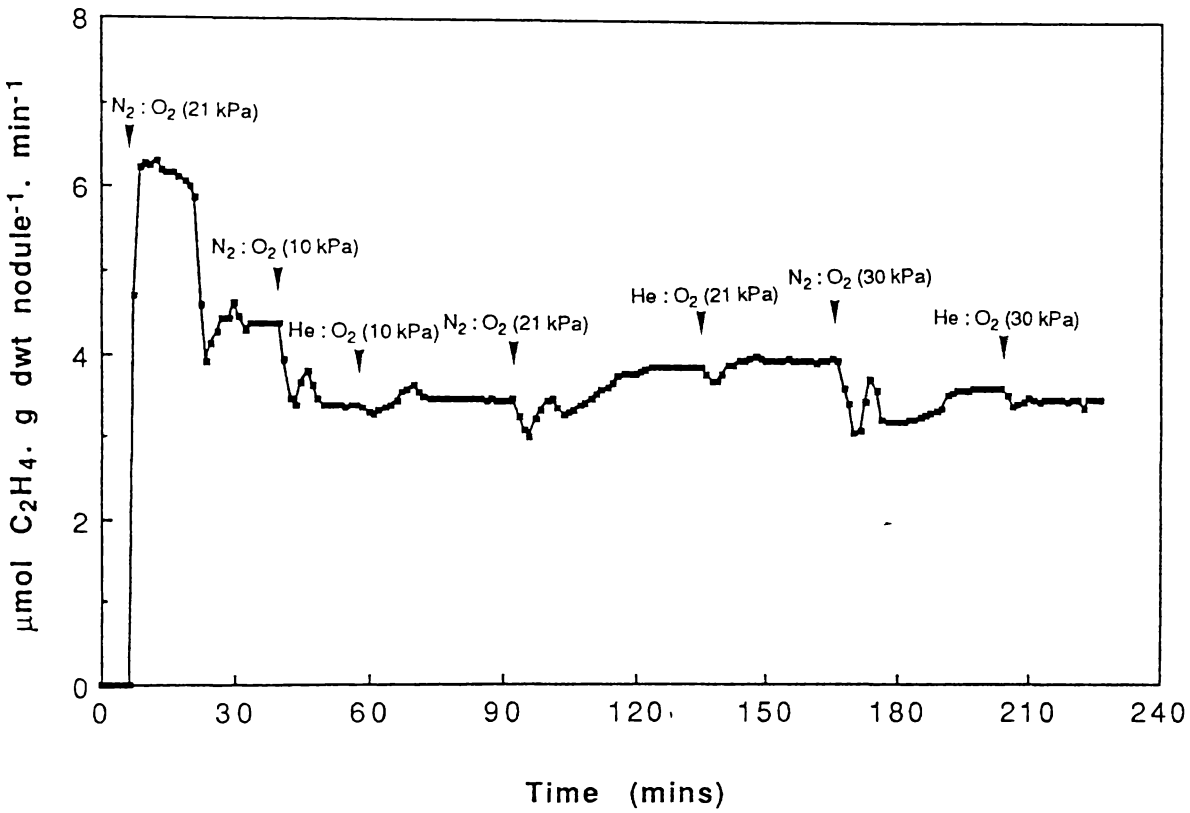

Figure 8.9 Effect of alternating nitrogen and helium in the gas stream on nitrogenase activity in *Coriaria arborea*. Gas changes were made as indicated by arrows, over a pO_2 range of 10 kPa O_2 to 30 kPa O_2.

8.6 Discussion

Previous work indicates that, in some respects, *Coriaria* is physiologically similar to the legumes. *Coriaria* exhibits a non-recoverable acetylene-induced decline, has a broad pO_2 optimum for nitrogenase activity and readily adapts to changes in ambient pO_2 by virtue of a variable diffusion resistance mechanism, all legume characteristics. Results from this study highlight further similarities, namely the comparable energy cost for nitrogen fixation between *Coriaria* and legumes and the similar effect of defoliation on nodule diffusion resistance.

Calculation of the amount of energy consumed in nodules in terms of the rate of CO_2 evolved from nodule respiration : nitrogenase activity gave a ratio for *Coriaria* of 4.19 : 1. Tjepkema and Winship (1980) have made similar calculations for detached nodules of field grown plants representing several actinorhizal and legume species. In those actinorhizal species tested, *Alnus rugosa*, *Ceanothus americanus*, *Comptonia peregrina*, *Elaeagnus umbellata* and *Myrica gale*, the ratio for most plants lay between 2.8 to 3.8 while most of the legumes tested fell within the range 3.4 to 4.5. Although the energy cost of nitrogen fixation for *Coriaria* in this study was measured using a different technique to that employed by Tjepkema and Winship (1980), the results are similar and indicate there is little difference between actinorhizal and legume species in this respect. These results compare with a theoretical estimate for CO_2 evolution : nitrogenase activity of 1.2 : 1 (Tjepkema and Winship 1980) indicating a significant amount of energy must also be used for other nodule processes such as growth, cell maintenance and amino acid synthesis and transport and possibly for oxygen protection.

It should be noted however that CO_2 evolution may also underestimate the true energy consumption of nodules since some CO_2 may be fixed in the synthesis of carbon skeletons for amino acid synthesis (Tjepkema and Winship 1980). Nitrogen and acetylene also differ in their properties as substrates for nitrogenase. For example,

acetylene reduction is associated with little accompanying hydrogen evolution compared with the hydrogen evolution during reduction of nitrogen. Therefore an alternative measure is needed before absolute measures of the energy requirements of nitrogen fixation in root nodules can be made. Energy consumption could also be calculated from the rate of oxygen uptake associated with nitrogenase activity rather than CO_2 evolution. Measurements of oxygen uptake and CO_2 evolution in legumes (Minchin and Witty 1990) show the ratio between the two to be approximately one under most conditions and thus oxygen uptake could provide a reliable indication of energy consumption.

Defoliation of *Coriaria* caused considerable decrease in both nitrogenase and respiratory activity although the effect was delayed so the decline did not commence until about two hours after shoot removal. Whether this decline in activity was due to a limitation of carbohydrate supply from photosynthesis was not determined since no measurements of carbohydrate reserves in nodules were made. It seems sensible however, to assume that carbohydrate limitation does contribute to the decrease in *Coriaria* nodule activity following defoliation. Walsh *et al.* (1987) showed root respiration and nitrogenase activity in nodulated soybean was highly dependent on the continued supply of photosynthate from the shoot since leaf excision, stem girdling and stem chilling all caused a rapid decrease in nodule activity. However, similar experiments examining the effect of defoliation on lucerne and birdsfoot treefoil (Cralle and Heichel 1981; Fishbeck and Phillips 1982) showed changes in carbohydrate reserves after shoot removal did not coincide with and were of a different magnitude to those changes in nitrogenase activity. Thus the findings were not fully consistent with the supposed role of carbohydrate availability as the main factor limiting nitrogenase activity following defoliation.

Hartwig *et al.* (1987) showed nitrogenase activity of white clover was more severely limited at low pO_2 and more responsive to high pO_2 following defoliation. They suggested the large beneficial effect of additional oxygen on acetylene reduction of defoliated plants indicated a lack of oxygen at the site of nitrogenase was the major factor

limiting activity and this oxygen-limitation was due to an increase in nodule diffusion resistance. Lag-phase measurements showed *Coriaria* also increased diffusion resistance following defoliation and, like the observed decrease in nitrogenase and respiratory activity, the increase in diffusion resistance was delayed. However the increase in nodule diffusion resistance was relatively small and therefore a depletion in carbohydrate reserves probably also contributed to the decline in nodule activity.

In contrast to other studies, the effect of defoliation on *Coriaria* was considerably delayed and not very severe. Hartwig *et al.* (1987) showed defoliation of white clover plants led to a 95 % reduction in nitrogenase activity within six hours while Walsh *et al.* (1987) report disruption of phloem supply to roots in soybean caused a sharp decrease in nitrogenase activity and CO_2 evolution within 20 to 30 minutes. In *Alnus incana*, nitrogenase activity dropped rapidly and was almost zero within five hours of defoliation (Huss-Danell and Sellstedt 1985). A possible explanation for the less severe effect of defoliation on *Coriaria* may be that *Coriaria* nodules contain large reserve pools of carbohydrates. Walsh *et al.* (1987) provided evidence soybean nodule carbohydrate reserves were only exploited under conditions in which external phloem-supplied carbohydrate was limited. However, soybean nodule carbohydrate reserves are relatively small, and therefore metabolism is severely restricted once these reserves are used. In *Coriaria*, though, nodule carbohydrate reserves may be large enough to support the respiratory demand of active nodules for some hours following removal of the shoot.

Despite some physiological similarities, *Coriaria* nodules are structurally very different from their legume counterparts. No cortical diffusion barrier is evident in *Coriaria* nodules so that they, like most actinorhizal nodules, are considerably more aerated than legume nodules (Tjepkema 1979). This observation is reinforced by the response of *Coriaria* nitrogenase activity to changes in oxygen diffusivity when the atmosphere is changed from nitrogen to helium. The results indicate at least a small part of the diffusion pathway is an air-filled component. Previously vacuum infiltration of *Coriaria* nodules

with India ink and histological work (Silvester and Harris 1989) illustrated the external and internal periderm layers are suberised and impermeable to gas diffusion. Therefore the small gap remaining between the internal periderm and the vascular bundle would appear to be the most logical site for an air-filled component of nodule diffusion resistance. This zone is also a possible site for a variable diffusion resistance mechanism which would mean that at least some of the intercellular spaces in this region are water-filled provided, of course, that the variable diffusion resistance is regulated by an increase in the water content of intercellular spaces. However, if the gap is the site of both the air-filled component of the diffusion pathway as well as the variable diffusion resistance mechanism, then not all the intercellular spaces can be water-filled. It is possible, as external pO_2 is increased, the number of water-filled intercellular spaces could rise resulting in an increase in diffusion resistance. If this hypothesis is correct, when the balance gas is changed from nitrogen to helium, the change in nitrogenase activity would be less obvious at higher pO_2 since little of the diffusion pathway would be air-filled at high oxygen concentrations. However no such trend was observed over the pO_2 range tested in this work.

The impermeability of the external periderm in *Coriaria* nodules was confirmed by evidence that the lenticel region is the only site for entry of oxygen into the nodule. Restricting gas diffusion by blocking the lenticel caused a rapid decline in nodule activity presumably due to oxygen limitation. Expansion of the lenticel region to facilitate nodule aeration when plants were grown at low pO_2 (Silvester and Harris 1989) also supports the lenticel as being the sole entry port for gas diffusion particularly since no other difference in nodule structure was observed at above and below atmospheric growth pO_2.

Myrica also exhibited a change in nitrogenase activity in response to altered oxygen diffusivity. Infected cells in *Myrica* occur in groups scattered throughout the nodule cortex and India ink and microelectrode studies (Tjepkema 1983) indicate the pockets of infected cells are sites of low pO_2. It may be the air-filled component of diffusion in

Myrica is associated with non-infected tissue within the cortex or possibly with diffusion via nodule roots which presumably involves a pathway of considerable length. The high concentration of haemoglobin measured in *Myrica* nodules by Tjepkema and Asa (1987) has been taken as an indication of the presence of an external diffusion barrier similar to the situation in legumes. The presence of such a barrier would appear to be at odds with the interpretation of the N_2 : He work presented here unless an air-filled component of diffusion resistance is minor, relative to the overall diffusion resistance of *Myrica* nodules.

Results from the N_2 : He work indicated *Casuarina* has no air-filled portion in its gas diffusion pathway . This result is consistent with the high haemoglobin content measured for these nodules (Tjepkema and Asa 1987) and the results of various morphological studies (Berg and McDowall 1988; Zeng *et al.* 1989) which have shown lignified, infected cell walls in *Casuarina* nodules are the most likely site for a diffusion barrier.

Alnus also exhibited no response to changes in the balance gas. Unlike *Myrica* and *Casuarina*, *Alnus* has only a very low haemoglobin content (Tjepkema and Asa 1987) and the nodule appears to be well aerated. However Silvester *et al.* (1988*b*) provided evidence that *Frankia* vesicle walls in *Alnus* respond to different growth pO_2 and thus the vesicle envelope may provide diffusion resistance in these nodules. Since the vesicle envelope represents the final component in the diffusion pathway, any air-filled components of the diffusion pathway outside the vesicle would be unlikely to have a bearing on nodule diffusion resistance.

The results presented in this chapter have further illustrated the similarities and differences between actinorhizal and legume species and in particular the comparisons between *Coriaria* and legumes. Despite the comparable energy cost of nitrogen fixation in the two symbioses and the similarity in the effect of defoliation, the nature and site of diffusion

resistance is very different between the two groups of plants and also varies considerably among actinorhizal species themselves.

CHAPTER NINE

DISCUSSION

9.1 Introduction

All aerobic nitrogen-fixing organisms face a dilemma in having to protect nitrogenase from oxygen inactivation while maintaining a high flux of oxygen to support respiratory activity close to the site of nitrogenase enzyme. The magnitude of this dilemma is realised when it is considered approximately 21,000 Pa O_2 is present in air and this must be reduced to pressures of around 1 Pa at the site of nitrogenase. However, nitrogen-fixing organisms such as *Frankia* must not only protect nitrogenase but also create a balance between oxygen diffusion and oxygen use that is flexible, in order to maintain nitrogenase activity as environmental conditions change. Thus oxygen is a major factor regulating morphology and physiology in *Frankia* in culture and in symbiosis with actinorhizal plants. Identification of oxygen protection mechanisms in *Frankia* in culture and in symbiosis, particularly with *Coriaria arborea*, has therefore been the major focus of this study.

9.2 *Frankia* vesicles and oxygen protection

Work presented in this thesis provides further insight into the functioning of the *Frankia* vesicle as a barrier to oxygen diffusion providing protection for nitrogenase enzyme located within. This intrinsic oxygen protection mechanism allows *Frankia* to adapt nitrogenase activity to a range of oxygen concentrations above and below atmospheric which is in marked contrast to asymbiotic *Rhizobium* which can only support nitrogen fixation activity under microaerophilic conditions (Tjepkema and Evans 1975).

A major breakthrough in this study was the development of a technique for maintaining *Frankia* in continuous culture (chapter three). Using a low dilution rate, cultures were able to sustain a maximum rate of growth and nitrogenase activity for periods of more than 30 days. Maintenance of cultures in steady state was shown to be linked to the large proportion of active vesicles in continuous culture populations compared with batch culture where the vesicle population rapidly degenerates. It is important to note that, rather than prolonging the active life of individual vesicles, continuous culture maintained a large population of active vesicles by continually forming provesicles to replace those which are damaged i.e. "ghost" vesicles.

Continuous cultures permitted the effect of oxygen on vesicle structure and development to be studied independent from any "normal" endogenous changes occurring during vesicle ontogeny. Oxygen was shown to have no effect on the timing of vesicle induction but oxygen is however the sole factor regulating vesicle wall thickness so that thicker vesicle envelopes are formed at high pO_2 levels. This response to oxygen was observed even under conditions where nitrogenase activity was inhibited (in Ar : O_2) and when no activity could be detected (in *Frankia* cultures grown on nitrate) and confirms the vesicle envelope as a major barrier to oxygen diffusion. The effect of oxygen on vesicle wall thickness operates by regulating the maximum wall thickness attained by vesicles at any given oxygen concentration rather than by affecting the rate of thickening. Thus, provesicles appear to have similar wall thickness regardless of growth pO_2 and vesicles appear to develop thicker walls at an equivalent rate at all growth pO_2 levels until wall thickness reaches a certain upper limit correlated to the oxygen concentration. Measurement of a higher hopanoid content in *Frankia* grown at 60 kPa O_2 compared with 5 kPa O_2 supported observations of thicker-walled vesicles at high pO_2 levels. The role of the vesicle wall as a diffusion barrier was also confirmed by similar levels of activity measured in cultures grown at a range of oxygen concentrations. However, modification of the vesicle wall to oxygen and resultant adaptation of nitrogenase activity does seem to

have a limit since activity at very low oxygen concentrations appeared to be severely oxygen-limited. This result suggests a threshold effect of minimum vesicle wall thickness exists (chapter five).

Despite conclusive evidence that hopanoids comprise a large proportion of the vesicle envelope, there has been little consideration of how such molecules combine structurally to form lipid monolayers. Monolayer organisation of lipids has also been found in *Sulfolobus solfatarius*, a thermophilic archaebacteria, but the lipids in this organism are bipolar (Gliozzi *et al.* 1986). Most biological membranes are bilayers held together by the hydrophilic - hydrophobic nature of the component phospholipids. However neither bacteriohopanetetrol nor its phenylacetate monoester have hydrophilic - hydrophobic properties and neither are they polar molecules which would cause these molecules to form bilayers, Instead the hopanoids found in *Frankia* are classed as partially rigid amphiphilic molecules with dimensions adapted for organisation into monolayers (Ourisson *et al.* 1987). It is unknown how hopanoids are organised to form monolayers or how the repeated monolayers are bound together to form the vesicle envelopes up to 50 layers thick (Parsons *et al.* 1987). Side-chains and the addition of groups on to these side-chains and the presence of small amounts of polar lipids e.g. phosphatidylinositol and diphosphatidylinositol (Tunlid *et al.* 1989), may allow some form of bonding between molecules and between individual monolayers.

Questions also exist concerning synthesis of lipid envelopes in *Frankia* vesicles. The envelope is laid down outside both the vesicle cell membrane and the cell wall but synthesis of component lipids presumably occurs within the vesicle. Synthesis of fatty acids and hopanoids from acetyl-CoA requires both ATP and reductant (NADPH). Formation of one palmitic acid molecule for example requires seven ATP and 14 NADPH while synthesis of hopanoids generally requires larger amounts of ATP (Bloch and Vance 1977) which is generated by mitochondria within the vesicle. Lipid molecules must then be transported through the cell wall and laid down by aposition on the inside of the

existing envelope structure. It is possible hopanoid molecules are transported across the cell wall as smaller units e.g. as squalene, and that cyclisation reactions and addition of side-chains may occur using enzymes located in the cell wall.

Evidence suggests an increase in external pO_2 triggers the synthesis of further lipids to form a thicker vesicle envelope. Formation of a thickened vesicle wall takes at least 36 h (section 5.5) therefore this protection mechanism is a long term adaptation to oxygen concentration rather than a short-term response. However, the ATP requirements of lipid synthesis presumably necessitates an increase in respiration rate which in turn would consume additional oxygen and so help protect nitrogenase until the vesicle wall is thickened to provide adequate diffusion resistance. Short-term adaptation in *Frankia* cultures presumably involves switch-off of nitrogenase as evidenced by detection of oxygen transients in *Frankia* exposed to stepwise increases in pO_2 (Silvester and Winship 1990). Increases in respiratory activity may also provide some short-term protection of nitrogenase when oxygen concentration is increased although little work has been conducted on respiration of *Frankia* cultures using continuous flow assay.

The hopanoid content of *Frankia* measured in this study is high compared with values given for a range of other bacteria (Rohmer *et al.* 1984). Lipid and, in particular, hopanoid synthesis is a slow process requiring large amounts of ATP. Therefore synthesis of hopanoids for vesicle envelopes may account for the slow growth rates measured for *Frankia* in derepressed culture (Murry *et al.* 1984*a*; Zhang and Torrey 1985). Formation of vesicles, in order to protect nitrogenase from oxygen inactivation, therefore represents something of a trade-off for *Frankia* in terms of growth. However, other factors must also contribute to slow growth since no significant difference was measured in growth rates of *Frankia* at oxygen concentrations ranging from 5 kPa O_2 to 40 kPa O_2 despite a presumably lower hopanoid requirement at 5 kPa O_2 where vesicle walls are thinner.

9.3 Vesicles and expression of nitrogenase activity

Oxygen was also shown to have an effect on timing of appearance of nitrogenase activity in cultures, with *Frankia* grown at low pO_2 levels having a shorter lag time before the onset of activity than *Frankia* at higher oxygen concentrations. This effect is probably indirect and due to vesicles at low pO_2 reaching a wall thickness sufficient to protect nitrogenase sooner than vesicles at high pO_2 which must form thicker-walled vesicles to adequately protect nitrogenase. Although synthesis of nitrogenase enzyme in *Frankia* is regulated by a feedback mechanism involving the products of ammonium assimilation (Gauthier 1983), it would be interesting to determine whether oxygen has an effect on induction of nitrogenase synthesis or if nitrogenase expression in cultures at high pO_2 is delayed at some later step in the formation of the active enzyme complex. For example, nitrogenase formation could be blocked at the point of RNA transcription, at synthesis of the protein subunits or at the binding of protein subunits to form the enzyme complex. Alternatively a third subunit may bind to the nitrogenase complex rendering it inactive until the oxygen environment is adequately regulated by the vesicle. It is also possible catalytically active nitrogenase is formed but is immediately inactivated until such time that vesicles have sufficiently thickened walls to protect the enzyme. Presence of nitrogenase enzyme in vesicles could be tested by immunolabelling techniques (Huss-Danell and Bergman 1990) and work on the *Frankia* genome may provide further information on signals which control nitrogenase expression.

A feedback mechanism similar to that regulating nitrogenase synthesis may also control vesicle number. In cultures grown on nitrogen-free medium in $N_2 : O_2$ vesicle number reaches a steady state maximum but in $Ar : O_2$ grown *Frankia* the vesicle number continues to increase. This may be because no products of ammonium assimilation are present in $Ar : O_2$ grown cultures. Oxygen has no effect on vesicle number since *Frankia* cultures at different growth pO_2 levels had similar vesicle counts.

Various studies have shown close correlation between vesicles in culture and the presence of nitrogenase activity (Murry *et al.* 1984*a*; Tjepkema *et al.* 1980; 1981). This led to the understanding that, under most growth conditions, vesicle formation and nitrogenase activity are concomitant. However some exceptions to this rule have been reported. Murry *et al.* (1985) showed *Frankia* sp. strain HFPCcI3 had active nitrogenase but no vesicles when grown at low pO_2. Presumably this was because the very low oxygen concentration rendered a diffusion barrier, normally provided by the vesicle wall, unnecessary. More recently there have been reports of a few *Frankia* strains forming vesicles when grown on ammonium but having no nitrogenase activity (Meesters *et al.* 1985; Tisa and Ensign 1987). Similarly CcI3 formed vesicles when grown on nitrate but no significant levels of nitrogenase activity could be detected (chapter four). Whether nitrogenase enzyme was present but catalytically inactive in nitrate -grown vesicles is unknown but could be determined using immunolabelling techniques, These results indicate vesicles and nitrogenase activity are not as closely correlated as was first thought and it is unlikely that gene sequences encoding for vesicle and nitrogenase formation are triggered by the same signals.

Considerable research into structural genes for the nitrogenase complex (*nif* genes) and factors regulating expression of these genes in *Frankia,* has been conducted (An *et al.* 1985; Normand *et al.* 1988; Simonet *et al.* 1988; Normand and Bousquet 1989). *Nif D* and *nif K* genes, coding for the a and b subunits of the MoFe protein, and *nif H*, for the Fe protein have been found in all *Frankia* strains examined. In *Rhizobium meliloti* and *Klebsiella pneumoniae*, nitrogenase expression is in part controlled by the *nif A* gene, which itself is induced by low oxygen tension. A *nif A* probe from *R. meliloti* hybridised to cloned DNA from *Frankia* sp. strain ArI3 which indicates a *nif A*-like regulatory gene may be operating in *Frankia* (Simonet *et al.* 1988).

9.4 Comparison with cyanobacteria

The properties of vesicles which allow *Frankia* to express nitrogenase in aerobic environments are paralleled by heterocysts of cyanobacteria. *Frankia* can be regarded as the heterotrophic analogue of cyanobacteria, the filaments acting as the nutrient exploiting component and vesicles as the nitrogen source. Both organisms represent perhaps the ultimate in morphological and physiological differentiation among prokaryotes; in both cases vesicles or heterocysts are usually formed in the absence of a ready nitrogen source and are generally repressed whenever an alternative nitrogen source is supplied. Also, both the vesicle and the heterocyst represent strong oxygen diffusion barriers which are modified by ambient pO_2 so that nitrogenase activity can be maintained over a wide range of oxygen levels. This is in marked contrast to the third prokaryote capable of symbiotic nitrogen fixation with higher plants, *Rhizobium*, which is totally dependent on the host plant for oxygen protection of nitrogenase.

9.5 Oxygen diffusion in *Coriaria* nodules.

Oxygen is central to regulation of nitrogenase activity in actinorhizal and legume symbioses. Numerous reports have investigated the effect of above or below atmospheric oxygen levels on actinorhizal (Winship and Tjepkema 1983, 1985; Rosendahl and Huss-Danell 1988; Silvester *et al.* 1988*a,b* ; Silvester and Harris 1989) and legume (Sheehy *et al.* 1983; Witty *et al.* 1983; Hunt *et al.* 1987; Denison and Layzell 1991; Minchin and Witty 1990) associations while other experiments have shown a number of environmental factors may also affect nitrogenase activity e.g. exposure to acetylene or argon, defoliation, application of nitrate. In most cases however these effects can be overridden by modification of the ambient oxygen concentration. In legumes (Witty *et al.* 1983) and in *Coriaria* (section 7.5) for example, the acetylene-induced decline can be relieved by an increase in external pO_2 while in *Myrica* a decrease in oxygen concentration causes a

reduction in the magnitude of the decline (Tjepkema and Schwintzer 1990, 1991). Thus oxygen appears to be central to control of nitrogenase activity.

All legumes have similar nodule structure, and protection of nitrogenase in legume nodules is largely due to a combination of bacteroid respiration, the oxygen carrying and buffering capacity of leghaemoglobin, fixed diffusion resistance provided by the inner cortex (Bergersen 1982) and a variable diffusion resistance mechanism (Sheehy *et al.* 1983). Together these mechanisms reduce atmospheric oxygen concentrations to approximately 1 Pa in the bacteroid zone (Appleby 1969; Tjepkema 1971) and can also help maintain nitrogenase activity in the face of changes in external pO_2. In contrast, work on actinorhizal plants has shown considerable variation in nodule structure between genera, which is reflected in the array of oxygen protection strategies apparent. *Coriaria* has an unusual nodule structure compared with both legumes and other actinorhizal genera but physiologically *Coriaria* is probably more similar to legumes.

Coriaria nodules, in contrast to legumes, are much more aerated as indicated by India ink studies (Silvester and Harris 1989) since they have no fixed diffusion barrier outside the infected zone. Although the suberised periderm layer is impermeable, it does not completely surround the nodule and oxygen may enter the infected tissue through the small gap between the internal periderm and endodermis. Furthermore, no significant concentration of haemoglobin is present in *Coriaria* (Tjepkema and Asa 1987) confirming oxygen diffusion into infected tissue is not restricted. Nodule respiration rates in *Coriaria* do increase in response to elevated oxygen concentrations (Harris 1989) but the increases observed are small. It is unlikely therefore that such increases in respiration rate could be solely responsible for protection of nitrogenase. This implies that some form of diffusion resistance must be present in *Coriaria* nodules.

Infiltration of India ink into the intercellular spaces between infected cells in *Coriaria* nodules suggests the bulk of fixed diffusion resistance must lie at the infected cell wall or

vesicle envelope. Support for the infected cell wall as a diffusion barrier comes from observation of heavy suberisation of infected cell walls in nodules grown at high pO_2 (Harris 1989). Vesicle walls however showed no change in thickness in nodules grown at different oxygen concentrations (Silvester and Harris 1989).

It is important to note that fixed diffusion resistance in nodules is not necessarily due to a cell structure or lack of intercellular spaces but may be provided by the presence of water in intercellular spaces. Alternatively, fixed diffusion resistance may be provided by the presence of glycoprotein in intercellular spaces. Glycoprotein has been localized in intercellular spaces within the inner cortex of soybean nodules using monoclonal antibodies and immunolabelling techniques. More glycoprotein was observed occluding air spaces in the inner cortex of plants grown at 40 kPa O_2 than in plants adapted to lower oxygen concentrations indicating varying the glycoprotein level may be a means of long-term adaptation to pO_2 (James *et al.* 1991). A fixed diffusion barrier in *Coriaria* nodules could be located in the gap between the internal periderm and endodermis if these intercellular spaces are water-filled or are occluded by glycoprotein. However, while speculating on the site of fixed diffusion resistance in *Coriaria* nodules it is important to remember a small component of the diffusion resistance is air-filled as was indicated by N_2 : He work (chapter eight) and that infiltration of India ink into the infected zone (Silvester and Harris 1989) does not lend support for presence of a diffusion barrier in the gap region.

It is interesting to compare the consequences of the fixed diffusion barrier i.e. the inner cortex, in legume nodules with that in *Frankia*. In legumes the fixed diffusion barrier provided by the inner cortex means leghaemoglobin is necessary to facilitate oxygen diffusion into the infected zone. In contrast most actinorhizal nodules do not contain haemoglobin or an alternative oxygen carrier since they have no significant diffusion barrier outside the infected zone. The *Frankia* vesicle however is a nearly complete diffusion barrier, the only "gap" being where the vesicle stalk attaches to the subtending

hyphae i.e. the vesicle pore. Any gas diffusion through this region may be restricted since the vesicle cell is separated from the subtending hyphae by a septum (Fontaine *et al.* 1984) and the thickened lipid envelope extends down the vesicle stalk, particularly in *Frankia* grown at higher oxygen concentrations (section 5.5). In addition, the vesicle pore is constricted (Lancelle *et al.* 1985) to the extent that in septate symbiotic vesicles e.g. in *Alnus* species and members of the Elaeagnaceae, the lipid envelope is considerably thicker around the vesicle stalk than around the vesicle itself and the pore is virtually occluded. Thus the vesicle is effectively isolated from both the external environment and, to a large extent, the vegetative hyphal cells (Berg and Nichol 1990). This situation in vesicles parallels that in legume nodules which means an oxygen-carrying compound, such as leghaemoglobin, may also be required in vesicles. However no such compound has been detected in vesicles *in vitro* or *in vivo*. Despite the presumably large diffusion resistance provided by the vesicle envelope, diffusion of oxygen across this barrier would be expected to be quicker than diffusion across the inner cortex barrier in legume nodules since the distances involved are smaller. The vesicle envelope is 0.1 to 0.2 μm thick (Torrey and Callaham 1982) in contrast to the cortical barrier in legume nodules which is approximately 50 μm thick. Rearrangement of Fick's second law of diffusion, which relates diffusion of molecules over time to distance travelled, shows a diffusion rate is proportional to the square of the length of the diffusion pathway (Nobel 1970). Thus diffusion of oxygen across the vesicle envelope would be considerably faster than diffusion of oxygen into the bacteroid zone of legume nodules and an oxygen-carrier is therefore unnecessary in vesicles.

9.6 Mechanism of variable diffusion resistance

Similarities in the responses of *Coriaria* and legumes to oxygen, acetylene exposure and defoliation have already been noted (Silvester and Harris 1989. chapter seven, chapter eight). Lag phase measurements in *Coriaria* indicated that, like legumes, modification of

nodule diffusion resistance is the main factor responsible for changes in nitrogenase activity observed in response to these environmental parameters. In legumes, variable diffusion resistance allows a constant flux of oxygen across the cortical diffusion barrier and so maintains an optimum pO_2 in the bacteroid zone. By controlling oxygen flux a stable bacteroid respiration rate is maintained which prevents oxygen inactivation of nitrogenase when external pO_2 is increased. Variable diffusion resistance also helps maintain nitrogenase activity when external oxygen concentration becomes limiting (Weisz and Sinclair 1987). Physiological evidence suggests variable diffusion resistance fulfils a similar role in *Coriaria* nodules. However changes in diffusion resistance are not rapid enough to account for oxygen transients shown by *Coriaria* in response to stepwise increases in pO_2 (Silvester and Harris 1989) and these are most likely due to switch-off of nitrogenase (Silvester and Winship 1990) as was discussed in chapter eight. However a variable diffusion resistance mechanism could be involved in re-establishing optimum pO_2 in the infected zone following external pO_2 change.

Functioning of the variable diffusion resistance mechanism in *Coriaria* and legumes is still relatively unknown. In legumes, change in diffusion resistance is considered to be caused by changes in the number or distribution of air spaces in the inner cortex (Witty *et al.* 1986; 1987) although exactly how this may occur is unknown. Since oxygen diffusion through water is 10000 times slower than through a gaseous atmosphere, the mechanism probably involves variations in the length of the water-filled section of the diffusion pathway in nodules. Sheehy *et al.* (1983) proposed the mechanism could operate within narrow water-filled intercellular pores in legume nodules.

The involvement of water in regulating diffusion resistance in nodules is supported by work on the response of legumes to drought stress. Pankhurst and Sprent (1975*a*) observed that, when subjected to drought stress, air spaces in lenticels of soybean nodules closed as cells in the outer cortex lost turgor and collapsed. This presumably caused a decrease in nodule gas permeability (Pankhurst and Sprent 1975*b*). Data of

Weisz *et al.* (1985) later showed drought stress in soybean nodules did in fact cause increased diffusion resistance. Witty *et al.* (1987) microscopically observed disappearance of air-spaces at the inside of the inner cortex and within the infected zone of detached and halved *Pisum* and *Phaseolus* nodules. They report nodules from low-light stressed or wilted plants had few air spaces in these regions and exposure of cut nodules to oxygen increased the rate of air-space disappearance.

Modification of water content in intercellular spaces is a logical mechanism for control of diffusion resistance in *Coriaria* nodules. Lag-phase measurements in drought stressed *Coriaria* plants may be useful in determining the involvement of water in regulation of diffusion resistance. Drought stressed *Coriaria* plants would also be expected to show a less noticeable response to changes in the balance gas from N_2 to He during continuous flow assay if increases in diffusion resistance are due to an increase in the water-content of intercellular spaces.

The source of water to fill intercellular spaces is also unknown. Water filling apoplastic intercellular spaces may originate from the symplast and be associated with changes in the osmotic potential of cells. In legume nodules, most starch is localised in the inner cortex and changes in diffusion resistance in this region may be regulated by an osmotic mechanism involving starch / sucrose interconversion (Hunt *et al.* 1990). Several lines of evidence support a mechanism involving osmotic changes in legume and *Coriaria* nodules. Defoliation, which causes an increase in apparent nodule diffusion resistance in legume (Hartwig *et al.* 1987) and *Coriaria* nodules, has a direct effect on sucrose concentration in nodules. Increases in external oxygen concentration, which are accompanied by increases in nodule diffusion resistance in legumes (Sheehy *et al.* 1983; Witty *et al.* 1983; Hunt *et al.* 1987; Denison and Layzell 1991; Minchin and Witty 1990) and *Coriaria* (Silvester and Harris 1989), affect the adenyl energy charge which in turn exerts control over the sucrose pool within nodules. Hunt *et al.* (1990) showed nodules adapted to high pO_2 displayed a decline in sucrose concentration while pool sizes of 37

other sugars, amino acids and organic acids, did not change significantly. Conversely, low oxygen concentrations inhibit starch formation resulting in increased sucrose levels and a decrease in nodule diffusion resistance. Exposure to argon and acetylene also induces an increase in apparent nodule diffusion resistance in legumes (Witty *et al.* 1984; 1986; Minchin and Witty 1990) and *Coriaria* as indicated by lag-phase measurements. Hunt *et al.* (1990) report exposure to argon was accompanied by decreased nodule osmolarity and a decline in nodule sucrose concentration. Effects of acetylene and argon on sucrose concentration may be mediated by switch-off of nitrogenase assimilation and increase in the size of the ATP pool causing an increase in sucrose to starch conversion i.e. sucrose concentration decreases. A mathematical model devised by Hunt *et al.* (1990) indicated observed changes in sucrose concentration and osmolarity are sufficient to cause redistribution of water in nodules necessary to produce the measured changes in nodule resistance. Although there is some doubt whether changes in sucrose concentration can occur quickly enough within nodules to regulate variable diffusion resistance (Parsons 1989), Fernando *et al.* (1990) showed the sugar concentration within soybean nodules declined significantly within two minutes of an increase in external pO_2 from 20 to 30 kPa.

Parsons (1989) proposes an alternative mechanism for regulation of variable diffusion resistance in soybean nodules in which collapse of thin-walled boundary layer cells adjacent to the inner cortex releases water into intercellular spaces which would effectively increase diffusion resistance in the inner cortex. Although *Coriaria* nodules possess nothing equivalent to either a cortical diffusion boundary or a boundary layer, cells in the lenticel and between the lenticel and internal periderm are relatively thin-walled (Harris 1989). Thus, these cells and, in particular, cells in the gap region, may collapse in response to environmental changes and release water into intercellular spaces. Factors triggering loss of turgor pressure and collapse of cells may involve some form of osmotic mechanism. Sectioning of *Coriaria* nodules under a microscope or exposure of nodule

sections to changes in pO_2 in an oxygen controlled chamber, may allow any changes in cell turgor and resultant filling of air-spaces with water to be detected.

Rather than filling of intercellular spaces with water, regulation in nodule diffusion resistance may be mediated via changes in the volume or shape of cells causing occlusion of air spaces. Parsons (1989) suggests this as an alternative mechanism for the involvement of boundary layer cells in variable diffusion resistance in legumes. Exposure of cells in the boundary layer to increased pO_2 may cause them to actively accumulate ions, take up water and expand. This may be achieved using a mechanism similar to that involved in K+ ion movement in guard cells and subsidary cells which regulates opening of stomata (Zeiger 1983). Modification of stomatal opening generally occurs over time periods of ten-to-50 minutes in response to RH, or two-to-five minutes if induced by changes in CO_2 or a short interval of darkness (Barr 1971). The mechanism operating in guard cells could therefore account for modification of diffusion resistance in legume and *Coriaria* nodules since changes in diffusion resistance occur over a similar time frame (Hunt *et al.* 1987; Silvester and Harris 1989; chapters seven and eight).

9.7 Location of a variable diffusion resistance mechanism

Oxygen flux across diffusion barriers within a nodule is proportional to the concentration gradient between the outside and inside of any one barrier i.e. the greater the difference in pO_2 across a barrier the faster the oxygen diffusion rate. Presumably then a variable diffusion mechanism would be most effective in controlling oxygen flux if it operated across the site of maximum resistance, which in *Coriaria* nodules appears to be the infected cell wall. If both fixed diffusion resistance and the variable diffusion resistance mechanism are located at the infected cell wall, internal pO_2 of nodule tissue outside this barrier would be close to atmospheric. This model is consistent with India ink infiltration

data (Silvester and Harris 1989) and with the absence of haemoglobin in *Coriaria* nodules (Tjepkema and Asa 1987).

Alternatively, resistance at the infected cell wall may be fixed, and variable resistance may instead be located at the gap between the internal periderm and endodermis. The periderm layer is impermeable to gas diffusion, therefore the gap is the only port through which oxygen can diffuse into the infected tissue. The gap would thus provide a convenient site for operation of a variable diffusion resistance mechanism. It is also logical to have a variable diffusion resistance mechanism operating at a point earlier in the diffusion pathway than the fixed diffusion barrier, since variable diffusion resistance would be more effective regulating oxygen flux in response to small changes in external pO_2 than it would be providing the bulk of oxygen protection. In addition, from a structural viewpoint, a variable diffusion mechanism could be more easily operated in the gap region than at the infected cell wall since all oxygen diffusion would be localised in a small zone.

Obviously further information on the oxygen environment within *Coriaria* nodules is required before location of fixed and variable diffusion resistance can be stated with any degree of certainty. Valuable data could be gained from oxygen microelectrode probes of *Coriaria* nodules, a technique which has already been useful in pinpointing the location of diffusion resistance in legume nodules (Tjepkema and Yocum 1974). However this study has shown that a variable diffusion resistance mechanism does in fact exist in *Coriaria* nodules and probably has some similarities to the system operating in legume nodules. It is also possible variable diffusion resistance is involved in oxygen protection strategies of other actinorhizal species and *Datisca,* which has a similar nodule structure to *Coriaria,* may be a likely prospect. In many respects however *Coriaria* is vastly different from all other actinorhizal species and this may be a reflection of its taxonomic isolation. Its nodule morphology and endosymbiont anatomy is unique and this study shows nodule physiology is very different from that of other actinorhizal species studied thus far. In

fact, *Coriaria* nodule function bears greater resemblance to the legume - *Rhizobium* symbiosis than any other actinorhizal association.

9.8 Significance of a variable diffusion resistance mechanism in *Coriaria*

Why certain root nodules have evolved variable diffusion resistance mechanisms and under what environmental conditions these are an advantage, is as much a mystery as the mechanism itself. Obviously presence of variable diffusion resistance gives nodules the potential to counteract complications arising from small variations in oxygen supply which may limit or inhibit nitrogenase activity. Nitrogenase is an expensive enzyme in terms of biological energy required for its synthesis and activity. It is therefore in the organism's interests to protect nitrogenase from oxygen inactivation and to maintain an efficient rate of activity when oxygen is limited. Thus, under certain environmental conditions, which directly or indirectly cause fluctuations in oxygen supply, those nitrogen-fixing plants which have nodules that can adapt their diffusion resistance to external pO_2 may have an advantage over plants which only have fixed diffusion resistance (Sprent 1988).

A variable diffusion resistance mechanism may function to prevent oxygen inactivation of nitrogenase under circumstances where internal pO_2 is increased, for example if photosynthate supply is restricted. When photosynthate is limited, respiration and therefore oxygen utilisation is decreased and, if oxygen diffusion rates cannot be adjusted, oxygen may inactivate nitrogenase. *Coriaria* and legume species however, respond to defoliation and darkness by increasing diffusion resistance in order to maintain oxygen balance within the nodule. Variable diffusion resistance therefore is an advantage to perennial forage species as well as allowing plants to maintain active nitrogenase during darkness.

Alternatively variable diffusion resistance in *Coriaria* nodules could be an adaptation to maintain nitrogenase activity when flooding causes a reduction in oxygen level around the root system. By decreasing nodule diffusion resistance, nitrogenase would not become oxygen limited. Maintenance of respiratory activity to support nitrogenase is essential since limitation of ATP synthesis leads to a build-up of ADP, the product of utilisation of ATP by nitrogenase. The resultant imbalance in adenyl energy charge and high level of ADP can cause inhibition of nitrogenase (Layzell *et al.* 1990). Although *Coriaria* is not a bog plant, it often grows as a pioneer species along river banks and in disturbed sites which may be prone to flooding.

Drought affects legume nitrogen fixation by reducing oxygen diffusion and supplies of photosynthate from the shoot (Sprent 1988). Thus a variable diffusion resistance mechanism provides protection for nitrogenase when respiration is limited by drought. It is possible *Coriaria* uses its variable diffusion resistance mechanism to overcome similar problems although the effect of drought on *Coriaria* is not yet known.

Variable diffusion resistance in *Coriaria* nodules may also be an adaptation to protect nitrogenase and maintain activity in environments where temperature is variable. Winship and Tjepkema (1983) showed *Alnus* recovery from low temperature is much more rapid than from inhibition due to direct oxygen shock and therefore *Alnus* may possess a diffusion barrier with a permeability that is affected by temperature. Thus at low temperatures, which caused lowered respiratory protection, diffusion resistance may be greater.

Regulation of nodule diffusion resistance and maintenance of a low oxygen concentration within nodules may also be necessary for continued synthesis of the enzyme. If expression of *nif* genes in *Frankia* is regulated by an oxygen-sensitive *nif A* gene, as in *Rhizobium*, then expression of *nif A* and resultant synthesis of nitrogenase would be dependent on a low oxygen environment. Unless nodule diffusion resistance could be

modified to buffer small increases in external pO_2, nitrogenase synthesis in *Coriaria* may be inhibited.

Variable diffusion resistance may not be concerned with protection of nitrogenase but rather be a mechanism to control the rate of nitrogenase activity. For example, increased resistance would limit oxygen supply and ATP production and so cause a decrease in nitrogen fixation. Conversely, by relaxing diffusion, ATP production would no longer be oxygen-limited so allowing increased nitrogenase activity but still maintaining internal pO_2 low enough to prevent damage to the enzyme itself. Thus a variable diffusion resistance mechanism could function to either increase nitrogenase activity under favourable conditions or to decrease activity when environmental conditions are unfavourable or when an alternative source of nitrogen is readily available.

9.9 Future work

Probably the ultimate goal of nitrogen fixation research is to induce nitrogen-fixing symbioses in plants which are presently incapable of association with a diazotrophic prokaryote. *Frankia*'s unique intrinsic oxygen protection mechanism means it is comparatively easy to manipulate and its ability to form associations with a taxonomically wide range of plants suggests it is a promising candidate for introduction into other plants. Work is already underway on the potential of *Betula*, a close relative of *Alnus*, as a recipient for host genes and host gene modifiers involved in the symbiosis with *Frankia* (Seguin and Lalonde 1988). Obviously, recent research on morphological, physiological and genetical aspects of *Frankia* symbioses has provided a sound basis for future investigation in this field.

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