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ISOLATION, CULTURE, AND BEHAVIOR OF FRANKIA STRAIN HFPCgI4 FROM ROOT NODULES OF CASUARINA GLAUCA

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Casuarina glauca (Casuarinaceae) is an important introduced tree species in Egypt, valued for windbreaks, land stabilization, and soil improvement associated with actinomycete-induced root nodules that fix atmospheric nitrogen. A strain of *Frankia* designated HFPCgI4 was isolated from root nodules collected in Egypt and its characteristics assessed both in pure culture and in symbiosis. Strain CgI4 grows well in synthetic nutrient medium with added NH_4^+ or, in the absence of combined N in the medium, forms vesicles and fixes dinitrogen adequate for growth. Hyphae, vesicles, sporangia, and spores characteristic of the genus *Frankia* were observed. This strain shows spontaneous spore release when grown in media lacking N. When tested for infectivity on actinorhizal host plants grown in unaerated water culture, CgI4 nodulates several species of *Casuarina* that fix atmospheric dinitrogen. Other genera in the Casuarinaceae, namely, *Allocasuarina* and *Gymnostoma*, were not nodulated under these conditions. Species of the genus *Myrica* and *Comptonia peregrina* (Myricaceae) were effectively nodulated by CgI4. The isolate may have use as an inoculant for forest plantations using species of *Casuarina sensu stricta*.

Introduction

Casuarina glauca Sieb. ex Spreng., commonly called swamp she-oak in Australia, is an erect, fast-growing, dioecious tree 10–20 m in height, sometimes reaching 30 m, found naturally in a narrow coastal belt in New South Wales in eastern Australia (MIDGLEY et al. 1983). This species prefers warm humid or subhumid climates and occurs commonly in estuarine locations, tolerating elevated saline and seasonally dry soils. The root system is relatively shallow, and plants may regenerate vigorously from root suckers. The roots are infected by the filamentous soil bacterium *Frankia* of the Actinomycetales which results in root nodules with attached, vertically growing nodule roots. Symbiotic nitrogen fixation contributes to the success of these plants on poor sites containing little or no soil nitrogen.

Seeds of *C. glauca* have been sent around the world from Australia, and tree plantations have been reported in many tropical countries including Egypt, Israel, Kenya, South Africa, Cyprus, Malawi, and in the United States in Florida, California, and Hawaii. This species has proved of special value in forestry for wind breaks, for land or dune stabilization or for amenity plantings (EL-LAKANY 1983; NATIONAL RESEARCH COUNCIL 1984). The success of *C. glauca* in low-N sites depends upon its being effectively nodulated by appropriate strains of *Frankia*. When *C. glauca* is introduced as an exotic plant, care must be taken to inoculate seedlings in the nursery or in the field. In the past such inoculation has been made with crushed nodule suspensions, with soil, or with leafy litter from around nodulated plants.

In the absence of available strains of *Frankia* isolated and cultured from *C. glauca* we undertook such an isolation, using nodules from plants growing in Egypt. The following account presents a characterization of a new isolate from *C. glauca*, its growth characteristics in culture, and its infectivity and effectivity among species of the genus *Casuarina* and other genera in the Casuarinaceae.

Material and methods

METHOD OF ISOLATION

Root nodules were collected from a large specimen of *Casuarina glauca* Sieb. ex Spreng. growing in clay soil in the Botanical Garden in Kafr El-Sheikh governate, Egypt. The nodules were washed with tap water, mixed with clean moist sand in polyethylene bags, and refrigerated.

The fresh nodules were dissected into individual lobes, surface sterilized for 20 min in 30% H_2O_2 with a drop of detergent, and then washed several times in sterile distilled water. Each lobe was transferred to a tube containing 5 ml sterile Difco yeast extract-dextrose broth and incubated for 2 wk at 28 C to test for microbial contamination. Nodules free of contaminant were dissected into small pieces and transferred to 125-ml flasks containing 50 ml of sterile BAP medium (MURRY et al. 1984) modified to contain 10 mM pyruvate as described by TZEAN and TORREY (1989) and incubated at 28 C.

Within 10 d filamentous outgrowths were observed. Lobe pieces covered with *Frankia* fila-

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ments were homogenized and diluted 1:100 (v/v) with sterile distilled water, and 1 ml of this suspension was transferred to a 10-cm petri plate containing 15 ml melted agar DPM medium (BAKER and O'KEEFE 1984). The plate was agitated, allowed to solidify, and incubated at 28 C. After 3 wk single colonies could be removed by Pasteur pipette, homogenized, and transferred to a flask containing 50 ml liquid B medium (modified from MURRY et al. 1984). One such culture, designated HFPCgI4 (catalog number HFP 020804), hereafter referred to as CgI4, was selected for intensive study and was subcultured approximately every 3 wk in B medium.

CULTURAL STUDIES

The following formulations of liquid nutrient media were used in cultural studies of the new *Frankia* strain: modified M6B (CALLAHAM et al. 1978), DPM (BAKER and O'KEEFE 1984), YCz (Czapek's modified by WAKSMAN 1961, supplemented with 0.4% yeast extract), Qmod (LALONDE and CALVERT 1979), L/2 (LECHEVALIER et al. 1982), BAP (MURRY et al. 1984) with either pyruvate or propionate, NZA (modified S medium of LECHEVALIER et al. 1983), and B medium (MURRY et al. 1984).

To obtain rapidly growing hyphal cultures devoid of differentiated structures, CgI4 was transferred to B medium supplemented with 2mM NH_4Cl and subcultured every 2 d for 3 wk. Filamentous samples were examined under Nomarski interference contrast optics and found to be free of vesicles, sporangia, or spores. The hyphae were harvested by centrifugation at 2,500 rpm with washing in sterile distilled water, homogenized with a Potter-Elvejhem homogenizer, and inoculated into the different media. Five replicate tubes containing 10 ml of liquid medium for each treatment were inoculated with 0.01 ml packed cell volume (PCV) of hyphal homogenate and incubated at 28 C.

Microscopic examination was made at 5, 10, 15, and 30 d of cells transferred to 1 ml of 3% glutaraldehyde for 3 h to which 1 ml of 50% glycerol was added. Examination of samples transferred to glass microscope slides after 12 h was made with the microscope using phase or interference contrast optics.

Protein determinations were made at 15 and 30 d after inoculation using the Bio-Rad protein assay following the method described by MURRY et al. (1984). The 10 ml contents of each tube were centrifuged, washed with distilled water, and sonicated using a Braunsonic 1510 sonicator for 15–20 sec at 100 watts. To determine μgm protein per ml, 0.5 ml samples of sonicated hyphae were subjected to the Bio-Rad assay with five replicates per treatment and the absorption (A) read at 595 nm compared to the reagent blank. A standard curve was established with bovine serum albumen as the reference protein.

INFECTIVITY AND EFFECTIVITY STUDIES

Frankia strain CgI4 was tested for its capacity to infect different species of actinorhizal plants. The following host plants were inoculated with CgI4 homogenates and observed for nodulation: *Allocasuarina campestris, A. decaisneana, Alnus incana* ssp. *rugosa, Casuarina cunninghamiana, C. equisetifolia, C. glauca, Ceanothus americanus, Comptonia peregrina, Datisca glomerata, Elaeagnus angustifolia, E. umbellata, Gymnostoma papuanum, Hippophaë rhamnoides, Myrica cerifera, M. gale*, and *Shepherdia argentea*.

Seeds were sown in sand between upper and lower layers of vermiculite in flats, watered with 1/4-strength Hoagland solution (HOAGLAND and ARNON 1950), and grown in a growth chamber with 16-h light at 28 C and 8-h dark at 19 C. When plants were about 5 cm tall (1–3 mo depending on species), young plants were removed from the sand, the roots rinsed with deionized water, and transferred to water-culture jars containing 1/4-strength Hoagland solution lacking nitrogen. Three jars were used for each species with three plants per jar.

Strain CgI4 was grown in BAP medium for 3 wk, washed in distilled water with centrifugation, and homogenized. The inoculum was applied dropwise along the roots of each seedling using 0.01 ml PCV of culture per plant. Three uninoculated jars served as negative controls. Plants were grown in the greenhouse without aeration. Observations of nodule formation were made at regular intervals and nodule number recorded. Acetylene reduction assays were made at the end of each experiment, following the methods described by MURRY et al. (1984) based on the method of BURRIS (1974).

In one experiment CgI4 was grown in nine different media, and at the end of 3-wk growth, these cultures were tested separately as inocula for seedlings of *C. glauca*, using standard procedures.

Comparisons were made among a number of known *Frankia* strains isolated from host plants in the genus *Casuarina* with respect to their cultural behavior and their infectivity and effectivity as related to *Frankia* strain CgI4. The following isolates were studied: CcI3 (HFP 020203) isolated from root nodules of *Casuarina cunninghamiana* by ZHANG et al. (1984), Cjl-82 (ORS 021001) isolated from a hybrid between *Casuarina junghuhniana* and *C. equisetifolia* by DIEM et al. (1983), JCT 287 (not cataloged) isolated from *C. equisetifolia* by SHIPTON and BURGGRAAF (1983), and Cc01 isolated from *Casuarina cunninghamiana* in China (LI and DING 1986) kindly provided for our studies by D. BAKER.

Results

Morphology

Like other *Frankia* strains, CgI4 is filamentous, branched, and septate with hyphal diameters ranging from 0.63–1.88 μm depending on the medium (fig. 1). Differentiation of sporangia, vesicles, and the release of spores is dependent upon the cultural conditions and the medium. Sporangia form at the hyphal ends (terminal) or on short lateral branches or are sessile (figs. 2, 4). Maturation of spores within the sporangium begins at the distal end and proceeds basipetally (fig. 4). Mature spores average 1.5–2.0 μm in diameter. Immature spores within the sporangium stain blue with the carbol fuchsin-methylene blue stain (Baker 1967), and mature spores stain bright red (fig. 8). Unlike most *Frankia* strains described, spore release from within sporangial walls occurs spontaneously in B medium (figs. 3, 4, 5). This unusual behavior has been described in *Frankia* strains CeI5 and CgI1 isolated by Berg (cf. Tzean and Torrey 1989). Germinating spores were observed in B liquid medium within 3 wk of subculture.

Spherical terminal vesicles with diameters ranging from 2.5–3.5 μm showing internal septa form on short or long lateral branches (figs. 6, 7). The morphological expressions of CgI4 when cultured on a range of nutrient media were quite variable (table 1). Vesicle formation occurred in high frequency in media lacking combined N (DPM and B) but also in the NZA medium. Sporangia were also most abundant in DPM and B media where spore release was observed in older cultures (fig. 8).

Growth of CgI4, expressed as μgm protein/ml in the nine different media tested, was greater at 15 than 30 d in most cases (fig. 9). Decreases were attributable to autolysis late in the culture period. Among defined media BAP pyruvate and B media produced the greatest growth at day 15. Pyruvate served as a better carbon source than propionate for CgI4. Among complex media, NZA produced the greatest growth at day 15. On both M6B and YCz media CgI4 showed poor growth, suggesting that yeast extract may be inhibitory to growth.

No diffusible pigment was observed in any of the media tested; nondiffusible pigment formed by CgI4 differed from one medium to another, tending to increase with age. The hyphal filaments of CgI4 showed abnormalities with terminal and intercalary swellings in M6B, L/2, and Qmod media.

Infectivity and acetylene-reduction activity

The results of water-culture trials using *Frankia* strain CgI4 as inoculum were tabulated for a range of actinorhizal host plants (table 2). The occurrence of infection is recorded together with comparisons of acetylene-reduction activity in nodulated plants. Only *Casuarina* species and members of the *Myrica* family were nodulated in these trials. *Casuarina* seedlings showed nodule initiation within 2 wk; members of the Myricaceae nodulated within 4 wk. Nitrogenase activity varied considerably among the different associations, with *M. cerifera*, although nodulated, completely ineffective. It was interesting that *M. gale* showed the highest acetylene-reduction activity when sampled.

When CgI4 was grown in nine different culture media for 3 wk and then tested as inocula on *C. glauca*, all cultures were equally infective and effective, showing no significant difference either in number of nodules formed per plant or in acetylene-reduction activity per plant or per nodule dry weight.

Comparisons of the relative infectivity and effectivity of five different *Casuarina* isolates tested on three different *Casuarina* species grown in water culture show that all strains infected seedling roots of all three *Casuarina* species promptly and produced effective symbioses (table 3). This result agrees with those of Baker and Torrey (1990). Rates of acetylene-reduction activity by root nodules of the different *Casuarina* species showed no significant differences among the tested organisms with a confidence level of $P = .05$. *Casuarina equisetifolia* plants showed significantly lower rates of acetylene reduction compared with the other two species when inoculated with CgI4.

Discussion

Actinorhizal plants become significant components of an ecosystem when their roots are nodu-

Figs. 1–8.—Photomicrographs of *Frankia* strain CgI4 grown in liquid culture. Abbreviations: *f*, filamentous hyphae; *s*, spore; *sp*, sporangium; *v*, vesicle. Figs. 1–7 taken with Nomarski interference contrast optics at 1,150 ×. Bar = 10 μm. Fig. 8 taken with bright field illumination at 340 ×. Bar = 30 μm. Fig. 1, A colony of CgI4 grown in B medium. Fig. 2, Sessile and subsessile sporangia (sp_1 and sp_2, respectively). Arrows mark sporangial attachment. Fig. 3, Mature ruptured sporangia, showing the release of mature spores. Fig. 4, Mature sporangium showing rupture of side wall resulting in spore release (arrow). Differentiation of spores occurred from top of sporangium toward the base during maturation. Fig. 5, Sporangium after spore release, showing the residual sporangial wall (arrow) with some mature spores still inside. Fig. 6, Vesicles of CgI4 grown in B medium, formed on short and long lateral branches (arrows). Fig. 7, Vesicle of CgI4 grown in L/2 medium, showing internal septum (arrow). Fig. 8, Culture of CgI4 grown in B medium prepared as a bacterial smear and stained with the spore stain carbol fuchsin-methylene blue. The mature spores (double arrows) that have been released stained red while the immature spores (single arrow) still within the sporangial wall, the hyphal filaments, and the vesicles stained blue.

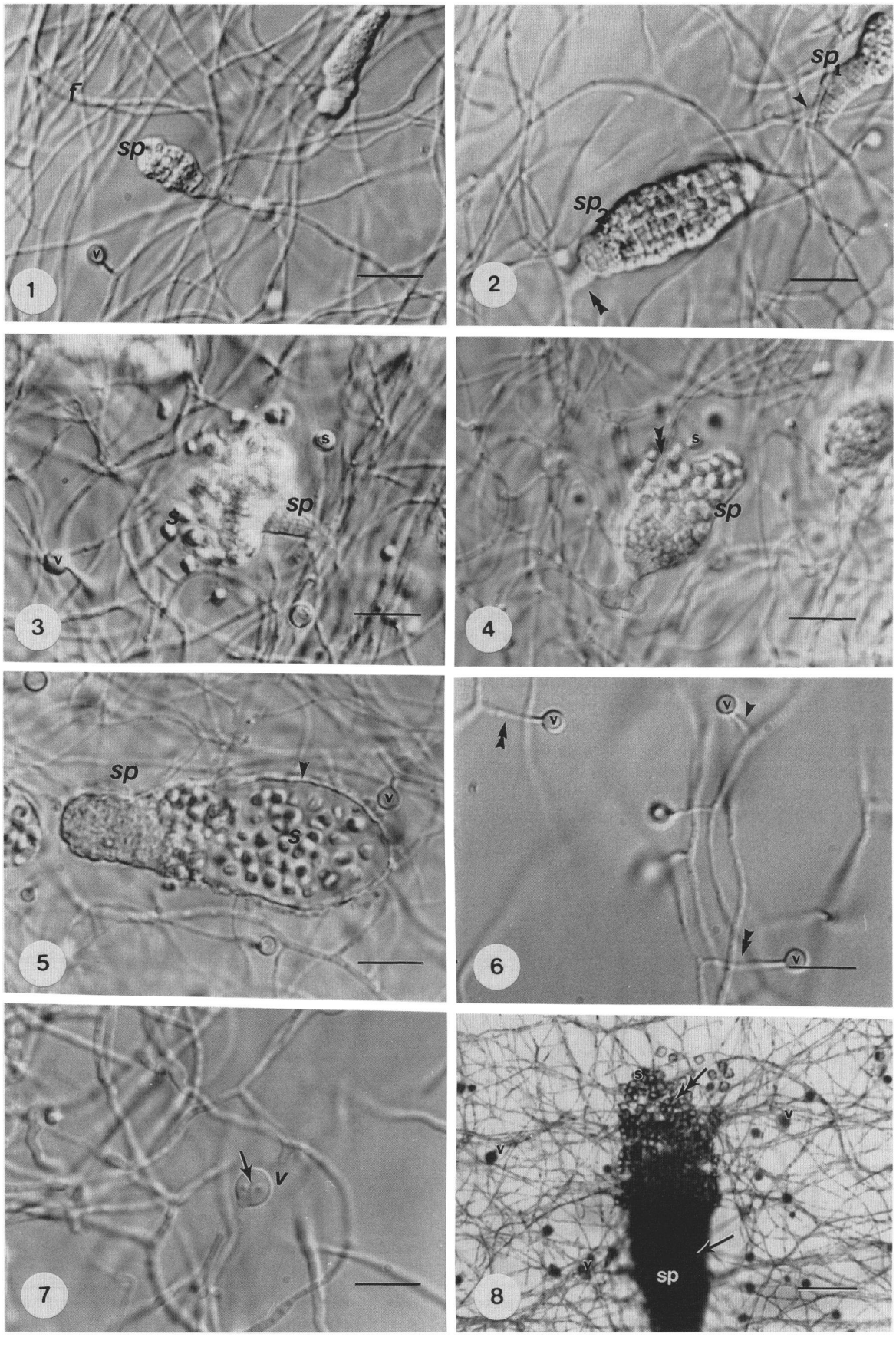
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TABLE 1

MORPHOLOGICAL CHARACTERISTICS OF FRANKIA STRAIN CgI4 GROWN IN DIFFERENT CULTURE MEDIA OVER 25 D

	MORPHOLOGICAL EXPRESSION IN CULTURE* (Time in days)							
	Vesicles				Sporangia			
CULTURE MEDIUM	5	10	15	25	5	10	15	25
M6B	−	−	±	±	−	−	−	−
YCz	−	−	±	±	−	−	−	±
L/2	−	−	±	±	−	−	−	±
Qmod	−	±	±	±	−	−	−	±
BAP_{pyr}	−	±	+	+	−	−	±	±
BAP_{prop}	−	±	±	+	−	−	±	±
NZA	−	+++	+++	+++	−	−	±	±
DPM	+++	+++	+++	+++	−	±	+	+++
B	+++	+++	+++	+++	±	++	++	+++

* Frequency per field: − = 0; ± = 1–5; + = 6–10; ++ = 11–20; +++ = >20.

lated by the soil bacterium *Frankia* in an effective dinitrogen-fixing symbiotic association. *Casuarina glauca* is one of the three species of *Casuarina* most widely disseminated in tropical countries for use in forestry plantations. Usually inoculation of seedlings in nursery plantations or in field planting is necessary, using crushed nodule suspensions or soil from around effectively nodulated plants.

Isolation and culture of strains of *Frankia* from root nodules of *Casuarina* initially proved difficult (DIEM et al. 1983; ZHANG et al. 1984). Now a number of *Frankia* strains are available that infect *Casuarina* species and produce effective symbiotic associations (cf. CHAUDHARY and MIRZA 1987; TORREY and RACETTE 1989; BAKER and TORREY 1990). Strain CgI4 should take its place among the handful of strains now available internationally for inoculation studies among members of the Casuarinaceae and is a potential strain for mass inoculation for commercial forestry plantations.

Rates of acetylene reduction in different species of *Casuarina* (tables 2, 3) show considerable variation depending on the strain of *Frankia* used as inoculum. Careful field trials to select the most effective host-microsymbiont combination will be needed before extensive plantations are established.

Frankia strain CgI4 grows readily in defined synthetic nutrient medium such as modified BAP or B medium (MURRY et al. 1984). In the latter

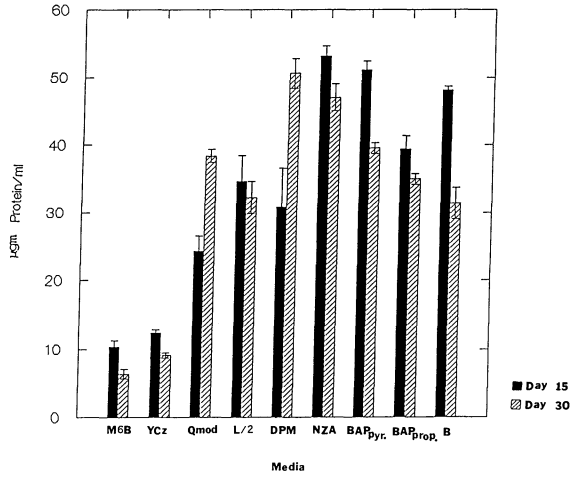

FIG. 9.—Bar graph showing growth of *Frankia* strain CgI4 in different liquid nutrient media after 15 and 30 d, expressed as µg protein/ml using the Bradford method. Initial inoculum = 7.57 µg protein/ml. Vertical lines represent standard errors.

TABLE 2

EFFECTIVITY OF FRANKIA STRAIN CgI4 IN ASSOCIATION WITH DIFFERENT SPECIES OF ACTINORHIZAL PLANTS GROWN IN UNAERATED WATER CULTURE

Host species	Effectivity µmol C_2H_4/h/g nodule dry wt ± SE
Casuarina glauca	10.21 ± 3.3
C. cunninghamiana	22.50 ± 3.0
C. equisetifolia	4.35 ± 1.3
Comptonia peregrina	9.20 ± 8.0
Myrica gale	105.53 ± 3.0
M. cerifera	0

NOTE.—CgI4 was grown in liquid B medium with pyruvate for 2–3 wk, washed, homogenized, and applied dropwise to roots at 0.01 ml PCV/seedling. Acetylene-reduction assays were conducted on nodulated whole root systems at 11–12 wk after inoculation. Other species tested that showed no nodulation under the same conditions included *Allocasuarina campestris, A. decaisneana, Alnus incana* spp. *rugosa, Ceanothus americanus, Datisca glomerata, Elaeagnus angustifolia, E. umbellata, Gymnostoma papuanum, Hippophaë rhamnoides, Shepherdia argentea.*

TABLE 3

A COMPARISON OF INFECTIVITY AND EFFECTIVITY AMONG FIVE DIFFERENT FRANKIA STRAINS ON THREE SPECIES OF CASUARINA

Frankia STRAIN	TIME TO APPEARANCE OF FIRST NODULE (d)			NUMBER OF NODULES/ SEEDLINGS AT 5 WK (mean ± SE)			EFFECTIVITY μmol C_2H_4/h/g NODULE DRY WT (mean ± SE)		
	Species 1	Species 2	Species 3	Species 1	Species 2	Species 3	Species 1	Species 2	Species 3
Cj1-82	14	17	19	23 ± 4.6	9 ± 1.8	11 ± 1.5	51.0 ± 9.0	84.9 ± 15.3	37.4 ± 5.3
JCT 287	15	14	19	12 ± 2.9	5 ± 0.9	5 ± 0.4	50.9 ± 11.7	82.0 ± 16.7	30.2 ± 4.2
Cc01	18	14	19	32 ± 2.9	18 ± 3.9	18 ± 1.5	48.6 ± 11.7	60.9 ± 11.7	29.0 ± 2.1
CcI3	15	15	18	17 ± 2.5	11 ± 2.3	16 ± 1.4	51.5 ± 12.8	91.3 ± 10.0	45.7 ± 4.2
CgI4	14	17	19	22 ± 3.1	9 ± 1.3	10 ± 2.1	70.9 ± 10.4	66.5 ± 9.9	23.64 ± 4.2

NOTE.—Nine seedlings grown and inoculated in water culture were tested for each species associated with each strain. Acetylene reduction activity was determined 5 wk after inoculation. Species 1 = *Casuarina glauca*; species 2 = *C. cunninghamiana*; species 3 = *C. equisetifolia*.

medium, which lacks NH_4^+, *Frankia* CgI4 rapidly differentiates vesicles and fixes N_2 from the atmosphere to sustain its growth.

Spontaneous spore release from mature sporangia formed in culture is an especially interesting character. Release of CgI4 spores occurred especially in B medium or in DPM medium at 25–30 d. Both of these media lack combined N, and the growth that occurs (fig. 9) results from use of dinitrogen fixed by vesicles formed in culture. Spore release seems to occur by specific sporangial wall rupture (figs. 4, 5). Spore release may be triggered by low N in the external environment. We have observed germinating spores in older cultures of CgI4 but have no measure of their contribution to the growth of the culture with time.

CgI4 exhibits interesting host specificities, rapidly and effectively nodulating seedling roots of the *Casuarina* species tested (table 2) but failing to nodulate seedlings of *Allocasuarina* species and *Gymnostoma papuanum* within the same family Casuarinaceae under the same conditions. Such specificity which occurs elsewhere in this host family (TORREY and RACETTE 1989) reflects very narrow and precise requirements for the necessary root-hair infection steps to occur and deserves further study. Like HFPCcI3, an isolate from *C. cunninghamiana* (ZHANG et al. 1984), CgI4 nodulates several different species in the family Myricaceae, an anomaly in host specificity that remains to be understood.

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