An effective strain of *Frankia* from *Casuarina* sp.

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A strain of *Frankia*, designated Cj1-82, from root nodules of a hybrid of *Casuarina junghuhniana* and *C. equisetifolia* has been isolated in pure culture. When grown under standard culture conditions, Cj1-82 exhibited the following characteristics: cushionlike colonies with short, wide hyphae and, in addition to typical sporangia, intercalary elongated sporangialike structures (SLS) which could be disrupted into sporelike units. No vesicles were found. When inoculated into the rhizosphere of *C. equisetifolia*, Cj1-82 produced vesicles. Reinfection of seedlings of *C. equisetifolia* was achieved repeatedly with inocula prepared from a suspension of Cj1-82. Sequences of infection of root hairs were described. Fourteen days after inoculation, nodules were apparent on the roots. Nodules were shown to be effective in nitrogen fixation as assessed by the acetylene-reduction technique.

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Une souche de *Frankia*, Cj1-82, a été isolée à partir d'un hybride de *Casuarina junghuhniana* et *C. equisetifolia*. En culture pure, Cj1-82 présente des caractéristiques suivantes: colonies en forme de coussin avec des hyphes courtes et larges, et, une plus des sporanges typiques, des structures intercalaires et allongées (SLS) ressemblant à des sporanges, structures pouvant se fragmenter en des unités semblables à des spores; pas de vésicules. Dans la rhizosphère de *C. equisetifolia* Cj1-82 produit des vésicules. La réinfection des plantules de *C. equisetifolia* a été obtenue avec une suspension de Cj1-82 utilisée comme inoculum. Les différentes séquences de l'infection des poils absorbants des racines sont décrites. Les nodules sont visibles sur les racines quatorze jours après l'inoculation. Soumis au test de la réduction d'acétylène, ces nodules se montrent capables de fixer l'azote.

During the last 2 years, we have isolated five strains of *Frankia* from nodules formed on seedlings of *Casuarina equisetifolia* inoculated with crushed nodules sampled from various parts of the world (Diem *et al.* 1982*a*). These strains, which exhibited the morphological features of the genus *Frankia* and fixed nitrogen *in vitro* (Gauthier *et al.* 1981), were nevertheless unable to nodulate the host plant. Recently we reported the isolation of an effective strain (Cj1-82) briefly described by Diem *et al.* (1983). In this paper, we present complementary data on the morphology and the infecting ability of this strain.

Material and methods

Strain Cj1-82 was isolated from nodules collected directly from a 1-year-old seedling of *Casuarina* grown in the nursery of the Thai Forest Service in Bangkok. According to Johnson and Briggs (1962), the tree is a hybrid of *C. junghuhniana* and *C. equisetifolia*.

Isolation procedure and cultivation

Cj1-82 was isolated according to the slightly modified method of Lalonde and co-workers as described elsewhere in this symposium (Diem and Dommergues 1983). Cj1-82 was then subcultured in Qmod (Lalonde and Calvert 1979) solid and liquid medium at 28–30°C without shaking.

Plant infection tests

Seedling cultivation in petri dishes

Seedlings of *C. equisetifolia* (6–8 weeks old) were placed in plastic petri dishes (9 cm diameter), with the roots lying horizontally on the bottom and the stem introduced through the hole pierced in a piece of plastic stuck to the rim of the dish (Fig. 1). The lid, painted black, had a slot near the rim to let out the aerial parts of the seedling. Roots were immersed in 30 mL of 0.1-strength nitrogenfree Crone nutrient solution (Lalonde 1979) in which $Fe_3(PO_4)_2 \cdot 8H_2O$ was replaced by Fe EDTA (4 mg/L). The nutrient solution was renewed every week and, when necessary, distilled water was added to compensate for evaporation. Two weeks after the transfer of seedlings to the petri dishes, roots were inoculated by adding a few drops of a dense suspension of *Frankia* Cj1-82. This inoculum had been obtained by gently crushing ca. 10 colonies of *Frankia* Cj1-82 picked up from a solid Qmod medium (Lalonde and Calvert 1979) in 2 mL of sterile distilled water. Inoculation was also performed by placing colony fragments directly on young root hairs.

The petri dishes with inoculated (20) and noninoculated (10) seedlings were then placed in a controlled-environment chamber (day (16 h light) : night (8 h dark); day temperature (28°C) : night temperature (20°C)). By 7–10 days after inoculation, deformed root hairs with attached inoculum were stained with trypan blue in lactophenol (0.1%) for microscopic observation. Two to 3 weeks after inoculation, the petri dishes were placed under a dissecting microscope so that initiation of nodules could be observed.

Seedling cultivation in tubes

One-week-old seedlings of *C. equisetifolia* were transferred into test tubes, permitting the shoot to grow outside (Gibson 1963). The agar slant contained 0.25-strength nitrogen-free

Hoagland solution according to Knowlton *et al.* (1980) supplemented with (in grams per litre) activated charcoal Merck ref. 2186, 20 g; $CaCO_3$, 1 g; agar, 16 g. The tubes were replenished up to 5 mm from the top with $\frac{1}{16}$-strength Hoagland solution with nitrogen (NH_4SO_4, 0.017 g/L). When the plants were 3–5 weeks old, the Hoagland solution ($\frac{1}{16}$ strength) with nitrogen was replaced by the same solution without nitrogen. Inoculation was performed by adding to each tube a few drops of a suspension of Cj1-82 homogenate in distilled water. The culture of Cj1-82 used for inoculation was obtained on Qmod liquid medium. Twelve inoculated tubes and 12 noninoculated tubes were then placed in a controlled-environment chamber as described above.

Assessment of acetylene-reduction activity

Three weeks after the appearance of the nodules, acetylene-reduction activity was assessed as usual (Hardy *et al.* 1968).

Results and discussion

Morphological and cultural characteristics of strain Cj1-82 grown in pure culture

After growth for 1 month on agar Qmod medium (with or without lecithin), Cj1-82 formed compact cushionlike 0.1- to 0.4-mm-diameter colonies that appeared to be embedded in mucilaginous material. Only hyphae located in the outermost periphery of the colonies could be stained with trypan blue solution (Fig. 2). Hyphae were short and wide (1–1.5 μm) with many septations, and they frequently developed into intercalary elongated sporangialike structures (SLS, Fig. 3). SLS probably resulted from the enlargement of the hyphae followed by the formation of transverse or oblique septa. When mature, SLS were globose or subglobose in certain areas (Fig. 3); most were arranged in elongated torulose chains of cells. In contrast with sporangia of *Frankia* from *Casuarina* described earlier (Diem *et al.* 1982*a*), SLS did not break out easily but disrupted only under pressure into single or groups of unicellular sporelike units the shape and size of which differed from that of typical *Frankia* spores (Fig. 5).

Subcultures of Cj1-82 in solid Qmod and liquid medium sometimes contained diffuse colonies characterized by a dense center containing old hyphae, mature SLS, and a fluffy periphery with flexous and fine hyphae (ca. 0.5 μm diameter) bearing numerous typical spore-producing *Frankia* sporangia (Fig. 4).

Occasionally, club-shaped sporangia were arranged in helicoid chains (Fig. 6). Spores produced by typical sporangia were similar in shape and size to those produced by other strains of *Frankia* from *C. equisetifolia* (Diem *et al.* 1982*a*).

The cause of such a pleomorphism in the colony morphology is unclear. Quispel and Burggraaf (1981) explained the variation in the growth pattern of *Frankia* from *Alnus glutinosa* as resulting from "selection among multi-strain cultures, mutation or by phenotypic adaptation."

In contrast with other *Frankia* strains from *C. equisetifolia* (Diem *et al.* 1982*a*), Cj1-82 did not produce pigment, nor did it form vesicles in Qmod solid or liquid medium, which probably explains its inability to fix N_2 *ex planta* and to grow in nitrogenfree media. Growth rate of Cj1-82 was very low in Qmod medium with or without lecithin (generation time, 3–5 days). Heavy inoculum was always required to start the subcultures, which suggests that some specific nutrient was lacking in the media used or that only a limited number of components of the inoculum were able to form new *Frankia* colonies. This finding supports the concept of units able to form *Frankia* colonies (UFF) developed elsewhere (Diem and Dommergues 1983).

Observations of strain Cj1-82 in the rhizosphere of C. equisetifolia

In some cases, hyphae and SLS of Cj1-82 were replaced by granulelike cells (Fig. 7) which differed obviously from the typical spores observed *in vitro* or the structures observed and designated as granules by Van Dijk and Merkus (1976) within the nodules of *Alnus glutinosa*. Actually little is known about these granule-like cells, especially the time and the cause leading to their formation.

In contrast with its behavior in pure culture, Cj1-82 formed vesicles in the rhizosphere (Fig. 8). The vesicles were similar to those formed *in vitro* by other *Frankia* strains (Tjepkema *et al.* 1980; Diem *et al.* 1982*a*) and to those formed by *Frankia* growing in the vicinity of nodules of *C. equisetifolia* (Diem *et al.* 1982*b*). Since *Frankia* reputedly does not form typical vesicles within *Casuarina* nodules (Tyson and Silver 1979), the production of typical vesicles by Cj1-82 and by extra-nodular growing *Frankia* (Diem *et al.* 1982*b*) in the rhizosphere suggests that the behavior of *Frankia* from *Casuarina* varies widely according to its habitat inside or outside the nodule.

Infectivity and effectivity of strain Cj1-82

Infection tests of *C. equisetifolia* seedlings grown in petri dishes showed that 80% of the plants bearing young root hairs on their lateral roots at the time of inoculation formed one to four nodules visible at 14–21 days. Plants having no young root hairs did not nodulate, which indicated that the presence of receptive root hairs is a prerequisite for nodulation. This confirms observations on the infection of *Alnus* reported by Pizelle (1972). Infection tests performed on seedlings grown in tubes showed that 100% of inoculated plants formed nodules (10–20 nodules per plant) within 3 weeks (Fig. 15); noninoculated controls never nodulated in either assembly.

Using nodules obtained from seedlings grown in petri dishes, we reisolated strain Cj1-82 within 3 weeks on the double-layer Qmod (without lecithin) solid medium as

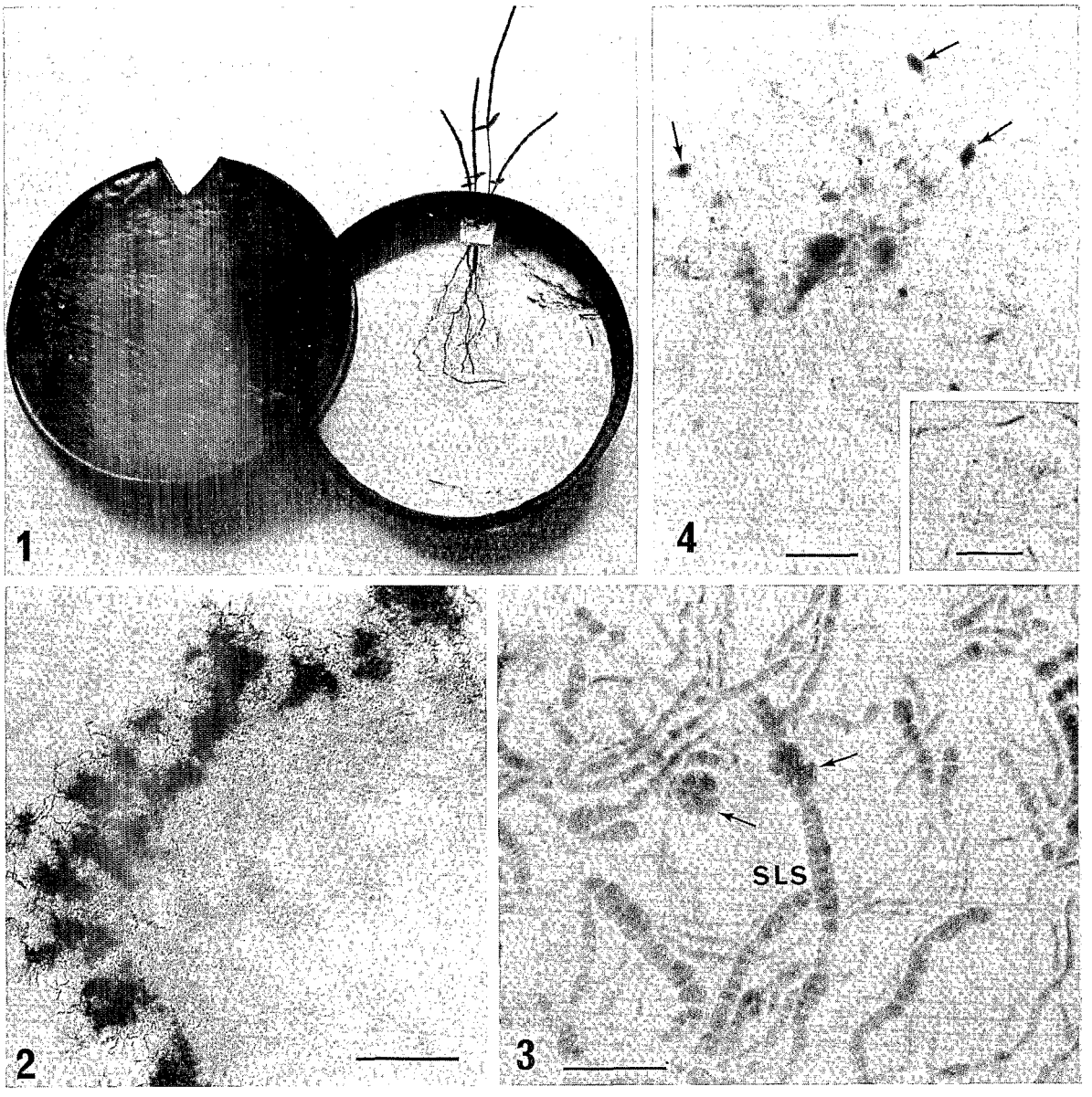

FIG. 1. Petri-dish assembly for seedling cultivation; 4-week-old seedlings. FIG. 2. Partial view of a cushionlike colony of *Frankia* Cj1-82 on agar Qmod medium; short peripheral hyphae are stained by trypan blue; the mucilaginous central part of the colony is not stained. Bar = 50 μm. FIG. 3. Cj1-82 on agar Qmod medium showing numerous intercalary elongated and globose or subglobose (arrows) sporangialike structures (SLS). Bar = 10 μm. FIG. 4. Filamentous colony of Cj1-82 bearing large typical sporangia (arrows) on agar Qmod medium. Bar = 30 μm. Inset: a typical spore-producing sporangium formed on peripheral hyphae. Bar = 10 μm.

described by Diem and Dommergues (1983). The recovered organism exhibited all the morphological characteristics of the original strain Cj1-82.

Acetylene-reduction activity exhibited by 3-week-old nodules was 10.8 μmol C_2H_4 per gram (fresh weight) per hour, indicating Cj1-82 was effective.

Preliminary steps of nodulation

Most observations were performed on roots of inoculated seedlings grown in petri dishes. Root hair deformation occurred 24–48 h after inoculation (Fig. 9). When the inoculum was a fragment of *Frankia* colony, root hairs converged towards this fragment, entrapping

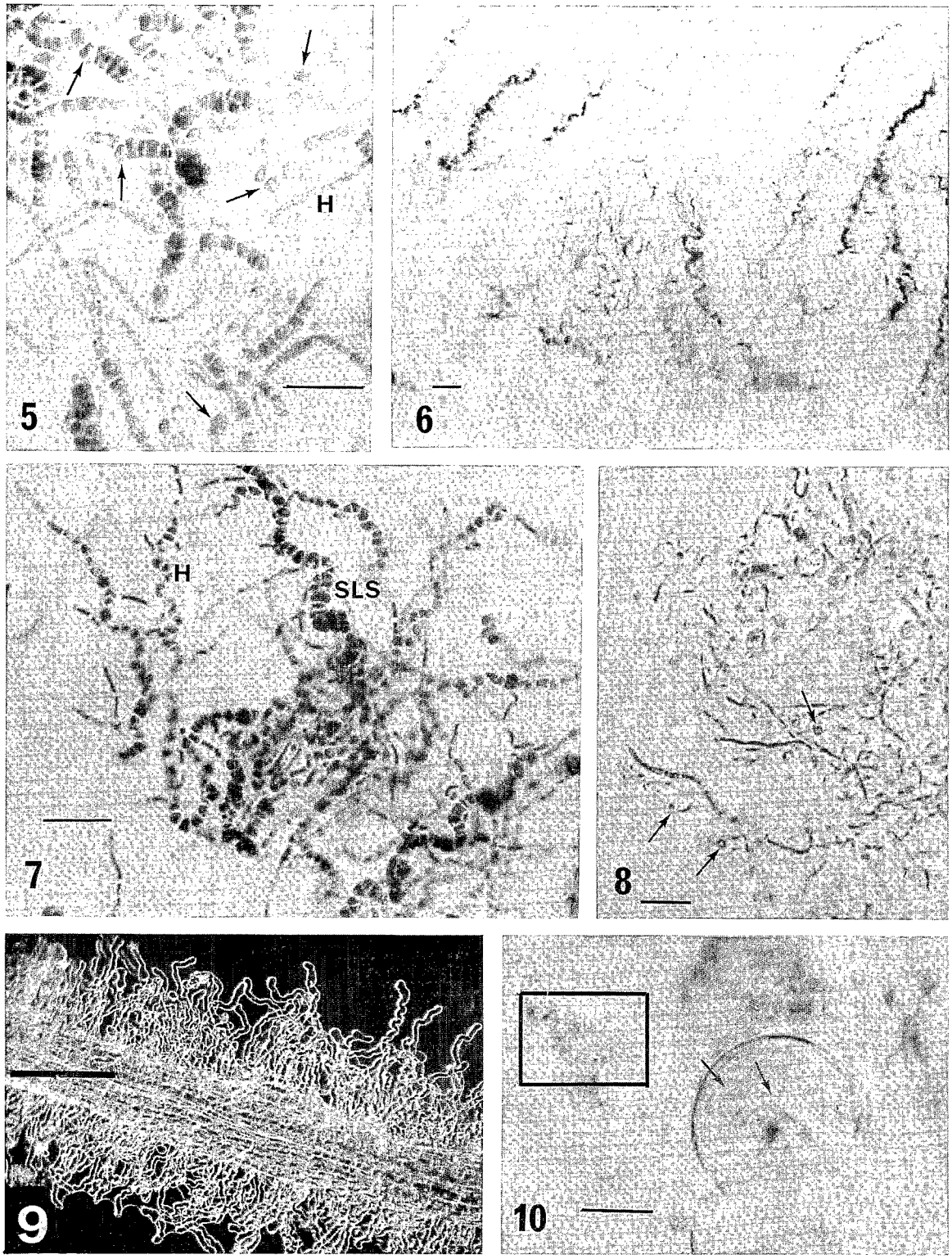

FIG. 5. SLS disrupted into sporelike units (arrows); short septate hyphae (H). Bar = 10 μm. FIG. 6. Helicoidal chains of club-shaped sporangia formed in some filamentous colonies of Cj1-82 on agar Qmod medium. Bar = 10 μm. FIG. 7. Hyphae (H) and SLS fragmented into granulelike cells in the rhizosphere of *C. equisetifolia* seedlings. Bar = 10 μm. FIG. 8. Vesicles

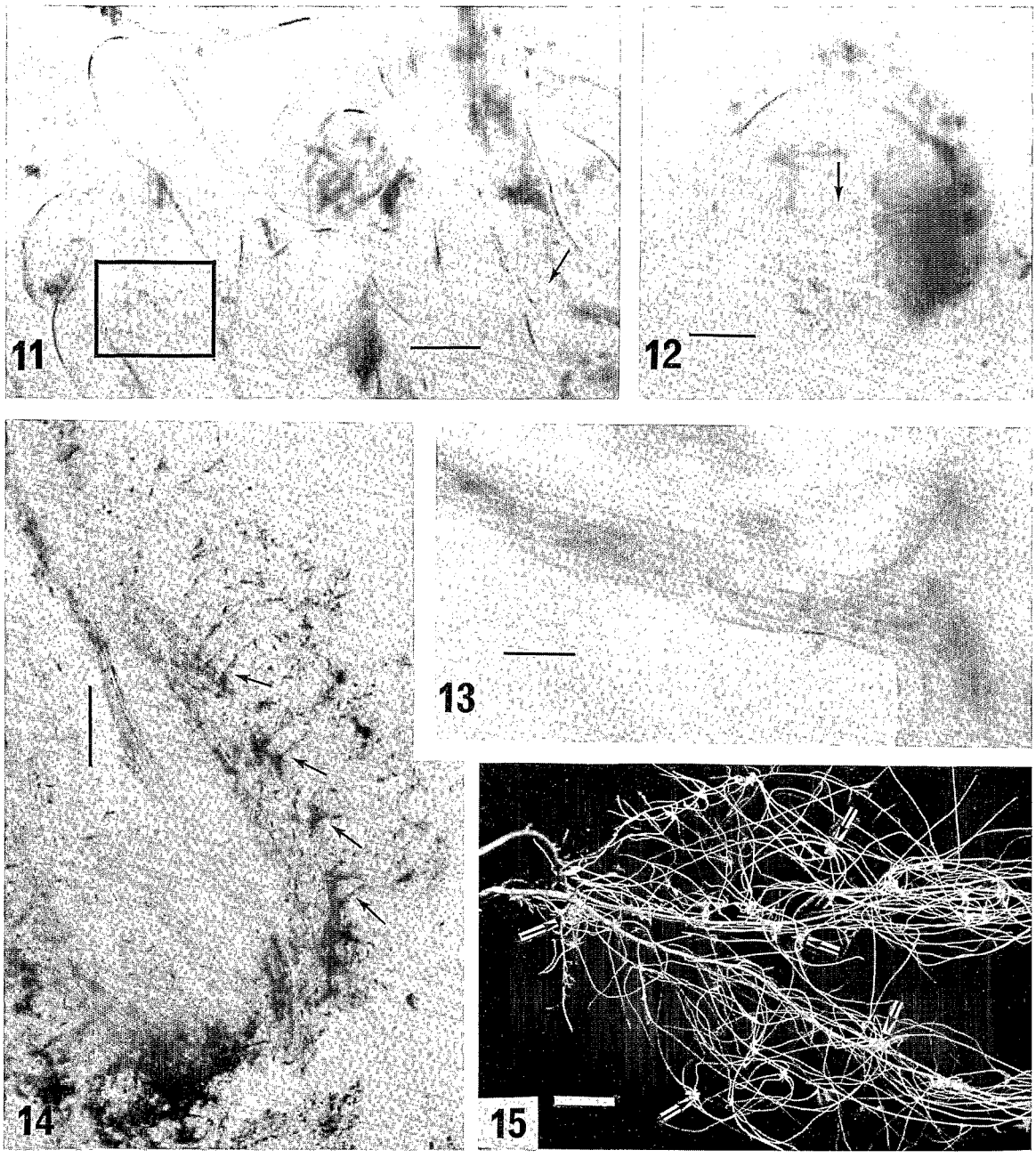

FIG. 11. Curling of root tips after inoculation; note the presence of granulelike cells (outlined area) and infection hyphae within a root hair (arrow). Bar = 10 μm. FIG. 12. Curled root hair tip showing convoluted material (arrow). Bar = 10 μm. FIG. 13. A root hair with several branched infection hyphae progressing towards its base. Bar = 10 μm. FIG. 14. Multiple infections occurring at the base of a lateral root of *C. equisetifolia*; only infected root hairs are stained by trypan blue (arrows). Bar = 100 μm. FIG. 15. *Casuarina equisetifolia* seedling root system from tube culture 6 weeks after inoculation with a suspension of Cj1-82; nodules (arrows). Bar = 1 cm.

(arrows) formed by Cj1-82 in the rhizosphere of *C. equisetifolia* seedlings. Bar = 10 μm. FIG. 9. Deformed root hairs of *C. equisetifolia* 48 h after inoculation with Cj1-82. Bar = 150 μm. FIG. 10. Curled root hair tip with initiating infection hyphae (arrows) within the curled area; granulelike cells from Cj1-82 (outlined area). Bar = 10 μm.

it, suggesting the involvement of an unknown attraction process. Later, the tips of some root hairs began to exhibit the curling that typically occurs prior to infection (Fig. 11), which always starts from the curled area. Preliminary observations with the light microscope did not allow us to determine the actual process by which Cj1-82 penetrated the root hairs; but since granulelike cells were detected in the vicinity of the curled root hairs (Figs. 10, 11, 12), we assumed that infection usually initiated by hyphae might be initiated also by the granulelike cells after they were enclosed in the fold of the root hair tips. This assumption is reminiscent of the theory that bacterialike cells are responsible for the infection of actinorhizal plants (Lalonde and Quispel 1977).

We have already mentioned that infection occurred in root zones bearing young and still-elongating root hairs. Only root hairs in that stage were able to curl and consequently provide *Frankia* with a niche allowing its penetration. These observations are in accordance with the principles governing the root hair curling and subsequent infection in legumes, as proposed by Bauer (1981) and Hubbell (1981).

By 7–10 days after inoculation, infection hyphae could be seen, originating from the fold enclosure (Fig. 10). In this area, convoluted hair wall material accumulated (Fig. 12), as reported by Callaham *et al.* (1979) for *Myrica gale* and *Comptonia*. Each root hair contained up to four infection hyphae (Fig. 13). The hyphae, encapsulated and often branched, progressed towards the base of the hair, then invaded the root cortical cells as described by Kant and Narayana (1977) and Callaham *et al.* (1979). The hyphae located in the root hairs or crossing, often in bundles, the wall between two infected nodule cells were encapsulated in a sheath apparently thicker than that of the hyphae coiled inside the nodule cells.

Ten days after root hair deformation, the infected root zone swelled as a result of the proliferation and hypertrophy of root cortical cells. Single infection was sufficient to initiate a nodule, but generally several root hair infections occurred leading to a single root nodule (Fig. 14). Nodulation was initiated not only at the base of lateral roots but also on main roots that had young root hairs. Nodulation on the main root was responsible for the deviation of the root axis.

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