

# Biological and Cultural Characteristics of the Effective *Frankia* Strain HFPCcI3 (Actinomycetales) from *Casuarina cunninghamiana* (Casuarinaceae)

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Accepted: 28 February 1985

## ABSTRACT

From homogenates prepared from surface-sterilized nodules of seedlings of *Casuarina cunninghamiana* grown aeroponically, a strain of *Frankia* designated HFPCcI3 was isolated and has been grown in pure filamentous culture in a defined synthetic nutrient medium. Vesicle and sporangium formation can be induced by removal of combined nitrogen from the medium. *Frankia* strain HFPCcI3 nodulates young seedlings of *C. cunninghamiana* and *C. equisetifolia* within three weeks of inoculation with an optimum root medium pH of 6–7 for nodulation and optimum temperature of 30–35 °C. The presence of combined nitrogen in the root medium inhibits nodulation with  $\text{NH}_4^+$  more inhibitory than  $\text{NO}_3^-$ . *Frankia* HFPCcI3 does not nodulate *Allocasuarina* species within the same family nor several other possible actinorhizal plants tested. Thus this strain is quite precise in its host specificity. The rate of acetylene reduction was greater in *C. cunninghamiana* than the closely related species *C. equisetifolia*. In neither of these host species were vesicles observed to occur within the infected root nodules which had been demonstrated to be actively fixing dinitrogen. Root nodules were shown to be active in acetylene reduction over a range of  $\text{O}_2$  concentration in the gaseous environment with an optimum at about 20 per cent  $\text{O}_2$ , the ambient  $P_{\text{O}_2}$  of the air. The mechanism(s) for oxygen protection of nitrogenase within the filamentous form of *Frankia* within these nodules remains to be explained.

Key words: *Casuarina*, *Frankia*, nodulation, nitrogen fixation.

## INTRODUCTION

The woody dicotyledonous family Casuarinaceae comprises trees and shrubs subdivided into four genera and nearly seventy species (Johnson, 1982). The group had its origins in Australia and the South Pacific Islands. The largest genus *Allocasuarina* occurs largely in Western and South Australia. The most familiar genus *Casuarina* is wide-spread throughout the tropics and sub-tropics of the world, having been disseminated by man for multiple uses. *Casuarina equisetifolia* may well become one of man's most useful fuel trees in the tropics (Midgley, Turnbull and Johnson, 1983).

The family is actinorhizal with roots nodulated by the filamentous soil bacterium *Frankia* of the Actinomycetales. The root nodules are sites of symbiotic dinitrogen fixation and are one of the features which allow Casuarinas to establish quickly and develop vigorously in poor sites with depauperate soils. Nodulation depends upon the presence of *Frankia* in the soil around the roots. Plants introduced into exotic locations or seedlings started in sterile or artificial soils, if they are to form nitrogen-fixing nodules, must be inoculated with nodule suspensions from effective plants or with suspensions of *Frankia* grown in pure culture after isolation and cultivation from the appropriate host strain.

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Because of the existing and potential importance of Casuarinas in tropical agriculture and forestry, efforts have been made in recent years to isolate and culture *Frankia* strains from root nodules of different genera and species in the family Casuarinaceae. Gauthier, Diem and Dommergues (1981) were the first to report the successful culture of *Frankia* strains from *Casuarina* root nodules. These strains were shown to fix dinitrogen in culture but failed to nodulate roots of seedlings of *Casuarina*. Unexpectedly, they nodulated seedlings of *Hippophaë*, a member of the quite unrelated family, Elaeagnaceae. More recently, Diem, Gauthier and Dommergues (1983*b*) reported the isolation of an effective strain of *Frankia* designated by them ORS1106 (formerly CJ1-82) from root nodules of *Casuarina junghuhniana*.

An effective strain of *Frankia* has been isolated in our laboratory from root nodules of *Casuarina cunninghamiana* plants grown aeroponically in our greenhouse. A brief report of this isolation has been made (Zhang, Lopez and Torrey, 1984). We report here an account of the behaviour of the new effective isolate, designated by us HFPCcI3 (Catalog No. HFP020203) in culture and the results of trials on seedlings of its normal host and other actinorhizal plants.

## MATERIALS AND METHODS

### *Isolation*

The method of isolation of HFPCcI3 was described by Zhang *et al.* (1984). Surface-sterilized nodules of *Casuarina cunninghamiana* were first inoculated in a nutrient broth to assure freedom from contamination and then individual nodule lobes were homogenized in a small volume of QMOD medium (Lalonde and Calvert, 1979), sealed in a flask and placed in a shaker. Filamentous outgrowth occurred in several weeks, the culture was rehomogenized and finally transferred to a synthetic defined nutrient medium referred to as BAP (Murry, Fontaine and Torrey, 1984).

### *Microscopic observations*

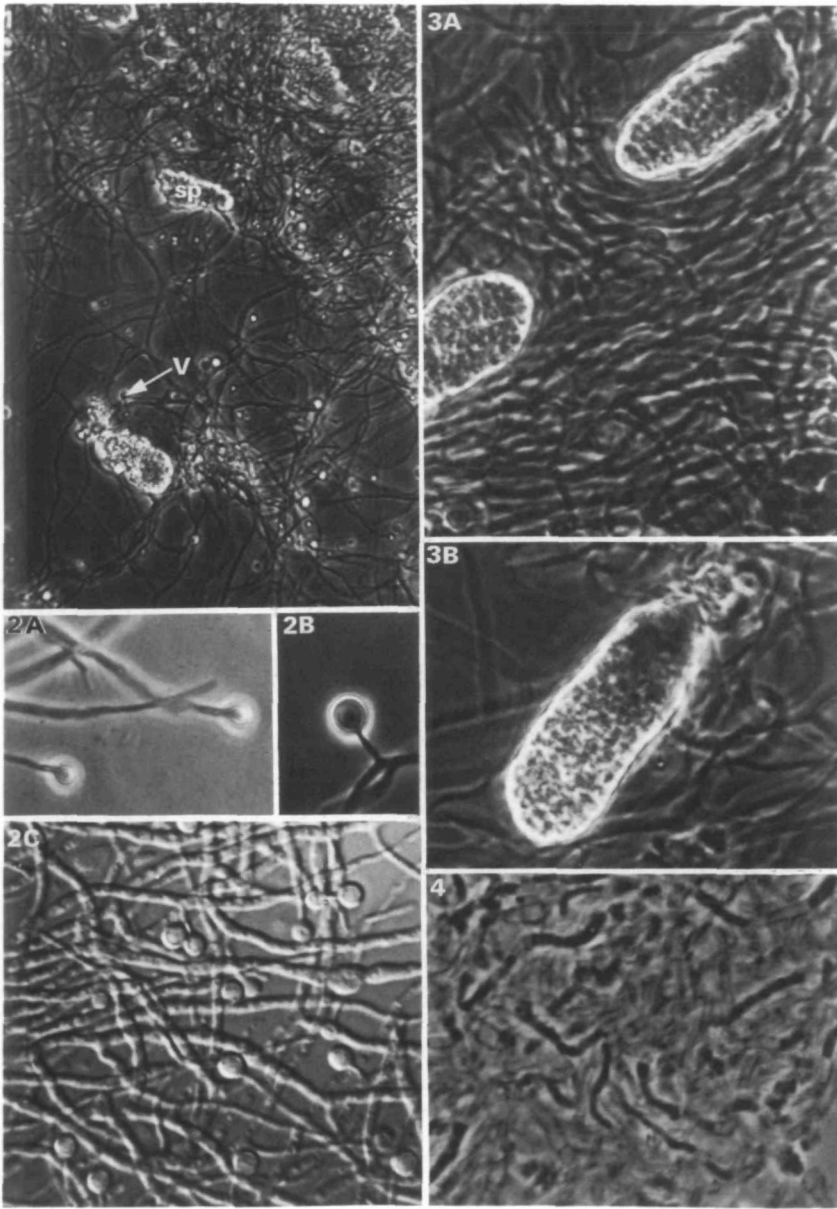
Living cultures of *Frankia* were pipetted onto glass slides and observed with a Zeiss compound research microscope equipped with phase or Nomarski phase-interference optics. To study the endophyte in the nodules, fresh root nodule lobes were fixed in acetic acid: absolute ethyl alcohol (1:3) for less than 24 h then transferred to 70 per cent ethanol. *Frankia* filamentous clusters were teased from infected cells with a needle, washed, pipetted onto a cover slip, squashed, stained with Azure B and mounted on a slide with Permount (Torrey, 1976).

### *Nitrogenase assays of nodules*

Assays for nitrogenase activity of whole root systems, excised nodules or aerobically grown cultures made use of gas chromatographic measurement of acetylene reduction (Burris, 1974). Excised nodules were placed in a serum vial, a moistened piece of filter paper was added and the vial filled with 10 per cent acetylene. The vials were incubated at 28 °C on a shaker for 30 min and the gas sampled by injection into the gas chromatograph. After the assay, nodules were weighed. Ethylene production from acetylene was measured with a Carle 9500 FID gas chromatograph fitted with a porapak R column (1.4 m) at 80 °C.

### *Measurement of the effect of O<sub>2</sub> on acetylene reduction in nodules*

Acetylene reduction was measured in single samples of detached weighed nodules in 130 ml serum bottles incubated at 28 °C. The nodules in the bottle were flushed with N<sub>2</sub>,



FIGS 1-4. Photomicrographs of *Frankia* HFPCc13 from living cultures. Fig. 1. Low-power photomicrograph using phase optics of culture of HFPCc13 grown on B medium, showing branched filaments, sporangia (sp), vesicles (v) and a few released phase-bright spores. Fig. 2. Vesicles formed in cultures of *Frankia*. A, Enlarged view of vesicles observed with Normarski optics. Note the vesicle stem attachment which terminates at a septum of the filament.  $\times 1040$ . B, High-power view of a vesicle observed with phase contrast. Note the halo produced by the vesicle envelope.  $\times 1040$ . C, A population of vesicles produced in B medium. Normarski optics.  $\times 1040$ . Fig. 3. Enlarged view of filaments, sporangium and vesicles of HFPCc13 cultured on B medium. Phase optics.  $\times 1040$ . Fig. 4. Culture of *Frankia* HFPCc13 grown in standing culture of QMOD medium, showing abnormal morphology associated with age and unfavourable conditions. Phase optics.  $\times 1040$ .

then O<sub>2</sub> was added to the concentration to be tested over the range of 0–40 per cent O<sub>2</sub> and acetylene added to a final concentration of 10 per cent (v/v). Ethylene concentrations were measured at each O<sub>2</sub> concentration at 5 min intervals for 20 min before adjusting the O<sub>2</sub> tension. Measurements were made in sequence from 0 to 40 per cent O<sub>2</sub>. In separate experiments with standard conditions, acetylene reduction remained linear for at least 3.5–4 h.

#### *Tests for infectivity*

Host plants to be treated for susceptibility to infection by cultured strains of *Frankia* were started from seeds sown in moist sand in small plastic flats watered with  $\frac{1}{4}$ -strength Hoagland's nutrient solution and grown in a growth chamber with mixed fluorescent and incandescent lamps on a long-day cycle of 16 h light and 8 h dark at temperatures of 33–28 °C respectively or other temperature cycles as noted. After four weeks, seedlings were transplanted to an aeroponic tank (Zobel, Del Tredici and Torrey, 1976), to water-culture in jars, or to sand culture in pots or Rootainers (Spencer-Lemaire Industries, Ltd., Edmonton, Alberta, Canada) and were provided  $\frac{1}{4}$ -strength Hoagland's solution lacking combined nitrogen (Hoagland and Arnon, 1950). Seedlings were inoculated with measured samples of homogenized two to four week old cultures of *Frankia* strains grown in BAP medium or with ground nodule suspensions from nodulated plants in the greenhouse. Control plants were not inoculated. Observations of nodule formation were made periodically and acetylene reduction assays conducted at the end of the experiment.

## RESULTS

#### *Morphological observations in culture*

*Frankia* strain HFPCc13 isolated from root nodules of *Casuarina cunninghamiana* and grown in liquid medium meets the morphological criteria characteristic of other *Frankia* strains already reported. Vegetative growth is filamentous with branching and occasional septations (Fig. 1). Mycelial mats appear white or colourless and no pigments are formed in the media tested. Filament diameters average around 1  $\mu\text{m}$  with occasional thickenings of the filaments to 2  $\mu\text{m}$ . Variation from this filamentous appearance depends on the components of the medium and the age of the culture.

When cultured in BAP medium (Murry *et al.*, 1984) which contains Na pyruvate at 10 mM and NH<sub>4</sub> Cl at 5 mM, only filamentous growth of HFPCc13 occurs in early periods of growth. When transferred to a similar medium which lacks combined nitrogen (medium B, Murry *et al.*, 1984), one observes vesicle formation occurring within a few days and progressing in increasing numbers over the culture period (Fig. 2A–C). The vesicles occur as spherical enlargements at the terminal endings of lateral branches or stalks. Typically, the vesicle stalks are about equal in length to the vesicle diameter which varies from 3 to 5  $\mu\text{m}$ ; occasionally the vesicles occur at the end of long unbranched filaments. Under Nomarski optics, the vesicles show the usual wide halo attributable to the presence of a vesicle envelope (Torrey and Callahan, 1982) and show internal differentiation with septations and particulate bodies evident. The stem attachment which extends from the spherical vesicle to the first septum of the hypha to which the vesicle is attached also shows the special optical characteristics of the envelope, which is lacking along the length of the vegetative hyphae. After vesicle induction occurs in B medium, as many as 10<sup>8</sup> vesicles/mg protein were observed. Such cultures showed substantial acetylene reduction activity (Murry, Zhang and Torrey, 1985) which was associated with the presence of vesicles.

Usually in B medium HFPCcI3 shows abundant sporangial formation, concomitant with vesicle formation (Fig. 3). Although it is apparent that lack of nitrogen triggers vesicle formation in culture, we are not yet certain what changes in cultural conditions trigger sporulation. In older cultures of BAP medium, presumably when nutrients begin to be exhausted, both vesicle and sporangial formation occur.

TABLE 1. Nodulation trials on seedlings of species of Casuarinaceae inoculated with suspensions of *Frankia* HFPCcI3

Host species	Environmental conditions	Duration of experiment (weeks)	Cultural method	Inoculation treatment	Seedlings in test (n)	Seedling nodules (n)
<i>C. cunninghamiana</i>	Growth chamber (16 h day 35 °C; 8 h night 28 °C)	4	Sand	+	8	8
		4	Sand	0	4	0
<i>C. equisetifolia</i>	Greenhouse (16 h day 32 °C; 8 h night 19 °C)	3	Water	+	6	6
		3	Water	0	3	0
<i>C. glauca</i>	Greenhouse (16 h day 32 °C; 8 h night 19 °C)	4	Water	+	9	9
		4	Water	0	3	0
<i>Allocasuarina lehmaniana</i>	Growth chamber (16 h day 24 °C; 8 h night 19 °C)	10	Water	+	9	0
		10	Water	0	3	0

Sporangia vary in size from early developmental stages of just sub-divided filaments to elaborate, swollen spore-filled structures up to 60  $\mu\text{m}$  in length. Under phase optics the mature spores are phase bright and the filaments dark. When photographed under Nomarski phase-interference optics, sporangia show the typical basipetal maturation of spores with internal differentiation and the development of phase brightness occurring first at the distal end of the sporangium. Spontaneous spore release from sporangia may occur in culture.

#### Growth of HFPCcI3 in culture

Experiments to determine the most favourable cultural conditions for growing *Frankia* HFPCcI3 were conducted in batch culture, first with complex media and then with defined media. Early trials with such media as M6B (Baker and Torrey, 1979), QMOD (Lalonde and Calvert, 1979), and similar complex media (cf. Baker and Torrey, 1980) failed to produce rapid growth; vegetative filamentous growth was frequently abnormal in appearance (Fig. 4).

With the use of synthetic media such as BAP with defined carbon and nitrogen sources, excellent growth rates were obtained and these were improved by using larger volume cultures, with stirring and/or air sparging. Results of growth studies and related acetylene reduction activity of HFPCcI3 in culture are reported elsewhere (Zhang, Murry and Torrey, 1985). Maximum growth with doubling times of approximately 24 h was achieved by culturing HFPCcI3 at 33 °C at pH 6.3 in BAP using pyruvate as primary carbon source and  $\text{NH}_4\text{Cl}$  as nitrogen source.

*Infectivity of Frankia HFPCc13*

In Table 1 are summarized typical experiments with seedlings of species of the Casuarinaceae. Successful nodulation of seedlings in all of these conditions was achieved quickly and in high percentages in members of the genus *Casuarina*. The first nodules were apparent at about three weeks and formed progressively with time. Primary nodules formed increasing numbers of nodule lobes with time. Efforts to nodulate *Allocasuarina lehmanniana*, a species of a closely related genus, grown in sand or water failed. Uninoculated plants consistently failed to nodulate.

TABLE 2. *Effects of pH and combined nitrogen on nodulation of seedlings of C. cunninghamiana grown in water culture with  $\frac{1}{3}$ -strength Hoagland's solution in the greenhouse with day temperature maximum 28 °C, night temperature 19 °C*

	Plants nodulated ( <i>n</i> )			Final pH
	Plants in test ( <i>n</i> )	3 weeks	6 weeks	
A. Effect of pH				
Initial pH				
5	6	3	6	5.1
6	9	8	9	5.6
7	9	9	9	6.4
8	9	4	9	7
B. Effect of combined nitrogen (initial pH = 7)				
Added N (g l <sup>-1</sup> )				
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>				
0.5	9	0	0	
0.2	9	1	6	
0.05	9	1	7	
KNO <sub>3</sub>				
0.5	9	2	3	
0.2	6	3	5	
0.05	9	4	8	
No added N	9	9	9	

Experiments to determine the influence of the pH of the root medium and the effect of the presence of combined nitrogen in the medium were set up with seedlings of *C. cunninghamiana* inoculated with *Frankia* HFPCc13. Table 2 shows that the most rapid and the highest percentage of nodulation occurred at pH 7 with seedlings provided no combined nitrogen in the nutrient solution. Ammonium nitrogen appeared to be more inhibitory than nitrate nitrogen although both reduced the rate and the amount of nodule formation.

Because of reports of alternate hosts for *Frankia* isolates from *Casuarina* (Gauthier, Diem and Dommergues, 1983; Zhang *et al.*, 1984), *Frankia* strain HFPCc13 was used to inoculate a number of other actinorhizal host plants studied in our laboratory. Inoculation trials with *Frankia* HFPCc13 on other hosts were made under a variety of conditions in growth chambers and in the greenhouse, in sand, in water culture or in aeroponics. In such inoculation trials, *Frankia* HFPCc13 consistently failed to nodulate the following possible host species: *Alnus rubra*, *Ceanothus americana*, *Elaeagnus*

*umbellata* and *Hippophaë rhamnoides*. Experiments with *Myrica gale* were inconclusive. In both greenhouse and growth chamber trials, some plants of *M. gale* became nodulated after inoculation with HFPCc13. Further studies are in progress to attempt to understand this anomaly.

TABLE 3. *Casuarina* seedlings grown in a common aeroponics tank with  $\frac{1}{4}$ -strength Hoagland's solution lacking combined nitrogen at pH 7 in the greenhouse

Host plant	Seedlings (n)	Nodulated plants (n)		Nodulation (%)	Nodules per seedling (n)	Acetylene reduction ( $\mu\text{mol C}_2\text{H}_4$ per g nodules per h)	
Expt. 1							
<i>C. cunninghamiana</i>	30	30		100	3.2*	16.87 $\pm$ 0.66	
<i>C. equisetifolia</i>	30	26		87	4.3	5 $\pm$ 0.6	
Expt. 2							
		(4 weeks) (8 weeks)				(4 weeks)	(8 weeks)
<i>C. cunninghamiana</i>	30	27	30	100	1.4†	12.92 $\pm$ 1.17	16.87 $\pm$ 0.66
<i>C. equisetifolia</i>	30	26	30	100	2.1	5.81 $\pm$ 0.62	5 $\pm$ 0.6

Seedlings were inoculated with *Frankia* HFPCc13 suspensions and observed eight weeks after inoculation.

\* Based on 10 nodulated plants. Earliest nodules were observed at 19 d.

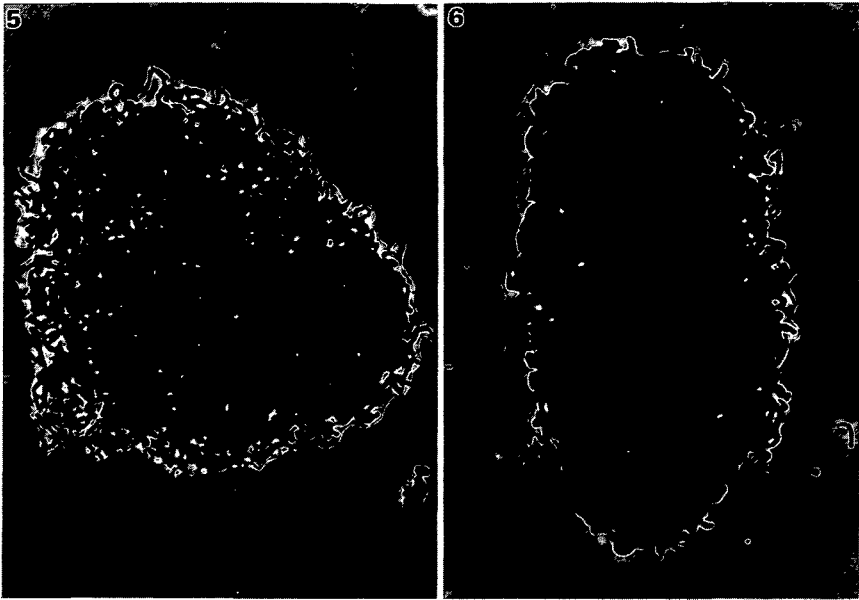
† Based on 30 plants observed at four weeks.

#### Effectivity of root nodules induced by *Frankia* HFPCc13

Acetylene reduction assays were conducted on excised root nodules of *C. cunninghamiana* at eight weeks after inoculation. Rates of activity averaged between 15 and 20  $\mu\text{mol C}_2\text{H}_4$  produced per g f. wt of nodule per h, suggesting effective rates of dinitrogen fixation.

Experiments were designed to compare the infectivity and effectiveness in nitrogen fixation of *Frankia* HFPCc13 on the two closely related species *C. cunninghamiana* and *C. equisetifolia* grown under comparable conditions in the greenhouse. Seedlings of similar age and development were placed on either side of an aeroponic tank with a common nutrient solution consisting of  $\frac{1}{4}$ -strength Hoagland's solution lacking nitrogen. Seedlings were each inoculated with a homogenized suspension of HFPCc13 and the seedlings observed. Table 3 summarizes the results of two experiments conducted sequentially. It is clear that nodulation is excellent with both species; only slightly fewer plants of *C. equisetifolia* were nodulated than *C. cunninghamiana*. However, the acetylene reduction rates were quite different, with nodules of *C. equisetifolia* showing an acetylene reduction rate approximately a third that of *C. cunninghamiana*. The appearance of the seedlings at later stages bore out this result with seedlings of *C. equisetifolia* growing less well, beginning to show yellowing and dwarfing. Unnodulated plants died.

Nodules of seedlings from *C. cunninghamiana* and from *C. equisetifolia* growing aeroponically were fixed and cytological squash preparations were made of nodule material known to have been active in acetylene reduction. In Figs 5 and 6 are shown the filamentous components of cells infected with *Frankia* HFPCc13 in each case. The preparations from the two different host species are similar; they both show the entire filamentous cluster of *Frankia* devoid of differentiated vesicles or sporangia. The same appearance of the endophyte was observed in older nodule lobes and in older nodules. In this case, unique so far as is known among actinorhizal plants, *Frankia* is active in fixing dinitrogen without benefit of the presence of vesicles. These observations confirm



FIGS 5-6. Photomicrographs of *Frankia* HFPCc13 within nodule cells. Fig. 5. Cytological squash preparation of the filamentous cluster from an infected cell of a root nodule of *C. cunninghamiana* seedling inoculated with *Frankia* HFPCc13. No vesicles or sporangia are seen. Fixed, stained with Azure B. Bright field optics.  $\times 880$ . Fig. 6. Similar squash preparation to that in Fig. 5 from infected cell of root nodule of *C. equisetifolia* seedling inoculated with *Frankia* HFPCc13. No vesicles or sporangia are seen.  $\times 880$ .

and extend those reported by others for *Casuarina* (Torrey, 1982; Berg, 1983). Studies on the nature of the  $O_2$  protection mechanisms in HFPCc13 are reported elsewhere (Murry *et al.*, 1985).

#### *The effect of $pO_2$ on acetylene reduction by root nodules induced by HFPCc13*

Among actinorhizal plants, members of the Casuarinaceae are unique in that *Frankia* within the root nodules do not develop vesicles yet are capable of fixing dinitrogen. Since the enzyme nitrogenase is known to be oxygen labile (Benson, Arp and Burris, 1979), the question arises as to the sensitivity of root nodules of Casuarinaceae to the oxygen in the gaseous environment surrounding the root nodules. Tests were made with root nodules from plants of *Casuarina cunninghamiana* inoculated in an aeroponic tank with HFPCc13. In such nodules, *Frankia* shows no vesicles, as shown earlier.

In Fig. 7 are shown the results of exposing the nodules to different oxygen concentrations. The optimum acetylene reduction occurred at or near ambient  $O_2$  concentration in the air (20 per cent) and was reduced either at higher  $O_2$  concentrations (presumably enzyme inactivation) or at lower  $O_2$  concentrations (presumably limiting aerobic respiratory processes providing energy for nitrogenase activity). These results confirm earlier reports by Bond (1961).

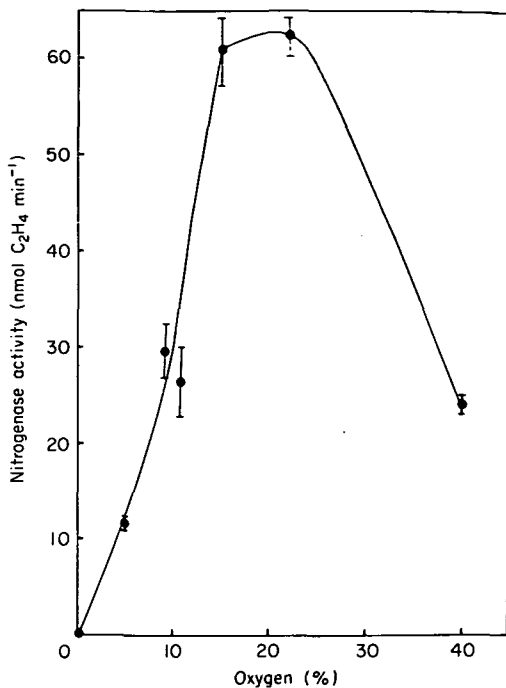


FIG. 7. Nitrogenase activity in detached nodules of *C. cunninghamiana* inoculated with HFPCcI3 is shown as a function of external O<sub>2</sub> concentration. In this experiment, nodule fresh weight was 0.129 g. The values shown are the mean  $\pm$  s.d.

#### DISCUSSION

The isolation of *Frankia* strains from root nodules of *Casuarina* species presented considerable difficulties and proved unsuccessful in earlier efforts using media containing yeast extract as the complex component (Torrey, unpublished). Success has been achieved by Diem, Gauthier and Dommergues (1982) and Diem and Dommergues (1983) and now by ourselves using the complex QMOD medium developed by Lalonde and Calvert (1979), a medium containing a number of complex components including organic nitrogen compounds and lipids. It is not known whether specific components in the latter medium favour growth of *Frankia* or whether some other feature of the medium allows colony formation or perhaps spore germination from nodule cells of the host plant; it is clear from this and similar experiences that there exists no one universal medium or method of successful isolation and culture of *Frankia* strains from different hosts.

Once established in pure culture, *Frankia* HFPCcI3 grew rapidly in a completely defined synthetic medium and responds in its morphogenesis to changes of nutrient components, especially to the absence of combined nitrogen (which elicits vesicle formation). Sporangial formation is also subject to changes in the medium but this response is much less well understood.

The environmental conditions which *Frankia* HFPCcI3 finds optimum for infecting the appropriate host plant are essentially similar to those reported earlier for this genus by investigators using inoculum prepared from nodule suspension (e.g. Bond, 1957; Coyne, 1973). As summarized by Torrey (1982, 1983), infection of *Casuarina* is best at relatively high temperatures around 30–35 °C. Combined nitrogen in the medium around the root inhibits the infection, with NH<sub>4</sub><sup>+</sup> being more inhibitory than NO<sub>3</sub><sup>-</sup>. The

interactions of combined nitrogen in the soil upon the association are complex as has been pointed out (Torrey, 1982).

Of particular interest is the specificity for host infection of the various *Frankia* isolates which have been reported. These interactions were discussed earlier for all the known *Frankia* isolates from nodules of *Casuarina* (Zhang *et al.*, 1984). The host specificity of *Frankia* HFPCc13 seems quite precise. The organism infects and produces N<sub>2</sub>-fixing root nodules on the *Casuarina* species tested. It fails to nodulate a close relative *Allocasuarina lehmanniana* and also *A. decaisneana*. Thus some sort of genetic distinction at the infection level is made by this organism which is unlike the situation in some other host families. In most cases, one infective isolate will nodulate all genera in the family as, for example, *Frankia* Eull isolated from *Elaeagnus umbellata* (Baker *et al.*, 1980) which nodulates all genera of the family Elaeagnaceae. That within the host family Casuarinaceae there may be more than one strain of *Frankia* for the different host genera was suggested by the experiments of Coyne (1983). This fact has now been demonstrated with the isolation of a *Frankia* from root nodules of *Allocasuarina* (Zhang and Torrey, 1985).

Specificity of *Frankia* HFPCc13 may go one step further in that fixation of dinitrogen seems to be favoured more in one species (*C. cunninghamiana*) than another (*C. equisetifolia*). Whether this subtlety of specificity will hold up in further experimentation remains to be seen. *Frankia* HFPCc13 clearly does not belong in the same group as the *Casuarina* isolates which are reported to nodulate members of the Elaeagnaceae but fail to nodulate *Casuarina* itself. The organism reported here seems to have the closest affinities to that reported by Diem, Gauthier and Dommergues (1982, 1983*b*) and referred to them as *Frankia* ORS1106.

The idea that strains of *Frankia* have greater compatibility for certain host species as compared with closely related species has been explored by Dillon and Baker (1982). Similar differences in effectivity between two hosts inoculated with one strain are apparent when HFPCc13 infects closely related species of *Casuarina*. An explanation of the difference in rates of acetylene reduction remains to be found. One might seek for anatomical and/or physiological differences in host nodules in the development of O<sub>2</sub> protection mechanisms, or in substrate availability for endophyte enzyme activity. Such differences might become important in selection of strains for inoculation for maximum nitrogen accretion by the symbiotic association.

The symbiotic relationship between *Casuarina* species and *Frankia* offers a particularly interesting further puzzle. The cultured microorganism can be induced to form vesicles and fix dinitrogen in aerobic conditions if combined nitrogen is removed from the medium. The same organism within nodule cells of the host fixes dinitrogen without differentiation of vesicles by the endophyte. If the vesicle structure normally provides protection of the enzyme nitrogenase from denaturation by molecular oxygen (Torrey and Callahan, 1982), such protection must be afforded to *Frankia* filaments within nodules of *Casuarina* in some other way. Furthermore, nitrogenase must be localized within the filamentous structure of *Frankia* in this case and not within vesicles. How the protection of nitrogenase from oxidative destruction within *Casuarina* nodules is maintained and the nature of the suppression of vesicle formation within these nodules makes this association one of continuing interest. Two possibilities have been suggested. Berg (1983) has demonstrated that infected cells of *Casuarina* nodules possess specially impregnated walls which might reduce oxygen accessibility to the endophyte. Several authors (Davenport, 1960; Tjepkema, 1983) have reported that *Casuarina* nodules contain haemoglobin-like components, suggesting the possibility they may function to regulate oxygen levels within nodule cells.

The availability of pure cultures of infective, effective strains of *Frankia* and *Casuarina* may prove to be a great benefit in the introduction and development of forestation involving the economically important tropical or sub-tropical trees *C. cunninghamiana*

and *C. equisetifolia*. Careful studies to select the bacterial strains with the greatest capacity for infection of appropriate and specific hosts and providing the highest rates of dinitrogen fixation should be made. Then pure cultures can be provided as inoculum for nursery plantations to assure nodulation before plants are placed in the field. These methods are already being tested (Diem, Gauthier and Dommergues, 1983*a*; Lundquist and Torrey, 1984). By selection of superior host trees inoculated with superior bacterial endophytes one can assure maximum effectiveness in forested plantations.

#### ACKNOWLEDGEMENT

This research was supported in part by Department of Energy Research grant DE-AC02-82ER-12036 and by the Maria Moors Cabot Foundation for Botanical Research, Harvard University. M. Murry provided data on oxygen effects on nodules as well as helpful advice and encouragement. The authors are also indebted to M. Fontaine, S. Lancelle, M. Lopez, R. Lundquist, K. VandenBosch and P. Young for advice and technical assistance and to F. O'Brien for secretarial expertise.

#### LITERATURE CITED

- BAKER, D. and TORREY, J. G., 1979. The isolation and cultivation of actinomycetous root nodule endophytes, pp. 38–56. In *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*, eds J. C. Gordon, C. T. Wheeler and D. A. Perry. Forest Research Laboratory, Oregon State University, Corvallis, OR.
- 1980. Characterization of an effective actinorhizal microsymbiont, *Frankia* sp. Avcl (Actinomycetales). *Canadian Journal of Microbiology* **26**, 1066–71.
- BENSON, D. R., ARP, A. J. and BURRIS, R. H., 1979. Cell-free nitrogenase and hydrogenase from actinorhizal root nodules. *Science* **205**, 688–9.
- BERG, H., 1983. Preliminary evidence for the involvement of suberization in infection of *Casuarina*. *Canadian Journal of Botany* **61**, 2910–18.
- BOND, G., 1957. The development and significance of the root nodules of *Casuarina*. *Annals of Botany* **21**, 373–80.
- 1961. The oxygen relation of nitrogen fixation in root nodules. *Zeitschrift für Allgemeine Mikrobiologie* **1**, 93–9.
- BURRIS, R. H., 1974. Methodology, pp. 9–33. In *Biology of Nitrogen Fixation*, ed. A. Quispel. North-Holland Publishing Company, Amsterdam, The Netherlands.
- COYNE, P. D., 1973. Some aspects of the autecology of Casuarinas, with particular reference to nitrogen fixation. PhD thesis, Department of Forestry, Australian National University, Canberra.
- 1983. Specificity between *Casuarina* species and root nodule organisms, pp. 205–10. In *Casuarina Ecology, Management and Utilization*, eds S. J. Midgley, J. W. Turnbull and R. D. Johnston. CSIRO, Melbourne, Australia.
- DAVENPORT, H. E., 1960. Haemoglobin in the root nodules of *Casuarina cunninghamiana*. *Nature (London)* **186**, 653–4.
- DIEM, H. G. and DOMMERGUES, Y., 1983. The isolation of *Frankia* from nodules of *Casuarina*. *Canadian Journal of Botany* **61**, 2822–5.
- GAUTHIER, D. and DOMMERGUES, Y. R., 1982. Isolement et culture *in vitro* d'une souche infective de *Frankia* isolée de nodules de *Casuarina*. *Comptes Rendus Académie Sciences (Paris) Series III* **295**–759–673.
- 1983*a*. Inoculation of *Casuarina* using a pure culture of *Frankia*. *Nitrogen Fixing Tree Research Reports* **1**, 18–19.
- 1983*b*. An effective strain of *Frankia* from *Casuarina* sp. *Canadian Journal of Botany* **61**, 2815–21.
- DILLON, D. T. and BAKER, D., 1982. Variations in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. *The New Phytologist* **92**, 215–19.
- GAUTHIER, D., DIEM, H. G. and DOMMERGUES, Y., 1981. *In vitro* nitrogen fixation by two actinomycetous strains isolated from *Casuarina* nodules. *Applied Environmental Microbiology* **41**, 306–8.
- 1983. Preliminary results of research on *Frankia* and endomycorrhizae associated with *Casuarina equisetifolia*, pp. 211–17. In *Casuarina Ecology, Management and Utilization*, eds S. J. Midgley, J. W. Turnbull and R. D. Johnston. CSIRO, Melbourne, Australia.
- HOAGLAND, D. R. and ARNON, D. I., 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347.

- JOHNSON, L. A. S., 1982. Notes on Casuarinaceae II. *Journal of the Adelaide Botanical Garden* 6, 73–87.
- LALONDE, M. and CALVERT, H. E., 1979. Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species, pp. 95–110. In *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*, eds J. C. Gordon, C. T. Wheeler and D. A. Perry. Forest Research Laboratory, Oregon State University, Corvallis, OR.
- LUNDQUIST, R. and TORREY, J. G., 1984. The propagation of *Casuarina* species from rooted cuttings. *Botanical Gazette* 145, 378–84.
- MIDGLEY, S. J., TURNBULL, J. W. and JOHNSTON, R. D., 1983. *Casuarina Ecology, Management and Utilization*. CSIRO, Melbourne, Australia. 286 pp.
- MURRY, M. A., FONTAINE, M. S. and TORREY, J. G., 1984. Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArI3 grown in batch culture. *Plant and Soil* 78, 61–78.
- MURRY, M. A., ZHANG, Z. and TORREY, J. G., 1985. Effect of O<sub>2</sub> on vesicle formation, acetylene reduction and O<sub>2</sub>-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from *Casuarina cunninghamiana*. *Canadian Journal of Microbiology* (in press).
- TJEPKEMA, J. D., 1983. Hemoglobins in the nitrogen fixing root nodules of actinorhizal plants. *Canadian Journal of Botany* 61, 2924–9.
- TORREY, J. G., 1976. Initiation and development of root nodules of *Casuarina* (Casuarinaceae). *American Journal of Botany* 63, 335–44.
- 1982. *Casuarina*: actinorhizal nitrogen-fixing tree of the tropics, pp. 427–39. In *Biological Nitrogen Fixation Technology for Tropical Agriculture*, eds P. H. Graham and S. C. Harris. CIAT, Cali, Colombia.
- 1983. Root development and root nodulation in *Casuarina*, pp. 180–92. In *Casuarina Ecology, Management and Utilization*, eds S. J. Midgley, J. W. Turnbull and R. D. Johnston. CSIRO, Melbourne, Australia.
- and CALLAHAM, D., 1982. Structural features of the vesicle of *Frankia* sp. CpI1 in culture. *Canadian Journal of Botany* 28, 749–57.
- ZHANG, Z. and TORREY, J. G., 1985. Studies of an effective strain of *Frankia* from *Allocasuarina lehmanniana* of the Casuarinaceae. *Plant and Soil* (in press).
- LOPEZ, M. F. and TORREY, J. G., 1984. A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. *Plant and Soil* 78, 79–90.
- MURRY, M. A. and TORREY, J. G., 1985. Culture conditions influencing growth and nitrogen fixation in *Frankia* sp. HFPCcI3 isolated from *Casuarina*. *Plant and Soil* (in press).
- ZOBEL, R. W., DEL TREDICI, P. and TORREY, J. G., 1976. Method for growing plants aeroponically. *Plant Physiology* 57, 344–46.