Sporulation in root nodules of actinorhizal plants inoculated with pure cultured strains of *Frankia*

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A low level of sporulation was seen to occur consistently within root nodules produced by inoculation with specific pure cultured strains of *Frankia*. The three *Frankia* strains, UFGCeI5 from *Casuarina equisetifolia*, UFGCgI1 from *Casuarina glauca*, and UFGMuI1 from *Myrica pubescens*, infect and produce root nodules on a range of actinorhizal host plants. Sporulation was detected in nodules of each host plant examined when the *Frankia* used for inoculation was one of these three strains. The amount of sporulation that occurred in any particular nodule was so low that it required identification in thin plastic sections prepared for examination at high magnification at the light microscope level. These strains, unlike other isolates studied, appear to be genetically predisposed to sporulate in the host root nodules. All three strains also show spontaneous spore release in culture.

Key words: actinorhizal plants, endosymbiont, *Frankia*, sporulation.

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On observe une sporulation faible mais régulière dans les nodules obtenues par inoculation avec des souches pures spécifiques de *Frankia*. Les trois souches de *Frankia*, UFGCeI5 de *Casuarina equisetifolia*, UFGCgI1 de *Casuarina glauca* et UFGMuI1 de *Myrica pubescens*, colonisent et produisent des nodules racinaires sur un ensemble de plantes hôtes actinorhiziennes. On retrouve la sporulation dans les nodules de chaque plante hôte examinée lorsque le *Frankia* utilisé pour l'inoculation est une de ces trois souches. La quantité de sporulation atteinte pour un nodule donné est si faible qu'il est nécessaire, pour les détecter, d'utiliser des sections minces enrobées dans le plastique et observées en microscopie optique aux forts grossissements. Ces souches, contrairement aux autres isolats étudiés, semblent génétiquement prédisposées à la sporulation dans les nodules racinaires de l'hôte. Les trois souches produisent également des spores de façon spontanée en milieu de culture.

Mots clés : plantes actinorhiziennes, endosymbiotes, *Frankia*, sporulation.

[Traduit par la rédaction]

Introduction

The filamentous bacterium *Frankia* (Actinomycetales) induces nodule formation on roots of a diverse array of woody dicotyledonous plants termed collectively actinorhizal plants. *Frankia*, when isolated and grown in pure culture, forms intrahyphal and terminal sporangia often in abundance depending on the nutrient conditions in the medium. In nodulated plants collected from the field, sporulation may or may not occur within the *Frankia*-infected root nodule cells. Nodules lacking sporulation are referred to as spore (−) and those exhibiting sporulation as spore (+). Our understanding of *Frankia* sporulation within host plant nodules is quite incomplete (cf. review by Torrey 1987). Serious efforts have been made in a number of laboratories to isolate and culture *Frankia* strains from spore (+) nodules from different host plants (Quispel and Tak 1978; Burggraaf *et al.* 1981; Quispel and Burggraaf 1981; Normand and Lalonde 1982; Burggraaf 1984; J. G. Torrey, unpublished studies). An isolate made by Zhang from spore (+) nodules of *Myrica gale*, designated as M^+gI5 and first described by Silvester *et al.* (1988), effectively nodulates *M. gale* seedlings in water culture, but no sporulation by *Frankia* within the nodules has been observed. Only one case of sporulation in root nodules induced on host plants by a pure cultured isolate of *Frankia* has been reported in the literature. Baker *et al.* (1980) observed both intercellular and intracellular sporulation in root nodules of *Elaeagnus umbellata* by a strain of *Frankia* designated EuI1b (catalogue No. DDB130120). The nodules induced by *Frankia* strain EuI1b failed to form symbiotic vesicles in the nodules and were ineffective in dinitrogen fixation.

VandenBosch and Torrey (1983, 1984, 1985) used nodule suspensions prepared from field-collected nodules of *M. gale* and *Comptonia peregrina*, both of the Myricaceae. Nodules shown to be spore (−) when collected induced spore (−) nodules on seedlings grown in water culture, whereas nodules produced by inoculation with spore (+) nodule suspensions showed spore (+) expression when grown to maturity in water culture. These results support the concept developed by Van Dijk (1978, 1979, 1984) that there are two genetically different bacterial types in *Frankia* and that this genetic difference of the bacteria is the primary basis for the expression of sporulation by *Frankia* within host plant root nodules, rather than host nodule environment regulating sporulation. A similar view led Normand and Lalonde (1982) to indicate spore-forming capacity in the host plant in any isolated strains by designating them as P (positive) or N (negative) in his strain designation (e.g., ACN3).

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TABLE 1. The occurrence of sporulation in nodules induced by inoculation of actinorhizal plants with a variety of *Frankia* isolates

Plant species	*Frankia* isolate	Catalogue No.	Nodule age (months)	Sample size	Occurrence of sporulation
Gymnostoma papuanum (rooted cuttings)	ArI3	HFP013003	16	8 nodule lobes	—
	EuI1c	DDB130130	16	9 nodules	—
	M^+gI5	HFP161105	16	10 nodules lobes	—
	EAN1	ULQ130100144	16	10 nodule lobes	—
	CpI1	HFP070101	16	10 nodule lobes	—
	GPI1	HFP021801	16	9 nodule lobes	—
	CcI3	HFP020203	16	9 nodule lobes	—
	CcI2	HFP020202	16	10 nodule lobes	—
G. papuanum (seedlings)	CeI5	UFG026605	6	26 nodule lobes	1–2 cells in each of 3 lobes
	CgI1	UFG028501	6	10 nodule lobes	1 cell in 1 lobe
	MuI1	UFG162201	6	10 nodule lobes	1 cell in each of 2 lobes
G. papuanum (seedlings in aeroponics)	CgI1	UFG028501	2.5	10 nodules	1–3 cells in each of 4 nodules
G. papuanum (seedlings)	CeI5	UFG026605	1.5	10 nodules	—
Casuarina glauca (seedlings)	CgI1	UFG028501	3	10 lobes of 1 nodule	1–3 cells in each of 3 lobes

NOTE: All nodules were examined in plastic sections.

The most direct proof of the genetic basis for sporulation in root nodules of host plants by *Frankia* would be to isolate, culture, and study pure cultured isolates of *Frankia* that possessed the stable character of sporulating within root nodules. Recently, Berg made a series of *Frankia* isolations from field-collected root nodules of *Casuarina* spp. and *Myrica pubescens*. Three strains have been made available for study: UFGCeI5 (catalogue No. UFG026605), UFGCgI1 (catalogue No. UFG028501), and UFGMuI1 (catalogue No. UFG-162201). These strains possess in common the unusual trait of spontaneous spore release in culture. In the course of cross-inoculation studies it was observed that seedlings grown and nodulated in water-culture trials with these strains of *Frankia* often produced nodules containing sporulation at a low frequency. Studies of this phenomenon are reported here.

Materials and methods

Nodules were harvested from plants of several actinorhizal species that had been inoculated with a variety of *Frankia* isolates. Many of the nodules that were examined came from plants that were originally established in water culture, or in one case aeroponics, for use in cross-inoculation trials between actinorhizal species and various *Frankia* isolates. After nodulation, plants were maintained with weekly replacement of one-quarter strength, N-free Hoagland's nutrient solution (Hoagland and Arnon 1950). Sampling for each species–isolate combination entailed examining 40–60 1-μm thick sections from approximately 10 nodules if the nodules were small, or 10 nodule lobes from several nodules if the nodules were large. Nodules were prepared for sectioning by fixing for 2–3 h in 2.5% glutaraldehyde in 25 mM phosphate buffer at pH 6.8, followed by dehydration in acetone and embedding in Spurr's low-viscosity resin (Spurr 1969). Sections were stained with 0.05% toluidine blue O in 1% sodium tetraborate. In many instances several nodules were also examined via hand sectioning with lactophenol–cotton blue staining. Table 1 lists the species–isolate combinations that were examined via thin sectioning, the age at which nodules were harvested, and the numbers of nodules or nodule lobes examined.

Based on the results of some of the sampling described above, an experiment was set up to test infectivity of and *in vivo* sporulation by the *Frankia* isolate UFGCgI1. Host plants that were tested included *Comptonia peregrina, Casuarina glauca, Elaeagnus angustifolia, Shepherdia argentea,* and *Myrica cerifera*. Seeds were germinated and seedlings established in water culture and inoculated with UFGCgI1 as described by Racette and Torrey (1989). For those species that nodulated, nodules were harvested at 6 and 10 weeks after nodule initiation was seen. Nodules were embedded and sectioned as described above. In this and all experiments, uninoculated controls were used to establish that nodulation which occurred was due to inoculation and not random contamination from the greenhouse environment.

In one experiment seedlings of *C. glauca* were grown in water culture, nodulated by *Frankia* strain UFGCgI1, and their roots exposed to continuous bubbling of a mixture of 35% O_2 in air. At the end of 4 weeks, nodules were fixed for 4 h at 25°C in 2% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2), postfixed in buffered 2% osmium tetroxide, dehydrated in an ethanol–acetone dehydration series, and embedded in Spurr's resin. Sections were poststained in uranyl and lead solutions for examination with transmission electron microscopy.

Results

The occurrence of *in vivo* sporulation by cultured *Frankia* strains is depicted in Figs. 1–7. Figures 1–3 show sporulation in *Gymnostoma papuanum* nodules by three different *Frankia* isolates: UFGCeI5, UFGCgI1, and UFGMuI1. The greatest

FIGS. 1–7. Sporulation within root nodules that were induced by cultured isolates of *Frankia*. Arrows indicate sporangia. Scale bar = 25 μm. Fig. 1. *Gymnostoma papuanum* × UFGCgI1 at 6 months after nodule initiation. Two adjacent cells contain scattered small sporangia. Fig. 2. *Gymnostoma papuanum* × UFGCeI5 at 6 months after nodule initiation. Sporangia were formed within a single cell. The sporangium indicated by the arrow on the left appears partially disintegrated, leaving individual "free" spores. Fig. 3. *Gymnostoma papuanum* × UFGMuI1 at 6 months after nodule initiation. A single sporangium is visible. Fig. 4. *Casuarina glauca* × UFGCgI1 at 3 months after nodule initiation. Several sporangia are closely arranged within a single cell. Note secondary wall thickening around infected cells (arrowheads) characteristic of *Casuarina* spp. Fig. 5. *Myrica cerifera* × UFGCgI1 at 6 weeks after nodule initiation. A single large cell is evident containing multiple sporangia surrounded by a darkly staining matrix. Fig. 6. *Shepherdia argentea* × UFGCgI1 at 10 weeks after nodule initiation. A single sporangium is present within a cell still colonized by *Frankia* hyphae and symbiotic vesicles. Arrowheads indicate senescent "ghost" vesicles. Fig. 7. *Elaeagnus angustifolia* × UFGCgI1 at 6 weeks after nodule initiation. Several sporangia occur within the intercellular space (IS) of the nodule. Arrowheads indicate mature vesicles within the adjacent infected cell.

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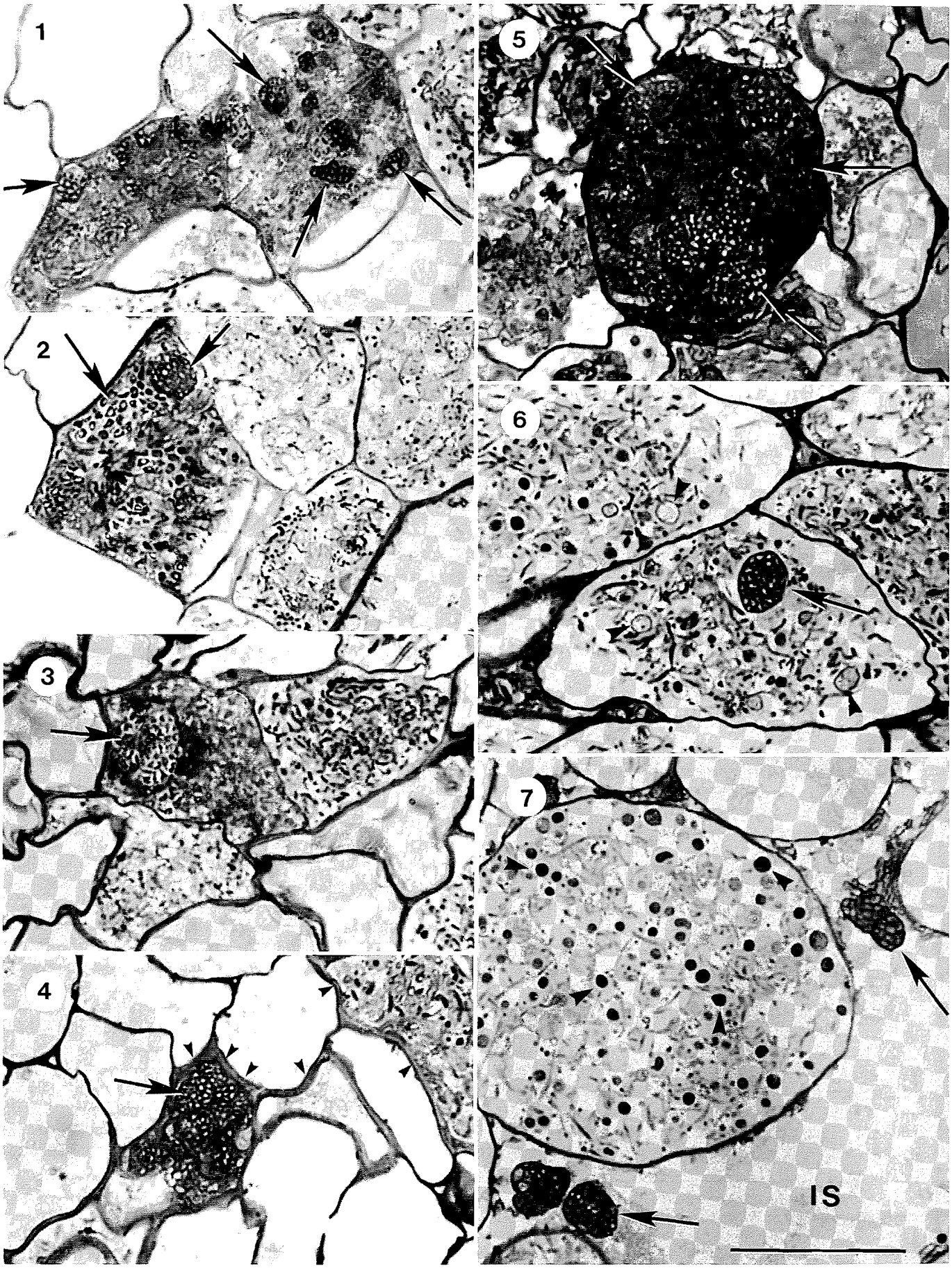

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TABLE 2. Infectivity, effectivity, and *in vivo* sporulation of *Frankia* isolate UFGCgI1 in various actinorhizal host plants

Plant species	Infectivity	Effectivity	Nodule age (weeks)	Sample size	Occurrence of sporulation
Comptonia peregrina	−				
Casuarina glauca	−*				
Elaeagnus angustifolia	+	+	6	10 nodules	2 nodules with 1 to several intercellular sporangia
			10	10 nodule lobes	None
Shepherdia argentea	+	−	6	6 nodules	Several intercellular sporangia in 1 nodule
			10	10 nodules	A single intercellular sporangium in each of 2 nodules
Myrica cerifera	+	+	6	10 nodules	In 1–2 cells in each of 5 nodules
			10	10 nodule lobes	In 1–2 cells in each of 3 lobes

*CgI1 nodulates *C. glauca* on rare occasions, producing effective nodules (see Table 1).

number of nodules examined from any one plant species came from *G. papuanum*, as this was the species in which sporulation was first noted. Sporulation was first seen during routine examination by hand sectioning of nodules induced by UFGCeI5. This was the only instance in which sporulation of a single cell in a nodule lobe was detected via hand sectioning, even in material that was later shown to have a low level of sporulation when examined via plastic sectioning. Reliable detection of low levels of sporulation therefore required embedding and thin sectioning of nodules. As can be seen in Table 1, sporulation was often absent in any given nodule or nodule lobe. In the case of *G. papuanum* inoculated with UFGCeI5 and harvested 6 weeks after nodule initiation no sporulation was observed, although sporulation was seen in a separate experiment at 6 months after nodule initiation. Sporulation in *G. papuanum* was also seen in 6-month-old nodules induced by UFGMuI1 and UFGCgI1 and in 10.5-week-old nodules induced by UFGCgI1. Nodules from a variety of other *G. papuanum–Frankia* isolate combinations (Table 1) failed to show any sporulation even at 16 months of age, consistent with all previous sampling of nodules induced by those isolates.

The case of sporulation in *C. glauca* inoculated by UFGCgI1 (Figs. 4, 8–10; Table 1) was based on the examination of a very small sample. In the nodule studied with light microscopy (Fig. 4), only 4 of 10 nodule lobes that were sectiond showed any sporulation by *Frankia*. As indicated by Table 2, UFGCgI1 is usually noninfective for *C. glauca*, but on several occasions this cross-inoculation resulted in the formation of a few effective nodules, accounting for the small sample size.

Sporulation by UFGCgI1 in *M. cerifera* nodules (Fig. 5) was similar to that seen in *G. papuanum*. The presence of a darkly staining material around the sporangia within some cells was seen in both species. The presence of sporulation at 6 weeks in *M. cerifera* and not in *G. papuanum* may relate to the more rapid progression of infection and nodule development in *M. cerifera* as compared with *G. papuanum*.

Shepherdia argentea nodules induced by UFGCgI1 were apparently ineffective, as the seedlings never recovered from nitrogen deficiency. Nodules generally remained small with few infected cells. Unlike the ineffective nodules of *E. umbellata* induced by EuI1b (Baker *et al.* 1980), there were vesicles present in some of the *S. argentea* nodules. Similar to *E. umbellata* though was the presence of both intercellular sporangia (not illustrated) and intracellular sporangia (Fig. 6), both of which occurred at very low levels.

Intercellular sporulation in *E. angustifolia* (Fig. 7) was seen in only two cases, both at 6 weeks after nodule initiation. At 10 weeks nodule lobes were quite large and the very low level of sporulation seen at 6 weeks could easily have been missed in sampling.

Sampling at the transmission electron microscope (TEM) level presented even greater difficulty because of the low frequency of sporulation. Figures 8–10 show TEM micrographs of sporulation by UFGCgI1 in root nodules of seedlings of *C. glauca*. All sporangia observed were intracellular, occurring in both living and already senesced nodule cortical cells.

Discussion

Sporulation of *Frankia* within field-collected nodules is not uncommon and all *Frankia* isolates have been found to be capable of sporulating in culture. Despite these facts the isolation and culture of a strain of *Frankia* that sporulates within a nodule has proven difficult (Torrey 1987). As the results show, low-level sporulation in nodules produced by inoculation with pure, cultured *Frankia* strains does occur with several specific *Frankia* isolates, namely UFGCgI1, UFGCeI5, and UFGMuI1. The level of sporulation that occurred in nodules grown under typical water-culture conditions was generally one to several sporangia, or one to two cells containing spores or sporangia, in 10–50% of nodules or nodule lobes examined.

For isolate UFGCgI1 the expression of low-level sporulation has been seen to occur across a range of host species. In three species, *M. cerifera*, *C. glauca*, and *G. papuanum*, sporulation was seen only intracellularly. In *E. angustifolia* intercellular sporulation occurred and in *S. argentea* both types were seen. This difference in the location of sporulation probably relates to the difference in the infection process between these species. All three species in which only intracellular sporulation was seen are infected via root hairs, with *Frankia* hyphae growing from cell to cell directly through adjacent cell walls. In the two species in which intercellular sporulation was seen infection occurs via intercellular penetration of the epidermis

FIGS. 8–10. Transmission electron micrographs of sporulation by *Frankia* isolate UFGCgI1 in root nodules of *C. glauca*, fixed after 4 weeks exposure of root systems to hyperoxia (35% O_2 in air). Fig. 8. At least five sporangia (*) were formed in this living cortical cell of a root nodule lobe. Some spores within one sporangium have matured (arrow). Scale bar = 5 μm. Fig. 9. A dead nodule cell that contained released spores (arrow). Scale bar = 5 μm. Fig. 10. An enlarged area from Fig. 8 showing simple fission of a prespore cell. Scale bar = 0.5 μm.

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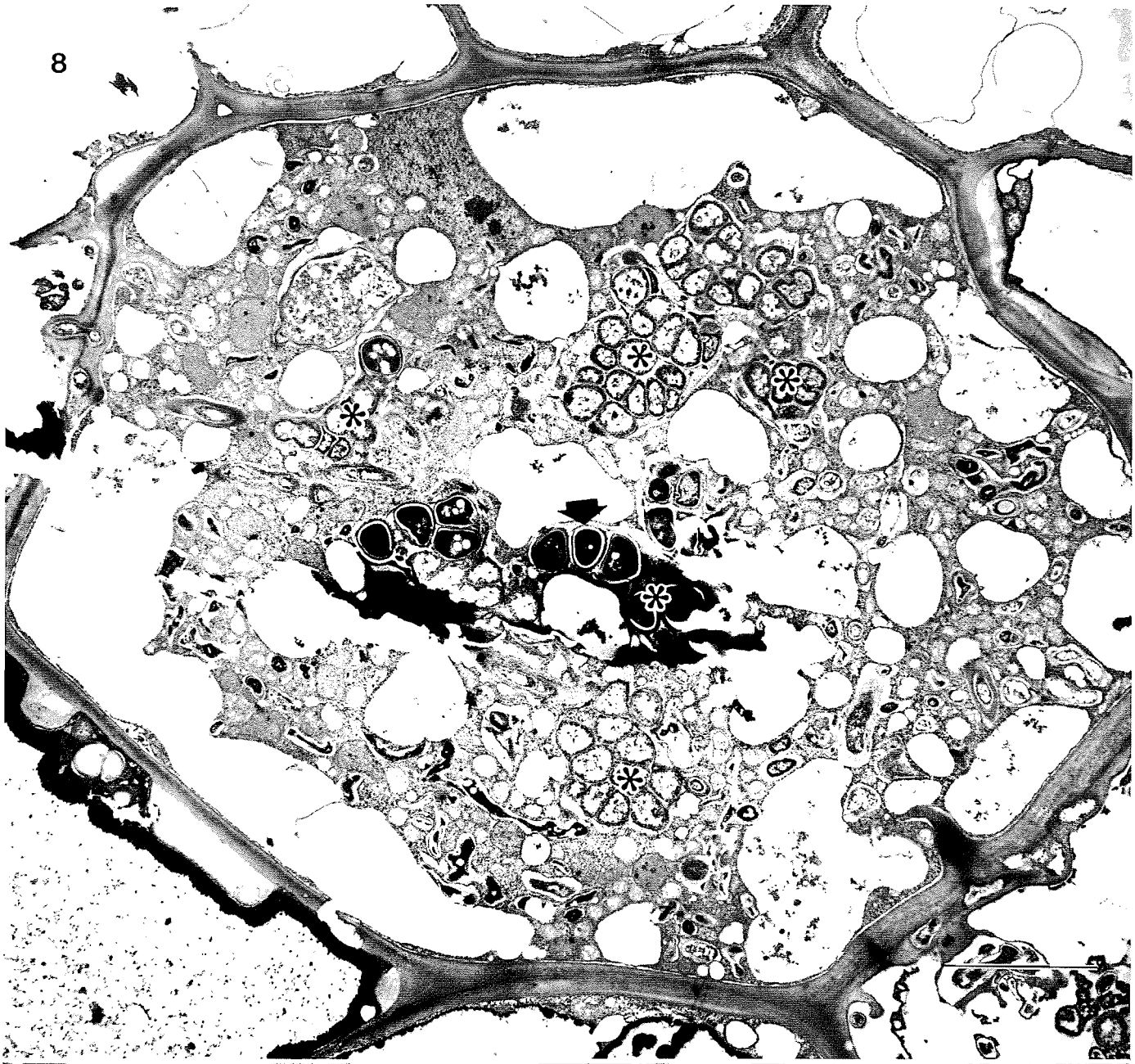

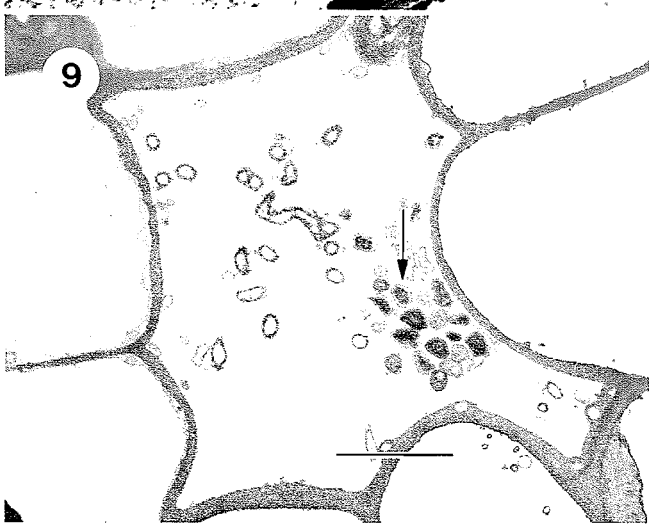

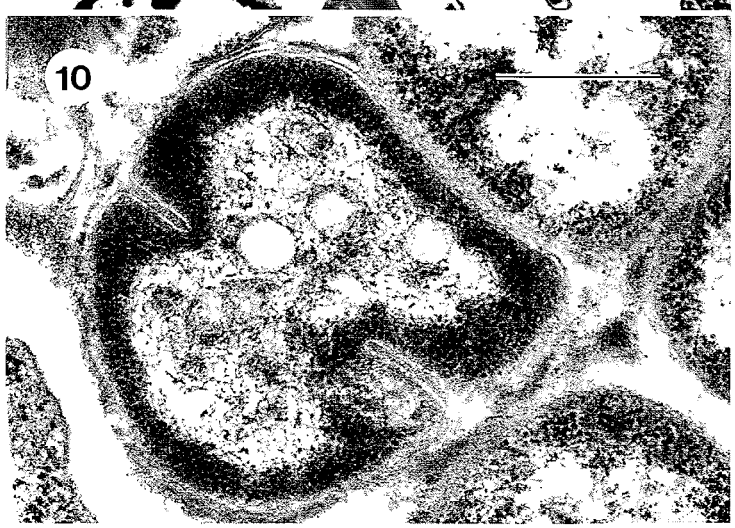

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and cortex, with each cell being directly infected by hyphae growing intercellularly.

The existence of these isolates that show low-level sporulation in the nodule supports the hypothesis that *in vivo* sporulation by *Frankia* is primarily controlled by the genetics of the actinomycete, not by the environment in which the nodule is growing. That these isolates can be used to obtain a fairly consistent, low level of sporulation within nodules may make them useful for the study of possible secondary environmental effects of sporulation within the nodule. The results of experiments attempting to alter *in vivo* sporulation by imposing different treatments upon the plant, such as the growing of plant roots under hyperoxic conditions, will be reported separately.

Although these three isolates do show sporulation in root nodules, the nodules would not be considered spore (+) under current usage of the term in the literature. Examination of these isolates in culture, looking for differences from other *Frankia* isolates, in parameters such as nutrient utilization, could provide a clue as to why it has been so difficult to isolate and culture *Frankia* from spore (+) nodules. These isolates also possess the unusual trait of spontaneous spore release in culture, and at least for UFGCgI1 and UFGCeI5 those spores have been found to germinate in high percentages (UFGMuI1 has not been studied in this regard). It is not known whether this trait in any way relates to their ability to sporulate in the host, but it would be of interest to examine for sporulation in nodules produced by inoculation with other isolates that show these spore-release and (or) spore-germination characteristics.

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