Frankia spores of strain HFPCgI4 as inoculum for seedlings of *Casuarina glauca*

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Spore suspensions of *Frankia* strain HFPCgI4 originally isolated from root nodules of *Casuarina glauca* were studied with respect to their capacity to germinate *in vitro* in chemically defined media and added root exudates. Spore germination in general was low and prolonged but could be increased by chemical additions to the basal medium and increased further (doubled percentages) by adding suitable dilutions of root exudates from *C. glauca* seedlings. Spores inoculated directly on seedling roots at 10^8 spores/mL caused seedling root hair deformation in *C. glauca* and some root nodulation (35%). Spore inoculation under axenic conditions elicited limited root hair deformation and no nodulation. Evidence is provided showing that *Frankia* spores of isolate HFPCgI4 serve as effective agents in root hair infection when applied in nonsterile water culture conditions. Seedling root exudates may facilitate the infection process by stimulating *Frankia* spore germination.

Key words: *Frankia*, nodulation, root hair infection, root exudate, spores.

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Les auteurs ont étudié des suspensions de spores de la souche de *Frankia* HFPCgI4, originalement isolées de nodules racinaires du *Casuarina glauca*, afin de déterminer leur capacité germinative *in vitro* dans des milieux chimiquement définis et additionnés d'exsudats racinaires. En général de germination des spores est faible et prolongée mais peut être augmentée par l'addition de substances chimiques au milieu de base, et encore plus (pourcentage doublé) par l'addition de dilutions appropriées d'exsudats racinaires provenant de plantules de *C. glauca*. L'inoculation direct de 10^8 spores/mL induit la déformation des poils absorbants de plantules de *C. glauca* ainsi que la nodulation racinaire (35%). L'inoculation de spores en conditions aseptiques induit une très faible déformation des poils absorbants sans aucune nodulation. Les résultats démontrent que les spores de l'isolat HFPCgI4 peuvent agir comme agent efficace pour l'infection des poils absorbants, lorsqu'elles sont inoculées en culture non-stérile, dans l'eau. Les exsudats racinaires de plantules peuvent faciliter l'infection en stimulant la germination des spores du *Frankia*.

Mots clés : *Frankia*, nodulation, infection des poils absorbants, exsudat racinaires, spores.

[Traduit par la rédaction]

Introduction

Seedlings of *Casuarina* species are nodulated by the filamentous soil bacterium *Frankia* via hyphal penetration of deformed root hairs followed by prenodule development and nodule lobe formation (Callaham et al. 1979). Symbiotic nitrogen fixation by root nodules of *Casuarina* permits this genus to become established in nutrient-poor sites and to serve multiple roles in land stabilization, soil improvement, and agroforestry (National Academy of Sciences 1984).

Of continuing interest is the following question: What is the morphological form of *Frankia* that persists in the soil and that serves as the infective particle for infection in the field? In culture under appropriate conditions and in root nodules of many actinorhizal plants *Frankia* shows four morphological forms, viz. hyphal filaments, sporangia that contain 1 μm diameter spores, and terminal hyphal swellings termed vesicles that are the site of the nitrogen-fixing enzyme nitrogenase. Berry et al. (1986) have shown in elegant detail in *Alnus rubra* that the course of infection involves hyphal penetration of deformed root hairs. Burleigh and Torrey (1990) prepared inocula from pure cultured *Frankia* consisting of hyphae, spores, or homogenized whole cells of *Frankia* that included hyphae, sporangia and spores, and vesicles. They found that infection and nodulation of seedlings of *Casuarina cunninghamiana* was greatest with pure spore suspensions based on packed cell volume of fresh inocula but that all three preparations caused nodulation. If the inoculum preparations were dessicated by air drying, only spores remained viable and capable of infection.

Tzean and Torrey (1989) studied spore germination *in vitro* in spore suspensions prepared from pure cultures of several strains of *Frankia* isolated from *Casuarina* root nodules. They reported great variation in the percentage of spore germination depending on the strain of *Frankia* studied. *Frankia* strain UFGCeI5 from root nodules of *Casuarina equisetifolia* showed germination of up to 75% of the spores depending on the chemical composition of the nutrient medium. *Frankia* strain HFPCcI3 from root nodules of *C. cunninghamiana* showed less than 0.01% germination even in the most complex medium.

The present study was undertaken to document the processes involved in seedling root infection and nodulation in *Casuarina glauca* using as inoculum spore suspensions prepared from the pure cultured strain HFPCgI4, originally isolated from root nodules of *C. glauca* (Mansour et al. 1990).

Materials and methods

Plant species and Frankia *strains used*

Seeds of *C. glauca* Sieb. ex Spreng. were collected from exotic plants growing in the Botanical Garden in Kafer-El-Sheikh, El-Garbeia, Egypt, and stored in glass jars at 4°C in the dark.

Frankia strains included UFGCeI5 (catalog No. UFG 026606) isolated by R. H. Berg from root nodules of *C. equisetifolia* (cf. Tzean

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and Torrey 1989) and HFPCgI4 (catalog No. HFP 020804) isolated by Mansour et al. (1990) from root nodules of *C. glauca*.

Spore germination trials

Frankia strain UFGCeI5 was subcultured at monthly intervals in modified BAP medium (as described in Tzean and Torrey 1989), and strain HFPCgI4 was subcultured in modified B medium (cf. Mansour et al. 1990). Both cultures showed spontaneous release of spores from the sporangia and could be collected as pure spore suspensions by filtration through Whatman No. 1 filter paper. The filtrates were centrifuged at 2500 rpm (1000 × *g*) for 1 h, the supernatant discarded, and the collected spores washed twice in distilled water. Spore suspensions in sterile distilled water were diluted to ~8 × 10^6 cells/mL based on hemocytometer counts and stored at 4°C in the refrigerator in the dark for not more than 2 months before use in germination trials. Longer storage led to some reduction in germination.

Spore-germination trials followed procedures developed by Tzean and Torrey (1989). Seven milliliters of medium containing 1% Bacto-agar were poured into 6-cm sterile plastic Petri plates and allowed to solidify. Inoculation of each plate was with 0.1 mL of spores, and the spores were spread on the surface with a glass spatula. The plates were inverted and incubated in the dark at 28°C for 65 h before counting. In some experiments counts of germinated spores were made after 65 h and again after 3 weeks. For counting, a Whipple disc divided into 100 squares was inserted in a 10× eyepiece of a compound microscope equipped with a 40× phase contrast objective. Plates were subdivided into 16 squares by drawing lines in a grid on the bottom of each plate. In each square two to three fields were scored for a total of between 30 and 60 fields containing 415–420 spores for each plate. The percentage of germinated spores was calculated for each plate. Each sample was based on mean values from five plates.

Preparation of root exudates

Seeds of *C. glauca* were surface sterilized by immersing for 20 min in 30% H_2O_2 with two drops of detergent, followed by several washes with sterile distilled water. Seeds were transferred to sterile Petri plates containing 2 mL of 1/8-strength Hoagland's solution lacking nitrogen and pH 7 (according to Machlis and Torrey 1956). The seeds were germinated in the dark at 28°C for 1 week and then exposed to light with a 16-h photoperiod (33:28°C, light:dark) in a growth chamber for an additional 3 weeks. The sterile nutrient solutions containing root exudates were collected in 10-mL sterile glass tubes. Each tube was tested for sterility by inoculating a 0.1-mL sample into Difco yeast extract dextrose broth and incubating at 28°C for 2 weeks. The exudate solutions from 43 seedlings were concentrated by lyophilization approximately 10-fold and then stored in a freezer at −20°C. This concentrated exudate was then diluted as shown in Table 2 for spore germination trials. Dilutions were such as to eliminate direct effects of nutrient salts.

Axenic versus nonaxenic seedling infection tests

Spore inoculation trials were made on both axenically and non-axenically grown seedlings of *C. glauca*.

Axenic seedlings

Seeds of *C. glauca* were surface sterilized for 20 min in 30% H_2O_2 with detergent, rinsed in sterile distilled water, and germinated in 2 mL of 1/8-strength Hoagland's solution lacking nitrogen in sterile Petri plates in the dark at 33°C. After 2 weeks seedlings were transferred aseptically to a modified Fahraeus-type slide apparatus (Berry and Torrey 1983). Each root was arranged on a microscope slide and covered with a cover slip. Before mounting, seedling roots were immersed in a spore suspension (10^8 spores/mL) prepared in 1.2% sterile sodium alginate solution followed by a dip in 1.0% $CaCl_2$ solution to provide a sheathing gel. For each treatment five jars each containing four slides were prepared. Observations of spore germination and root hair deformation were made under the microscope at frequent intervals (7, 10, 14, 21, and 30 days). An exactly parallel experiment as described above for axenic seedlings was carried out with modified Fahraeus slides but under nonaxenic conditions (see Table 3).

Nonaxenic seedlings

Seeds of *C. glauca* were sown in flats of washed sand with top and bottom layers of vermiculite, watered with 1/4-strength Hoagland's complete nutrient solution, and grown in a growth chamber. One month after planting, young seedlings were removed from the flat, and the roots washed twice with deionized water. Roots were immersed in spore suspensions in sodium alginate as described for axenic seedlings with or without $CaCl_2$ treatment. Seedlings were transferred to water cultures in jars or in glass test tubes. Plants were maintained under 16 h light : 8 h dark (33:28°C) for the period of the experiment.

Microscopic methods

Whole mount preparations of seedling roots on glass slides were observed unstained under the microscope using phase or Nomarski interference contrast optics. For studies of the route of *Frankia* infection, cleared root preparations were made. Root segments to be examined were fixed overnight at 4°C in 3% glutaraldehyde in 25 mM sodium phosphate buffer at pH 6.8, rinsed five times in deionized water, then boiled 30 min in 5% (w/v) potassium hydroxide. Following repeated rinsing in tap water and then distilled water, the roots were stained with lactophenol–aniline blue solution and mounted on glass slides for light microscope examination.

For scanning electron microscopy (SEM) root segments were fixed as described above, then rinsed in buffer solution and stored at 4°C in fresh buffer. Individual specimens were rinsed in distilled water, dehydrated through a graded alcohol series, and then were critical point dried using carbon dioxide, coated with gold–palladium, and examined in a JEOL 300 SEMat 25 kV.

Results

Spore germination in vitro

Frankia strain HFPCgI4, like strain UFGCeI5 (Tzean and Torrey 1989), showed spontaneous release of spores when cultured in liquid B medium. A series of spore germination trials were made to compare the nutrient medium conditions that most favored germination in spore suspensions of these two *Frankia* strains. The report by Tzean and Torrey (1989) on spore germination in CeI5 served as the basis for these comparative studies.

Table 1 shows that, when counted 65 h after planting, spores of CgI4 showed no germination in media that gave substantial germination percentages for CeI5 (up to 21% in modified BAP medium). Subsequent experiments (data not included) showed that CeI5 germinated optimally in modified BAP medium at pH 6.7 with sodium propionate at 5 mM. When L-proline was added as the nitrogen source at 5mM, CeI5 spore germination at 65 h was 44.7%, and we referred to this medium as best medium. In this same medium CgI4 spores showed zero percent germination when observed at 65 h.

In studies with *Frankia* strain CgI4 and other difficult to germinate strains (e.g., CcI3, cf. Tzean and Torrey 1989), long-term observations showed some spore germination. From a series of trials with CgI4 it was determined that a stable count of germinated spores could be made at 3 weeks. By that time CeI5-germinated spores had covered the plates with well-developed, overlapping colonies, so germination counts could no longer be made.

The effects of root exudates on spore germination

Root exudates from seedling roots of *C. glauca* were prepared aseptically as described in Materials and methods and

TABLE 1. Germination percentage of *Frankia* strains HFPCgI4 and UFGCeI5 on agar plates of BAP medium with modifications

Medium	CgI4	CeI5
BAP	0	17.58*b*
BAP–carbon (C) source	0	14.40*a*
BAP–nitrogen (N) source	0	21.10*c*
BAP–vitamin	0	18.48*bc*
BAP–C–N–vitamins (basal medium)	0	12.04*a*
Distilled water (control)	0	12.68*a*

NOTE: All media were adjusted to pH 6.7, and cultures were maintained at 28°C in the dark for 65 h. Means with the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

TABLE 2. Germination percentage of CgI4 spores plated on agar medium as listed and counted at 65 h and again at 3 weeks

	65 h	3 weeks
Experiment 1		
Basal medium*	0	3.3*a*
Basal medium + 1×10^{-2} dilution of RE†	0	4.7*b*
Basal medium + 1×10^{-4} dilution of RE	0	5.7*c*
Basal medium + 1×10^{-6} dilution of RE	0	5.6*c*
Experiment 2		
Basal medium	0	2.9*a*
Basal medium + 1×10^{-4} dilution of RE	0	6.5*b*
Best medium	0	7.9*b*
Best medium + 1×10^{-4} dilution of RE	0	14.0*c*

NOTE: Best medium is the basal medium plus 5 mM sodium propionate plus 5 mM L-proline at pH 6.7. Mean values with the same letter in separate experiments are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).
*See Table 1.
†RE, root exudate.

added at serial dilutions to basal and the best media. Table 2 summarizes two different experiments on germination of CgI4 spores with and without root exudates. Germination percentages were determined after 3 weeks.

Very low percentages of germination were observed in the basal medium with no chemical additions (2.9 and 3.3% in the two experiments). Germination of CgI4 spores was more than doubled by supplementing the basal medium with sodium propionate as the carbon source and L-proline as the nitrogen source.

Root exudates prepared at high dilution showed stimulating effects on spore germination in both the basal medium (expt. 1, Table 2) and when added to the best medium (expt. 2, Table 2). In best medium with added root exudate, spore germination quadrupled over that in the basal medium alone. These experiments strengthen the view that spore germination in the vicinity of seedling roots could be influenced by the ambient chemical environment, including products of the root released as exudates or diffusates.

Spore germination within the root environment

Quantitative studies of spore germination in the immediate vicinity of seedling roots proved not to be feasible. Therefore, careful microscopic observations were made under a range of conditions, and photographic records were produced during the time course of the experiments. Two types of experiments were performed. In one type, seedlings were grown axenically, inoculated with spores with or without an alginate carrier under axenic or nonaxenic conditions, and then mounted for observation in modified Fahraeus slides. In the second set of experiments the seedling roots were inoculated with spore suspensions in water culture under nonaxenic conditions.

TABLE 3. Events associated with nodulation in seedling roots of *C. glauca* cultured axenically and nonaxenically in modified Fahraeus slides after inoculation with spore suspensions of CgI4

Observations	Axenic	Nonaxenic
First spore germination (days)	10–14	10–14
First root hair deformation (days)	10–14	7
First root nodule observed (days)	—	21–30
Seedlings nodulated (%)	—	35

NOTE: Observations were based on five jars each with four plants (total 20), made at frequent intervals (7, 10, 14, 21, and 30 days).

Table 3 shows when early events occurred, i.e., spore germination and root hair deformation. Under nonaxenic conditions the appearance of root hair deformation was accelerated and in due course nodulation occurred. In axenically grown plants spore inoculation failed to result in nodulation. Seedling roots grown axenically in modified Fahraeus-type slides with 1/4-strength Hoagland's solution lacking nitrogen with or without alginate carrier failed to show nodulation following inoculation with spore suspensions.

Figures 1 and 2 show straight root hairs of seedling roots inoculated with a spore suspension at the root surface (Fig. 1), with spores ungerminated (time zero) and then germinated spores seen 1 week later (Fig. 2). Figure 3 shows at high magnification that spores germinate on the surface of the root hair and hyphal filaments elongate along the surface.

Extensive root hair deformation was observed in seedling roots inoculated with spore suspensions and grown in water culture nonaxenically (Figs. 4–7). Root hair deformations were of very low frequency in the absence of spore inoculation. Deformations after inoculation with spore suspensions included root hair branching (Fig. 5), shepherd's crooks (Fig. 6), and root hairs in the shape of corkscrews (Fig. 7). Most of these deformed root hairs did not show infection threads.

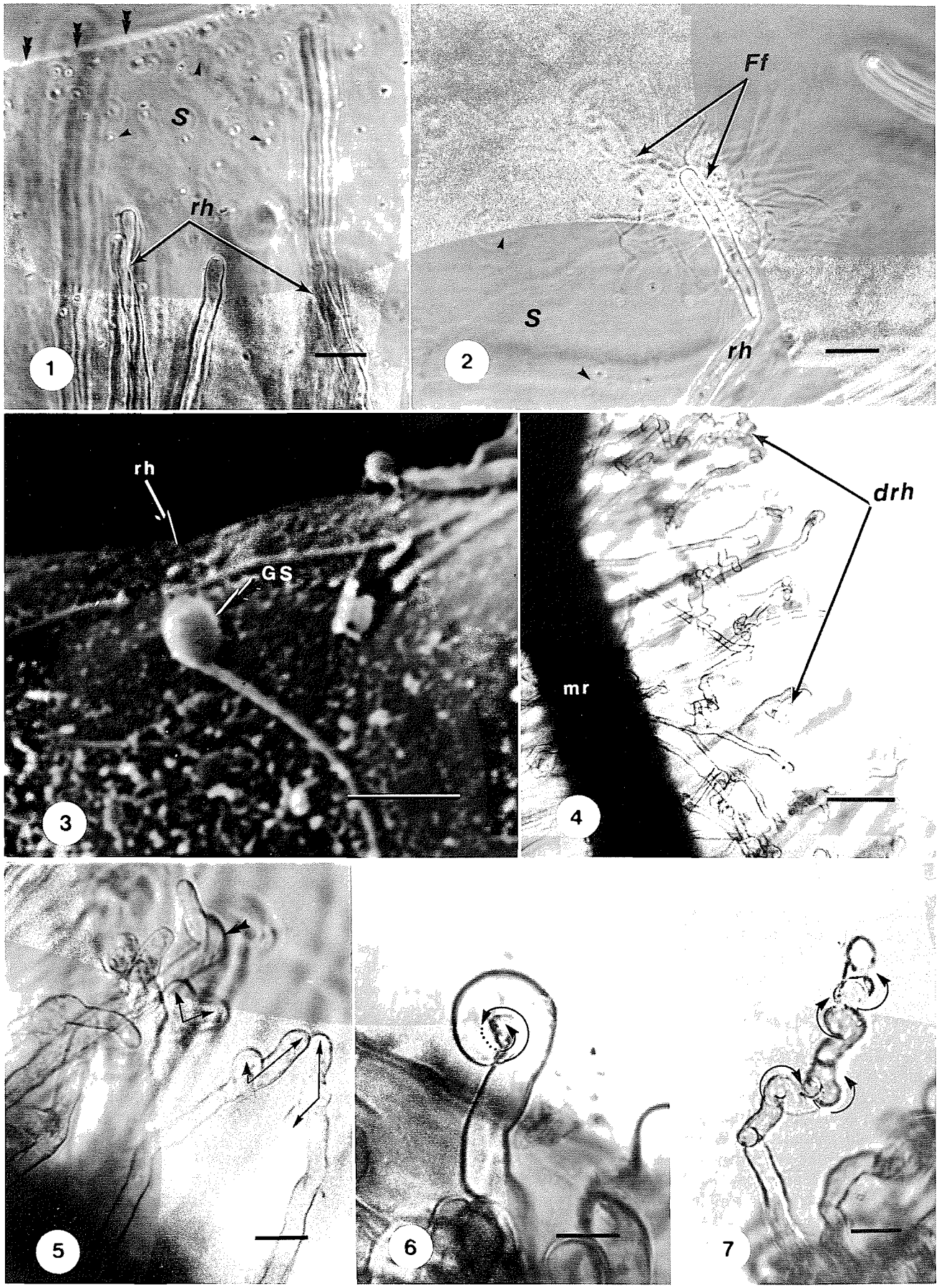
Root hair infection and nodule formation

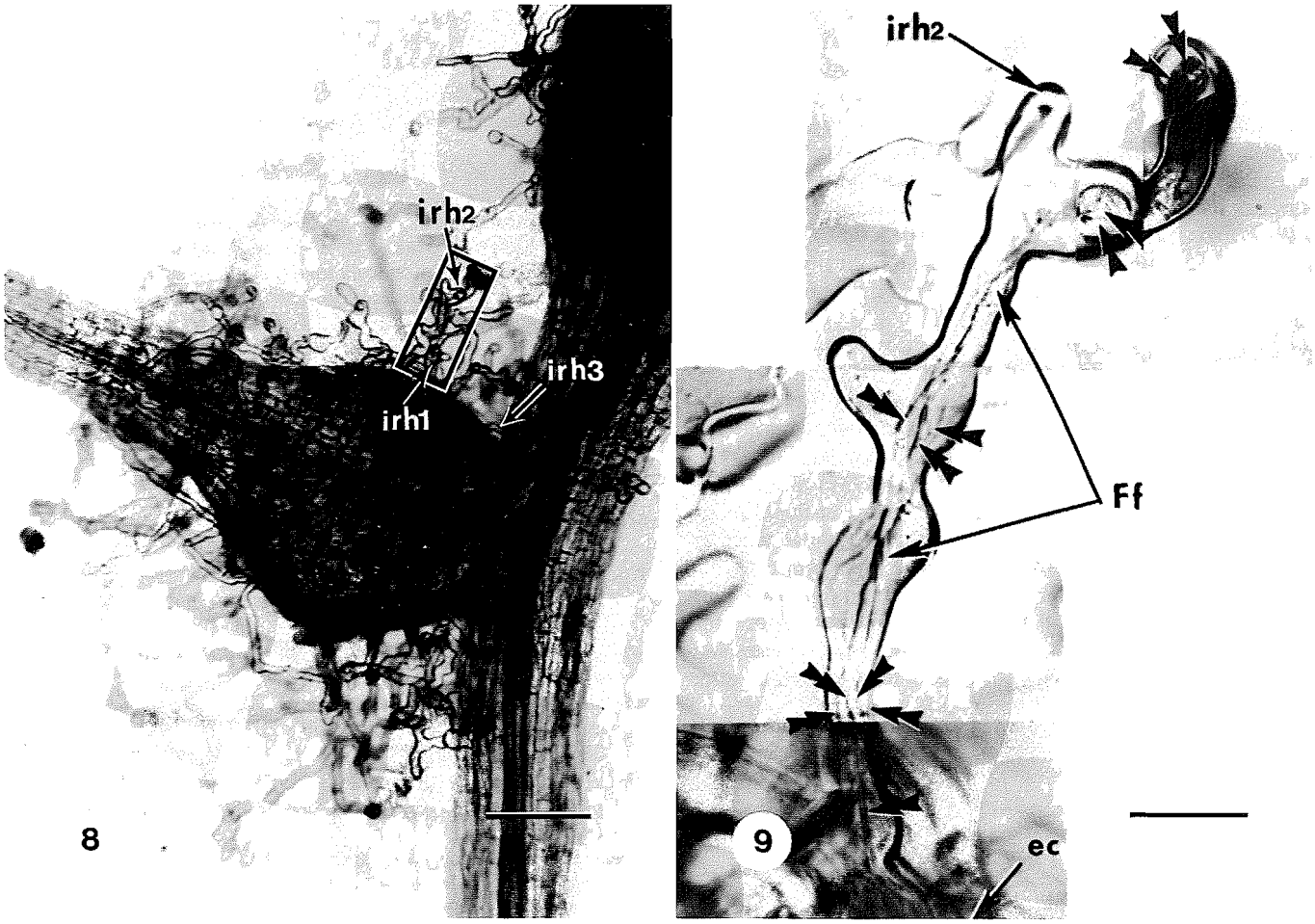
To see infection threads within deformed root hairs it was useful to clear the roots and stain the hyphal filaments within the hairs. Figure 8 shows a cleared seedling root at the juncture of a lateral root that was swollen by the development of a young nodule. Many deformed root hairs were visible, attached to the root axis and the lateral root. Three of these hairs were found to contain infection threads (marked *irh*1, *irh*2, and *irh*3 in Fig. 8). In Fig. 9 an enlargement of infected root hair 2 is illustrated, showing the path of the infection thread inside the root hair and into the epidermal cell of the swollen lateral root. This infection thread must have arisen from infection by a germinated spore.

Discussion

That spore germination in *Frankia* depends upon appropriate chemical stimuli was demonstrated in the studies of Tzean and Torrey (1989) and is confirmed in the present study. *Frankia* strain HFPCgI4 falls in the group of difficult to germinate spore types. Even under the most favorable conditions tested, spore germination in defined synthetic media seldom exceeded 10% of the population (Table 2) measured after 3 weeks in culture.

Yet spore suspensions of CgI4 proved to be suitable inoculum for nodule formation in *C. glauca* seedlings (Table 3). Chemical products released from seedling roots probably are





FIGS. 8 and 9. Photomicrographs of a cleared seedling root of *C. glauca* 10 days after inoculation with a spore suspension of *Frankia* HFPCgI4. Fig. 8. The swollen base of a lateral root is the site of origin of three different infected root hairs (*irh*1, *irh*2, *irh*3). Note the extensive root-hair deformation. Scale bar = 100 μm. Fig. 9. Enlarged photomontage of the boxed area in Fig. 8, showing infected root hair (*irh*2). *Frankia* filaments (Ff) can be seen originating near the branched tip (double arrowheads) and traversing the entire length of the root hair as double hyphal strands into the epidermal cell (*ec*) at the base of the root hair. Scale bar = 20 μm.

involved in stimulating spore germination and thereby facilitating infection and nodulation. Experiments designed to test the possible effects of root exudate on spore germination *in vitro* were uniformly positive (Table 2), even though the increase in germination percent was seldom more than double the control.

The evidence that root exudates may trigger responses in the infective bacterium in the soil in some respects parallels the situation studied in detail in *Rhizobium* (Peters et al. 1986 and reviewed by Halverson and Stacey 1986) in which specific products of root metabolism effect changes in soil bacteria leading to infection. Strikingly similar responses have been reported by Gianinazzi-Pearson et al. (1989) for spore germination and infection in mycorrhizal fungi.

The presence of spores in the environment surrounding the root elicits a response in the root by stimulating root hair deformation, a prelude to invasion of the root hair by hyphae of the compatible *Frankia* strain. Such root hair deformation is much greater in nonaxenic inoculation trials, showing that other soil microorganisms influence this early step in infection. The role of "helpers" described by Knowlton et al. (1980) and confirmed and extended in studies of *Alnus* by Berry and Torrey (1983) seems also to apply in the present studies of *C. glauca* nodulation. In the absence of helper microorganisms (axenic

FIGS. 1–7. Photomicrographs of root hairs on seedling roots of *C. glauca* inoculated with ~10^8 spores/mL of *Frankia* strain HFPCgI4. Fig. 1. Straight root hairs immediately after inoculation, showing elongating undeformed root hairs (*rh*) and spores (S) embedded in an alginate sheath (double arrowheads) surrounding the root. Scale bar = 20 μm. Fig. 2. *Frankia* filaments (Ff) from germinating spores (S) surrounding and attached to the surface of a root hair (*rh*). Scale bar = 20 μm. Fig. 3. Scanning electron micrograph of a root hair (*rh*) with a germinating spore (GS) on the root hair surface. Other *Frankia* hyphae from germinated spores run along the root hair surface. Scale bar = 2 μm. Fig. 4. Whole-root mount showing deformed root hairs (*drh*) on the main seedling root (*mr*) 7 days after inoculation with a spore suspension. Scale bar = 30 μm. Fig. 5. Root hair deformations following inoculation with a spore suspension. Arrows show root-hair branching. Curved and bulging root hairs (double arrowheads) are also seen. Scale bar = 20 μm. Fig. 6. A deformed root hair that forms a shepherd's crook. Arrows indicate rotation. Scale bar = 10 μm. Fig. 7. A root hair showing a corkscrew shape from multiple deformations caused by changed directions of growth (arrows). Scale bar = 20 μm.

conditions, Table 3) root hair deformation was delayed and, more important, nodule formation did not occur. Under axenic conditions, the incidence of root hair deformation was very low and no infected root hairs were seen, even though spore germination in the vicinity of the seedling roots was observed.

In their studies of axenically grown plantlets of *Alnus glutinosa*, Perinet and Lalonde (1983) reported a high incidence of success in effecting nodulation using homogenized suspensions of *Frankia* colonies grown in the complex medium Qmod. In their trials they attributed failures in nodulation to stress imposed on the host plants by the environmental conditions of the tests.

Further characterization of the infection process and its specificity would be facilitated by chemical studies of root exudates to determine whether specific products of root metabolism stimulate spore germination. It is interesting to note that L-proline, the amino acid present in our best medium for spore germination, was reported by Walsh et al. (1984) to be a significant component in the xylem sap in species of the Casuarinaceae tested. Similar studies might serve to show whether germinating spores of *Frankia* produce chemical products that lead to root hair deformation, thereby facilitating root hair infection. It seems reasonable to surmise that the higher the percentage of germinating spores in the root environment and the greater the occurrence of root hair deformation the better the chances for infection and nodulation to occur.

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