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Communication

Frankia Vesicles Provide Inducible and Absolute Oxygen Protection for Nitrogenase[1]

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ABSTRACT

When *Frankia* HFPCcI3 was grown in culture at oxygen O_2 levels ranging from 2 to 70 kilopascals O_2, under nitrogen fixing conditions, nitrogenase activity adapted to ambient pO_2 and showed a marked optimum close to growth pO_2. Vesicles were thin walled at low pO_2 and very thick walled at high pO_2. Freeze fracture transmission electron microscopy confirmed that *Frankia* produces vesicles with outer walls thickened by multiple lipid-like monolayers, in proportion to ambient pO_2.

Nitrogenase is extremely O_2 sensitive and the majority of nitrogen fixing organisms exist in ecological or symbiotic situations of reduced ambient pO_2 (5, 16). *Frankia* occurs as the nodule symbiont of actinorhizal plants, such as *Alnus*, *Myrica*, and *Casuarina*, and the response of nitrogenase to O_2 in these nodules contrasts strongly with that of legume nodules which contain *Rhizobium* (18). In general, *Frankia* nodules show a nitrogenase optimum at pO_2 levels close to atmospheric pO_2 and the nodules are well ventilated into the infected cell area (18). Legume nodules, on the other hand, often display increased nitrogenase activity at elevated pO_2 and the nodule structure provides a significant barrier to O_2 diffusion (1, 18, 21).

A further contrast between *Rhizobium* and *Frankia* is found in their response to O_2 in pure culture. Rhizobium is able to fix nitrogen *in vitro* only under conditions of very low O_2 (1, 16, 17), while *Frankia* in culture appears to be relatively insensitive to O_2 and optima for nitrogenase have been recorded at near atmospheric levels (11, 20).

The apparent key to the difference between *Frankia* and *Rhizobium* is the presence of thick walled vesicles in the former, both in culture and in symbiosis (20, 22). The vesicle is the presumptive site of nitrogenase activity and by analogy with the heterocyst of the cyanobacteria (6, 7, 13) the thick walled vesicle is considered to be the O_2 diffusion barrier.

Torrey and Callaham (22) showed that the outer wall of vesicles is a multilaminated structure with properties similar to many lipid monolayers. More recently Murry *et al.* (14) have demonstrated that nitrogenese activity in *Frankia* can adapt to a range of subatmospheric O_2 levels but the site of O_2 protection in *Frankia*, while assumed to reside in the thickened wall, has not been closely investigated. We therefore investigated the ability of *Frankia* to adapt to a much wider range of O_2 tensions and studied more closely the site of O_2 protection.

MATERIALS AND METHODS

Culture Conditions. *Frankia* strain HFPCcI3, isolated from root nodules of *Casuarina cunninghamiana* (23) was incubated in various pO_2 atmospheres in 1.1 L serum capped bottles containing 100 ml of nitrogenfree BAP medium (12) (10 mM Na propionate as carbon source). *Frankia* stock culture was added to the bottles aseptically, bottles capped with a rubber septum and flushed with appropriate sterile gas mixtures for 6 min at 2 L/min. Bottles were continuously shaken at 90 rpm on an orbital shaker at 30°C.

Gas Mixtures. Gas mixtures were made up by way of Tylan mass flow controllers in which O_2, N_2 and CO_2 were combined to give mixtures containing 2, 16, and 70 kPa O_2, with CO_2 at 0.2 kPa to facilitate propionate utilization, and the balance of N_2. These were distributed via a sterilized 0.22 μm filter membrane to the various bottles. O_2 electrode analysis of sample bottles confirmed that at the stirring rate provided the dissolved O_2 tension was about 10, 80, and 330% of air saturation, respectively.

Nitrogenase Assay. Nitrogenase activity was measured by acetylene reduction using 10 kPa acetylene in the gas phase and assaying for ethylene. Ethylene was detected using a Carle 9500 gas chromatograph fitted with a 1.2 m Poropak T, 60/80 mesh, column, run at 75°C. Assays were conducted in a continuous flow cuvette based on the open flow system described by Minchin *et al.* (9). The cuvette consisted of a 7 ml glass vial fitted with a rubber septum stopper, such that the stopper could freely slide up and down the tube to make a variable volume. In normal operation the cuvette volume was set at 5 ml with a 4 ml suspension of bacteria and a 1 ml gas phase above. Input gas was introduced to the bottom of the vial via a 25 gauge hypodermic needle and exited via a similar needle in the gas space and thence into a fine rubber tube, from which gas samples could be removed. The starting assay gas mixture for all assays contained 0.4 kPa O_2, 0.1 kPa CO_2, and 10 kPa acetylene, the balance of N_2. This mixture was pumped continuously by peristaltic pump, through the thermostated cuvette at 7 ml min^{-1}, such that the gas bubbled through the culture and exited via a tube where 0.1 ml samples were collected for analysis of O_2 and ethylene. In

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normal operation, a 4 ml aliquot of concentrated *Frankia* culture was transferred in a N_2 flushed syringe into the cuvette and analyses of exit gas started immediately. O_2 concentration was then increased stepwise by addition of an O_2/acetylene mixture into the input gas reservoir. O_2 concentration was measured on 0.1 ml samples injected into a Carle 8500 gas chromatograph fitted with a 1.2 m column of molecular sieve 5A.

Microscopy. Vesicles were viewed under dark field microscopy using a Reichert-Jung Polyvar photomicroscope. Vesicle brightness and apparent wall thickness was estimated on an arbitrary 0 to 5 scale in which 0 was a vesicle with wall brightness (thickness) equal to hyphae, and 5 was a vesicle in which wall brightness (thickness) is equivalent to 0.3 diameters (Fig. 2). Up to 100 vesicles were compared to a prepared visual scale and scored for apparent thickness.

Freeze fracture specimens were frozen in suspension and fractured under liquid N_2 in a pre-nicked plastic tube (2) and subsequently replicated in a cold block freeze fracture apparatus (3). Replicas were cleaned in chromic acid and photographed in a Philips EM301 transmission electron microscope.

RESULTS

Nitrogenase Activity. When *Frankia* CcI3 is grown at the wide range of pO_2 chosen (2, 16, and 70 kPa O_2) in the absence of combined N_2, significant growth occurs on all levels although growth was significantly better at the intermediate O_2 level. Aliquots of the main culture were tested for nitrogenase activity over a range of O_2 tensions and confirm that nitrogenase is present at all pO_2 levels and show the adaptation of nitrogenase activity to the ambient O_2 of the culture (Fig. 1). When grown at 2 kPa O_2 nitrogenase is active over a very narrow range of O_2 tensions being completely and irreversibly inactivated by levels above 3.5 kPa O_2 in the assay mixture. When grown at 70 kPa O_2 (3.5 times atmospheric) the culture shows a broad tolerance to O_2 in the range 2 to 85 kPa O_2. It is notable that maximum specific activity of nitrogenase (based on total cell protein) is similar across this wide range of O_2 levels.

Vesicle Structure. From analogy with the heterocyst, O_2 protection in *Frankia* has been described as a passive barrier localized in the vesicle wall (11). If such a barrier exists, the marked change in resistance that is implied by the changing pO_2 optimum of nitrogenase activity as shown in Figure 1 must be accompanied by vast changes in vesicle wall properties.

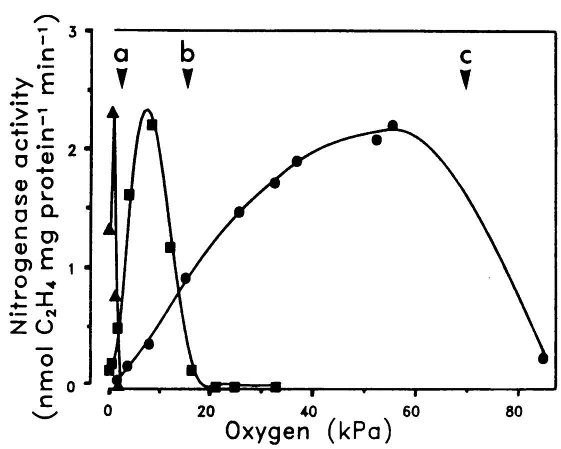

FIG. 1. Effect of pO_2 on nitrogenase activity in *Frankia* grown at three O_2 levels. *Frankia* was grown at 2, 16, and 70 kPa O_2 (dO_2 10, 80, and 330% of air saturation) and samples assayed for nitrogenase at various pO_2 levels starting from 0.4 kPa O_2. O_2 response curves of nitrogenase for cultures grown at 2 kPa (▲), 16 kPa (■), and 70 kPa (●) are presented. The arrows marked a, b, and c indicate the pO_2 level at which the culture was grown.

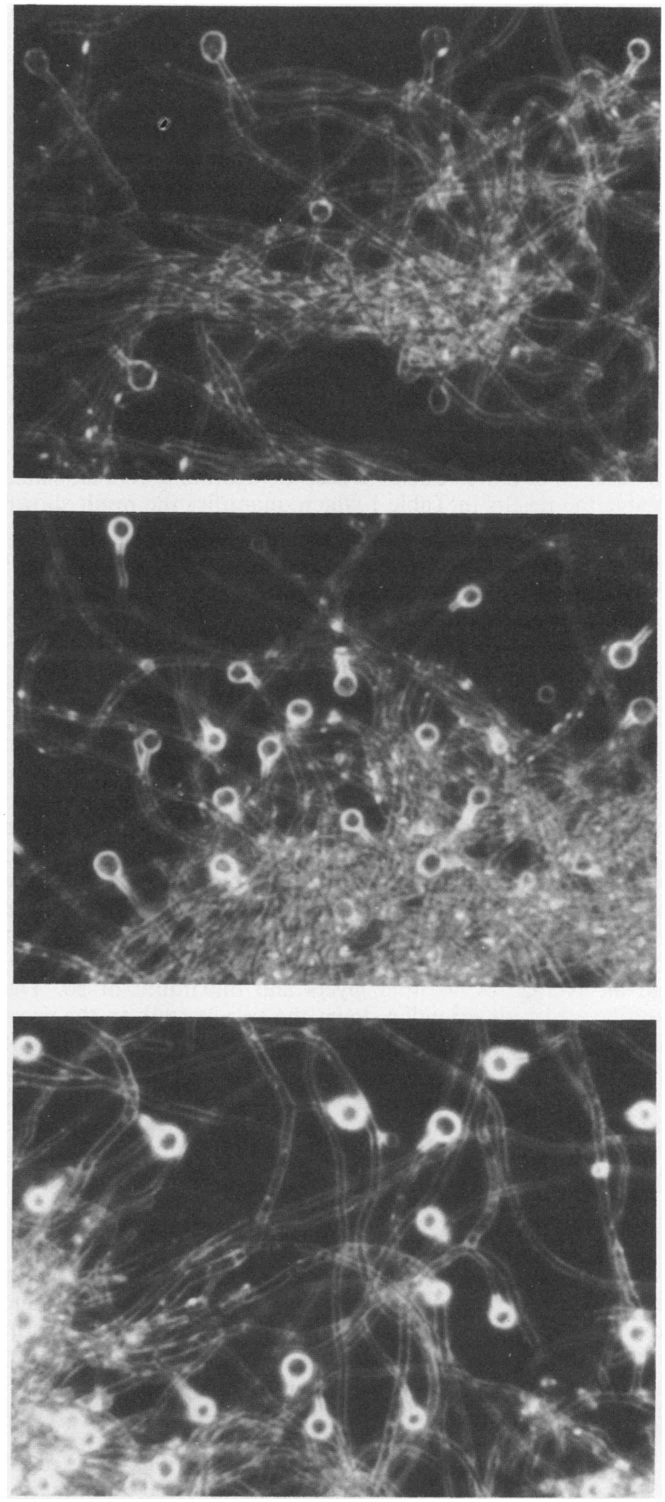

FIG. 2. Dark field photomicrographs of *Frankia* vesicles and hyphae grown at various O_2 levels. Differences are clear in vesicle brightness due to increase in wall thickness in cultures grown at 2 kPa O_2 (top), 16 kPa O_2 (middle), and 70 kPa O_2 (bottom). Vesicles are about 3 μm diameter.

Frankia vesicles are 2.5 to 3.5 μm in diameter and the birefringence produced by the structured walls in polarized light has been noted by Torrey and Callaham (22). This light effect is attributed to the structural layering of the vesicle envelope which was confirmed by freeze fracture EM (22). We observed a similar effect but found the vesicles appeared particularly bright under dark field microscopy. Vesicles from cultures grown at low O_2

Table I. *Apparent Wall Thickness of Frankia Vesicles when Grown at Various pO_2 Levels*

pO_2	Apparent Thickness	SE
kPa	*arbitrary units*	
2.0	1.38	0.06
16	1.75	0.09
70	3.17	0.11

are marginally brighter than associated filaments while at high O_2 the vesicles are dramatically bright with the effect extending back down the vesicle stalk to the cross wall (Fig. 2). The optical effects produced in dark field microscopy result from reflection of light from a lateral source and while the bright image produced may not accurately define the wall thickness, there is good reason to believe that it is at least proportional to wall thickness. A relative scale of apparent thickness was prepared and measurements of 80 or more vesicles in each O_2 treatment were tabulated to give the results in Table I which quantifies the result shown in Figure 2. Similar results were observed under both Nomarski interference and phase contrast optics but were most clear under dark field.

The vesicle wall of *Frankia* has been previously studied by freeze fracture (22) and freeze substitution TEM (8) and shown to include an outer multilayered wall which extends down the stalk of the vesicle. The layers appear as lipid monolayers, and on fracture face views up to 15 layers have been recorded (22) each with an estimated thickness of 3.5 to 4.0 nm or a total thickness of 60 nm. Freeze fracture images of our cultures were obtained and, while only a small number of fractures exposed the full thickness of the wall, they did confirm the result shown by dark field microscopy (Fig. 3). Cultures at 4 kPa O_2 had an average of 17 layers in the outer wall and in no case were more than 20 layers seen. Cultures grown at 40 kPa O_2 showed the very dramatic layering first observed by Torrey and Callaham (22) in cultures grown in air. Five fractures of vesicles at high O_2 had an average of 40 lipid layers and maximum of 50. The multilayered wall extended down the vesicle stalk to the cross wall and while no particular study was made of the cross wall it appeared to be unthickened. The freeze fracture results are based on a small number of measurements which completely exposed the wall, but despite this they confirm the increasing thickness of the wall as seen in dark field and identifies this change as an increase in outer envelope layers.

DISCUSSION

It has been stated (6) that "heterocystous cyanobacteria thus seem to be the only diazotrophs capable of genuinely aerobic growth in the dark under nitrogen fixing conditions." The term 'aerobic' in this context presumably means air saturated and in the light of both previous reports (11, 20) and the results presented here it is evident that *Frankia* must be added to this group of uniquely O_2 adaptive diazotrophs.

The adaptation of nitrogenase activity to various levels of O_2 below atmospheric has been noted before (14) and a number of workers have assumed that O_2 protection is a property of the thick vesicle wall. A remarkable feature of the system is the enormous range of pO_2 over which it operates and the inducible nature of the wall thickening solely in response to O_2 tension and provides more direct evidence that the vesicle wall is indeed the O_2 diffusion barrier in *Frankia*. Work in progress indicates that *Frankia* adapts rapidly to elevated O_2 shock by thickening the walls of both new and existing vesicles. The process takes 16 to 20 h during which synthesis of new nitrogenase enzyme and wall thickening occur.

The results presented here provide a clear note of warning to those working with nitrogenase in *Frankia*: In static cultures

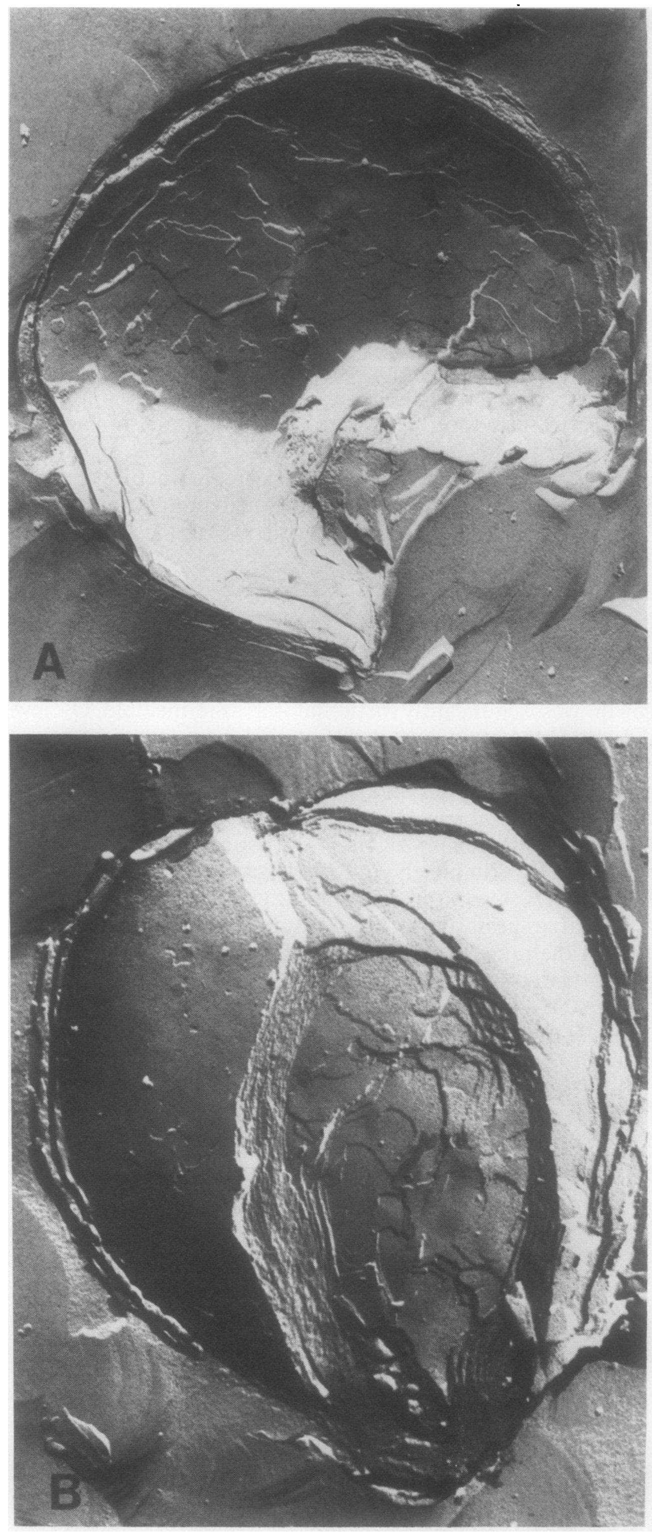

FIG. 3. Freeze fracture TEM micrographs of *Frankia* vesicles grown at 4 kPa O_2 (top) and 40 kPa O_2 (bottom). Vesicles are about 3 μm diameter.

Frankia rapidly and progressively adapts to the lowered pO_2 produced by respiration coupled with the high diffusion resistance of the unstirred water. We have found that disturbance of such cultures results in significant loss of nitrogenase as a result of higher pO_2 affecting the unadapted culture. Even with stirred cultures, and more so with static cultures, assays of nitrogenase need to be conducted at several O_2 tensions to establish maximum activity. This is especially true at low pO_2 where the O_2 profile of nitrogenase is particularly narrow (Fig. 1).

The comparison between cyanobacterial heterocysts and *Frankia* vesicles is striking. The work reported here combined with the earlier report (14) show a marked similarity to inducible O_2 adaptation in heterocystous cyanobacteria (4, 13, 15). Both cells have a multilayered wall which in the cyanobacteria is poorly developed at low O_2 tension (7).

O_2 protection provided by the multilayered wall of the *Frankia* vesicle provides a uniquely adaptable mechanism which allows the organism to function over the widest possible range of pO_2 levels, and up to at least three times atmospheric. Whereas in legume nodules it appears that the nodule structure (21) and presence of hemoglobin (1) are absolute requirements for the O_2 environment of *Rhizobium* bacteroids, the actinorhizal nodule is relatively aerated (18) and hemoglobin, while present in some cases, is not essential for nitrogenase activity (19). These results confirm that *Frankia* is unique among heterotrophic diazotrophs in possessing an intrinsic O_2 protection mechanism, able to adapt to an enormous range of O_2 tension and yet maintain a high specific activity of nitrogenase. Minchin (10) has recently reaffirmed the importance of this property of independence from external O_2 in considering the possibility of generating unique symbiotic systems and points to the cyanobacteria as amenable subjects for a leaf based system. Among root nodule systems *Frankia* appears to have properties that make it similarly suitable for consideration in unique symbioses. In comparison with *Rhizobium*, which is restricted essentially to one group of angiosperms and is totally dependent on nodule structure for O_2 protection, *Frankia* forms effective symbioses with a taxonomically diverse range of angiosperms and is able to control its own internal O_2 supply. We believe that these two properties make *Frankia* a prime subject for unique symbiotic systems.

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LITERATURE CITED

1. Bergersen FJ 1982 Root Nodules of Legumes: Structure and Functions. Research Studies Press, Chichester
2. Bullivant S, P Metcalf, KP Warne 1979 Fine structure of yeast plasma membrane after freeze-fracturing in a simple shielded device. *In* JE Rash, CS Hudson, eds, Freeze-Fracture: Methods, Artifacts and Interpretations. Raven Press, New York, pp 141–147
3. Bullivant S 1973 Freeze-etching and free fracturing. *In* JK Koehler, ed, Advanced Techniques in Biological Electron Microscopy. Springer-Verlag, Berlin, pp 67–112
4. Dromgoole FI, WB Silvester, BJ Hicks 1978 Nitrogenase activity associated with *Codium* species from New Zealand marine habitats. NZ J Mar Freshw Res 12: 17–22
5. Gallon JR 1981 The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms. Trends Biochem Sci 6: 19–23
6. Jensen BB, RP Cox 1983 Effect of oxygen concentration on dark nitrogen fixation and respiration in cyanobacteria. Arch Microbiol 135: 287–292
7. Kulasooriya SA, NJ Lang, P Fay 1972 The heterocysts of blue-green algae. III. Differentiation and nitrogenase activity. Proc Roy Soc Lond Ser B 181: 199–209
8. Lancelle SA, JG Torrey, PK Hepler, DA Callaham 1985 Ultrastructure of freeze-substituted *Frankia* strain HFPCc13, the actinomycete isolated from root nodules of *Casuarina cunninghamiana*. Protoplasma 127: 64–72
9. Minchin FR, JF Witty, JE Sheehy, M Muller 1983 A major error in the acetylene induction assay: decreases in nodular activity under assay conditions. J Exp Bot 34: 64–69
10. Minchin F 1986 Gaseous diffusion in cyanobacterial heterocysts. Nature 320: 483–484
11. Murry MA, MS Fontaine, JD Tjepkema 1984 Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3. Arch Microbiol 139: 162–166
12. Murry MA, MS Fontaine, JG Torrey 1984 Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI3 grown in batch culture. Plant Soil 78: 61–78
13. Murry MA, AJ Horne, JR Benemann 1984 Physiological studies of oxygen protection mechanisms in the heterocysts of *Anabaena cylindrica*. Appl Environ Microbiol 47: 449–454
14. Murry MA, Z Zhang, JG Torrey 1985 Effect of O_2 on vesicle formation, acetylene reduction, and O_2-uptake kinetics in *Frankia* sp. HFPCc13 isolated from *Casuarina cunninghamiana*. Can J Microbiol 31: 804–809
15. Pienkos PT, S Bodmer, FR Tabita 1983 Oxygen inactivation and recovery of nitrogenase activity in Cyanobacteria. J Bacteriol 153: 182–190.
16. Robson RL, JR Postgate 1980 Oxygen and hydrogen in biological nitrogen fixation. Annu Rev Microbiol 34: 183–207
17. Shaw BD 1984 Oxygen control mechanisms in nitrogen-fixing systems. *In* NS Subba Rao, ed, Current Developments in Biological Nitrogen Fixation. Edward Arnold, London, pp 111–134
18. Tjepkema JD 1979 Oxygen relations in leguminous and actinorhizal nodules. *In* JC Gordon, CT Wheeler, DA Perry, eds, Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Oregon State University, Corvallis, OR, pp 175–186
19. Tjepkema JD 1982 Hemoglobins in the nitrogen-fixing root nodules of actinorhizal plants. Can J Bot 61: 2924–2929
20. Tjepkema JD, W Ormerod, JG Torrey 1980 Vesicle formation and acetylene reduction activity in *Frankia* sp. CPI1 cultured in defined media. Nature 287: 633–635
21. Tjepkema JD, CS Yocum 1974 Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. Planta 119: 351–360
22. Torrey JG, D Callaham 1982 Structural features of the vesicle of *Frankia* sp. CpI1 in culture. Can J Microbiol 28: 749–757
23. Zhang Z, MF Lopez, JG Torrey 1984 A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. Plant Soil 78: 79–90