Characterization of an ineffective actinorhizal microsymbiont, *Frankia* sp. EuI1 (Actinomycetales)

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Accepted June 9, 1980

BAKER, D., W. NEWCOMB, and J. G. TORREY. 1980. Characterization of an ineffective actinorhizal microsymbiont, *Frankia* sp. EuI1 (Actinomycetales). Can. J. Microbiol. **26**: 1072–1089.

The actinomycete, *Frankia* sp. EuI1, isolated from root nodules of *Elaeagnus umbellata* is an infective endophyte but which lacks the ability to form an effective nitrogen-fixing symbiosis with its host. This ineffective organism can be distinguished easily from other frankiae, *in vitro*, on the basis of size, morphology, and the elaboration of a diffusible pigment. Cross-inoculation studies indicated that the host range of this symbiont is narrow and probably restricted to the Elaeagnaceae. In all cases of nodulation the symbiosis never developed nitrogenase activity and the microsymbiont never produced endophytic vesicles within the infected host cells. Sporangia were produced *in vivo* and *in vitro* so the morphogenetic block is apparently restricted to vesicle formation.

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L'actinomycète, *Frankia* sp. EuI1, isolée des nodules racinaires de *Elaeagnus umbellata* est une endophyte infectieuse qui n'a pas la capacité d'établir une symbiose efficace avec son hôte pour la fixation d'azote. L'organisme inefficace peut être rapidement distingué des autres *Frankia*, *in vitro*, de par sa dimension, sa morphologie et l'élaboration d'un pigment diffusible. Des études d'inoculations croisées indiquent que la gamme d'hôtes de ce symbiote est étroite et probablement restreinte aux Elaeagnacées. Dans tous les cas de nodulation, la symbiose n'a jamais produit d'activité nitrogénase et le microsymbiote n'a jamais formé de vésicules endophytes à l'intérieur des cellules hôtes infectées. Il y eut production de sporanges *in vivo* et *in vitro*, de sorte que le blocage morphogénétique est apparemment restreint à la formation de vésicules.

[Traduit par le journal]

Introduction

Studies of actinomycete–nodulated plant symbioses have lagged behind similar studies of the rhizobial–legume symbioses due to the relative difficulty of culturing *in vitro* the actinomycete microsymbiont. Recent successes in nodule actinomycete isolation in *in vitro* cultivation (Callaham *et al.* 1978; Berry and Torrey 1979) have renewed interest in this field of study. Baker *et al.* (1979) isolated an actinomycete from root nodules of *Elaeagnus umbellata* Thunb. by a procedure that was radically different from past isolation procedures (Baker and Torrey 1979). Briefly, the technique was to prepare a crushed nodule suspension from surface-sterilized root nodules, filter this suspension through Miracloth and glass wool, and apply the suspension to a discontinuous sucrose-density gradient composed of three layers: 60, 45, and 30% (w/v) sucrose. After sedimentation centrifugation the gradient was fractionated dropwise and certain fractions were used to inoculate pour plates of a yeast extract dextrose medium (Baker *et al.* 1979). The actinomycete which grew on the inoculation plates bore similarities to other *Frankia* species (Callaham *et al.* 1978; Berry and Torrey 1979) and was able to induce nodulation when applied to *Elaeagnus* seedlings. The induced nodules subsequently were shown to have no acetylene reduction activity and the young seed-

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0008-4166/80/091072-18$01.00/0

lings never recovered from nitrogen starvation. This paper describes further investigations of this ineffective organism, designated *Frankia* sp. EuI1, undertaken to better characterize its growth *in vitro*, its host range, and the root nodules which it induces.

Materials and methods

Culture in vitro

For routine culture of the frankiae, a simple yeast extract dextrose broth was used (Baker and Torrey 1979). The following additional media were used for characterization *in vitro*: Bennett's agar, one-half strength (B/2) (Gordon 1968); Czapek's agar (Waksman 1961), supplemented with 0.4% yeast extract (YCz); glucose asparagine agar (GUA) (Waksman 1961); glycerol asparagine agar (GYA) (Shirling and Gottlieb 1966); oatmeal agar (OAT) (Shirling and Gottlieb 1966); soil extract agar (SXT) (Gordon 1968); tap water agar (TAP); 1% Sigma agar; *Frankia* broth agar (FRB) (Baker and Torrey 1979); yeast malt (YM) (Shirling and Gottlieb 1966); nutrient agar supplemented with 0.2% Tween 80 (NTW); and *Frankia* broth agar, one-fifth strength, supplemented with 5% purified potato starch (J. T. Baker) (STR).

All growth media were inoculated using a pour-plate technique, incubated in the dark at 28°C, and examined at regular intervals up to 4 weeks. Growth characteristics recorded included growth, colony size and shape, pigmentation, and presence or absence of sporangia.

Cross-inoculation studies

The following species were tested as possible hosts: *Alnus rubra, Alnus viridis* ssp. *crispa, Ceanothus americanus, Comptonia peregrina, Elaeagnus angustifolia, Elaeagnus umbellata, Hippophaë rhamnoides, Myrica cerifera, Myrica gale,* and *Shepherdia argentea*. All plants on this list are woody dicots which in nature form actinomycete-induced root nodules capable of fixing atmospheric nitrogen. Six seedlings of each were placed in 400 mL water culture jars (three plants per jar) and supplied with a one-quarter strength nitrogen-free nutrient solution (Hoagland and Arnon 1950). After 1 week the plants were inoculated with a small amount of a suspension of *Frankia* sp. EuI1 which had been washed in distilled water and homogenized. The plants were observed over an 8-week period for the development of the nodules. The nutrient solution was completely renewed at weekly intervals.

Ultrastructural observations

Scanning electronmicroscopy (SEM)

Young developing nodules were excised from the root system and fixed in 2% glutaraldehyde in Sorensen's phosphate buffer for 3 h at 4°C. After washing in buffer the specimens were dehydrated through increasing concentrations of ethanol and then critical point dried using liquid CO_2 as an intermediate fluid. At this point the dried nodules were sliced with a double-edged razor blade to expose the interior of the nodule and then mounted on an SEM stub using "double-sticky" cellophane tape. The mounted specimens were "sputter-coated" with gold–palladium in a Technics Hummer II sputter-coater and then observed and photographed using an AMR-1000 scanning electron microscope at an accelerating voltage of 20–30 kV.

Samples of the actinomycete grown *in vitro* were prepared for SEM in a similar manner. A small amount of the organsim from broth cultures was filtered onto a 1.0-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) with vacuum. The membranes bearing the filtered fractions were fixed and prepared as above.

Light and transmission electron microscopy (LM and TEM)

Median slices, halved nodules, and intact small nodules were fixed in 2% glutaraldehyde in 0.025 *M* potassium phosphate buffer, pH 6.8, for several hours, rinsed in buffer, postfixed in 1% osmium tetroxide, rinsed again in buffer, dehydrated in an acetone series, and embedded in Spurr's resin (1969). The cultured actinomycete was fixed for 2 h at room temperature in 2% glutaraldehyde in the culture medium to minimize disruption of the fragile mycelium. The tissue was then transferred to a 12-mL centrifuge tube, rinsed in phosphate buffer, postfixed in 1% osmium tetroxide for several hours at room temperature, and washed in buffer; the tissue was gently centrifuged between these steps. The tissue was embedded in 2% agar to further minimize fragmentation of the mycelium and to eliminate further centrifugation. The agar slab was cut into 2- to 3-mm squares, dehydrated in acetone, and embedded in either Spurr's resin or Ladd's ultralow viscosity resin.

For bright-field optical microscopy, 0.5-μm sections were cut with glass knives and stained with 0.05% toluidine blue O in 1% sodium tetraborate. For epifluorescence microscopy similar sections were placed on glass slides, immersed in sodium methoxide (Erlandson *et al.* 1973) for 45 min to remove the plastic, rinsed in 1% periodic acid for 10 min, stained with the fluorescent Schiff (PAS) reagent (Culling 1974), examined with a Leitz epifluorescence microscope using a broad-band filter system (BG exciting filter and a K515 suppression filter), and photographed using Ilford HP5 or Kodak Tri-X film. Starch grains, cell walls, and other carbohydrates fluoresced a bright yellow–gold color. Living actinomycete cultures were examined using interference contrast optics. For TEM silver sections were cut with glass knives, stained for 30 min with a saturated solution of uranyl acetate in 50% ethanol followed by 5 min in 0.02% lead citrate (Venable and Coggleshall 1965), and examined with a RCA EMU 4C transmission electron microscope.

Results

Growth in vitro

Growth characteristics of *Frankia* sp. EuI1 are shown in Table 1. Growth was generally good on most media tested with the exception of GYA, YCz, OAT, and TAP, which supported little if any growth. Good growth of this organism was achieved on agar slants but the rate of growth was generally retarded in comparison with submerged culture. No growth of this organism was observed if the cultures were incubated anaerobically and it did not show the ability to liquefy gelatin. Pigmentation, a character peculiar to this strain, was not evident on any of the media tested after 4 weeks. However, cultures on B/2, FRB, and YM agar were routinely pigmented if incubated for 6–8 weeks.

One distinctive character of EuI1 *in vitro* was the ability to precipitate calcium oleate crystals from a medium which contained a fatty acid supplement such as Tween 80. This is not a character of other frankiae so far isolated (Baker and Torrey 1980) and may serve as a distinguishing feature. Such a

TABLE 1. Cultural characteristics of *Frankia* sp. EuI1 on agar pour plates at 4 weeks. All cultures were prepared by the pour-plate technique and incubated at 28°C

Media	Growth characteristics
B/2	Growth excellent, colonies compact, no pigmentation*, sporangia present
YCz	Growth negligible
GUA	Growth excellent, colonies diffuse, no pigmentation, sporulation extensive
GYA	No growth
OAT	No growth
SXT	Growth moderate to good, colonies compact, no pigmentation, sporangia present
TAP	No growth
FRB	Growth good, very dense compact colonies, surface colonies extend above agar, sporulation moderate, slight diffusable pigmentation*
YM	Growth minimal, very dense colonies, no visible sporulation, no pigmentation*
NTW	Growth good, dense compact colonies, no pigmentation, sporulation minimal, calcium oleate precipitation reaction
STR	Growth good, sporulation extensive

*Pigmentation (brown – blood-red diffusible pigment) occurs normally on these media after a longer period of incubation.

character has proved useful in the taxonomy of other actinomycetes (Goodfellow 1971).

Host specificity of the organism

Results of cross-inoculation studies are summarized in Table 2. The host range of *Frankia* sp. EuI1 is narrower than that of other frankiae (Berry and Torrey 1979; Baker and Torrey 1980; Torrey *et al.* 1980) and from the results in Table 2 appears to be restricted to members of the Elaeagnaceae. In all cases of nodulation of EuI1, nitrogenase activity (acetylene reduction) was not observed. Likewise, as is shown below, endophytic vesicles were never observed in the induced nodules.

Morphogenesis and cytology

EuI1 forms a dense mycelial mat composed of filaments whose diameter is consistently less than 1 μm (Baker *et al.* 1979) and typically 0.3 μm (Fig. 1). Spherical or ovate sporangia are borne on thickened sporangiophores attached as lateral branches or at the ends of the vegetative hyphae or filaments (Figs. 1–3). Initially the end of the hypha swells forming a spherical, ovoid, nonseptate structure (Figs. 2–4). Transverse septa form (Figs. 5 and 12) and are followed by the growth of longitudinal septa (Figs. 6, 12, and 13) to form compartments, each of which develops into one or more spores (Figs. 7–9). The spores increase in size, becoming spherical, to ovoid, 0.5–1.0 μm in diameter, and remaining unsculptured (Figs. 1 and 10). The spores appear to be arranged in pairs (Fig. 10), presumably because the two daughter spores are derived from a common cell (Figs. 13 and 14). The sporangia exhibit considerable variability in their shape and size. Usually spores that mature earliest are located at the distal end of the sporangium which is typically wider than the proximal end (Figs. 1, 8, and 13).

The cytoplasm of the hyphae and sporangia contains numerous ribosomes, glycogen-like inclusions, lipid-like droplets, and large nucleoid regions (Figs. 11–15*b*). The lipid-like droplets are larger and more numerous in spores (Figs. 14 and 15*a*) and often appear to be membrane bound (Fig. 12). Developing spores sometimes contain tubular structures (Fig. 15*b*). The cell wall of both hypha and developing spores is multilayered with a thin electron-translucent layer adjacent to the plasma membrane, bounded on the outside by a double- or triple-track envelope (Lechevalier and Lechevalier

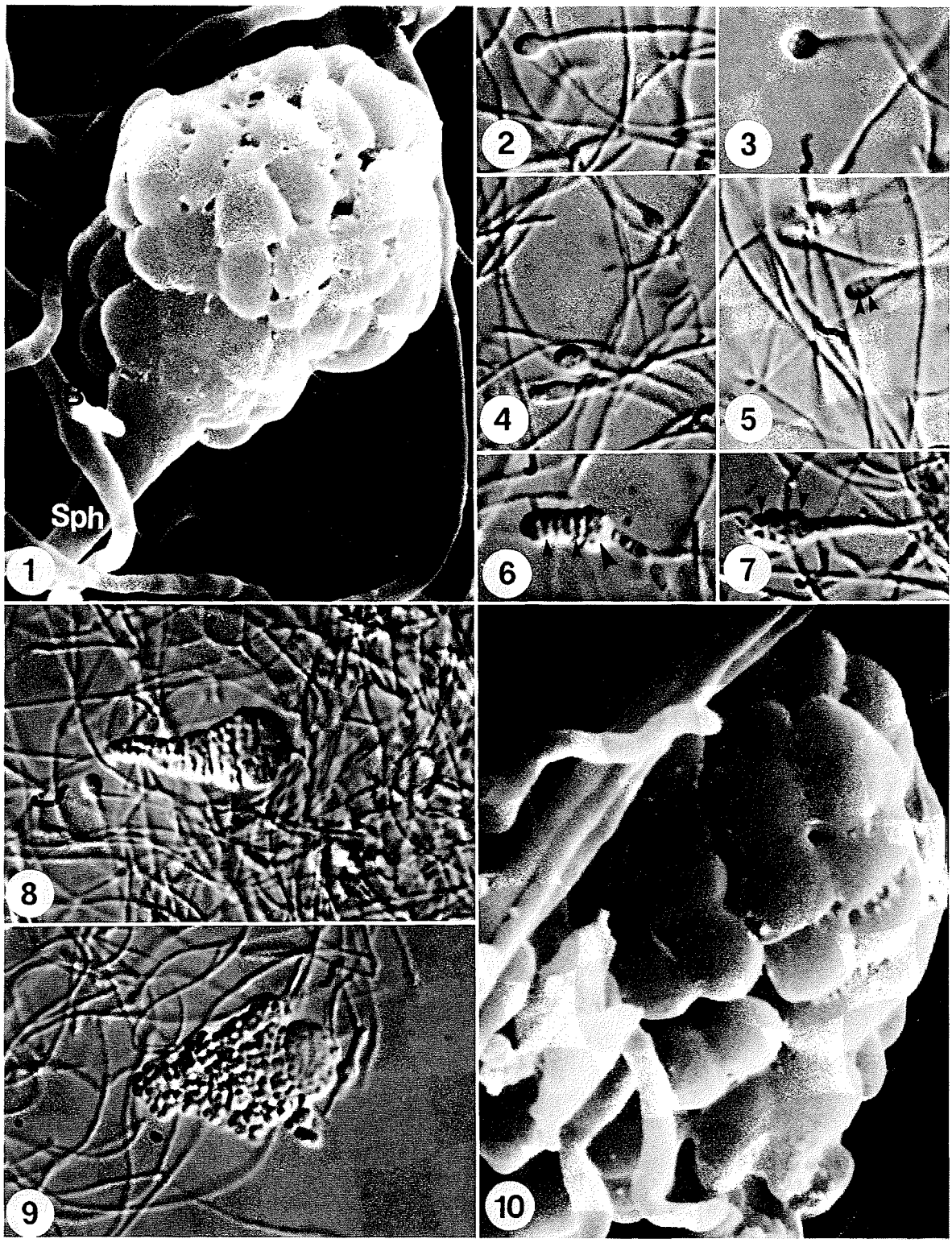
TABLE 2. Capacity of *Frankia* sp. EuI1 to infect host species

Plants not infected	Plants infected
A. rubra	*E. angustifolia**
A. viridis ssp. *crispa*	*E. umbellata**
C. americanus	*H. rhamnoides**
C. peregrina	*S. argentea**
M. cerifera	
M. gale	

*All plants developed ineffective symbioses.

ABBREVIATIONS USED: A, amyloplast; C, capsule; CW, host cell wall; G, Golgi; H, hypha; HB, hyphal branching site; L. lipid; M or *m*, mitochondrion; N, nucleus; NP, nuclear pore; Nu, nucleolus; Nuc, nucleoid region; P, proplastid; RER, rough endoplasmic reticulum; RV, ring-shaped vacuole; S, septum; Sp, sporangium; Sph, sporangiophore; V, vacuole; VA, void area; Ve, endophytic vesicle; W, endophytic wall.

FIG. 1. SEM of a mature sporangium showing the swollen sporangiophore and surrounding hyphal mycelium. Note hyphal branching site. × 13 640. FIGS. 2–9. LEM using interference contrast optics. Fig. 2. An early elliptically shaped nonseptate sporangium. × 1770. Fig. 3. Early spherically shaped nonseptate sporangium. × 1600. Fig. 4. Ovoid-shaped and elongate nonseptate sporangia. × 1600. Fig. 5. Ovoid-shaped sporangia, one of which has cross-septa (arrows). × 1670. Fig. 6. Developing sporangium containing cross-septa (small arrows) and a few longitudinal septa between developing spores (large arrow). × 2000. Fig. 7. Mature sporangium containing relatively few spores (arrows). × 1710. Fig. 8. Gradient of spore formation in a large sporangium. × 1510. Fig. 9. Spores being released from mature sporangium. × 1560. FIG. 10. SEM showing the paired arrangement of spores within a mature sporangium. × 10 800.



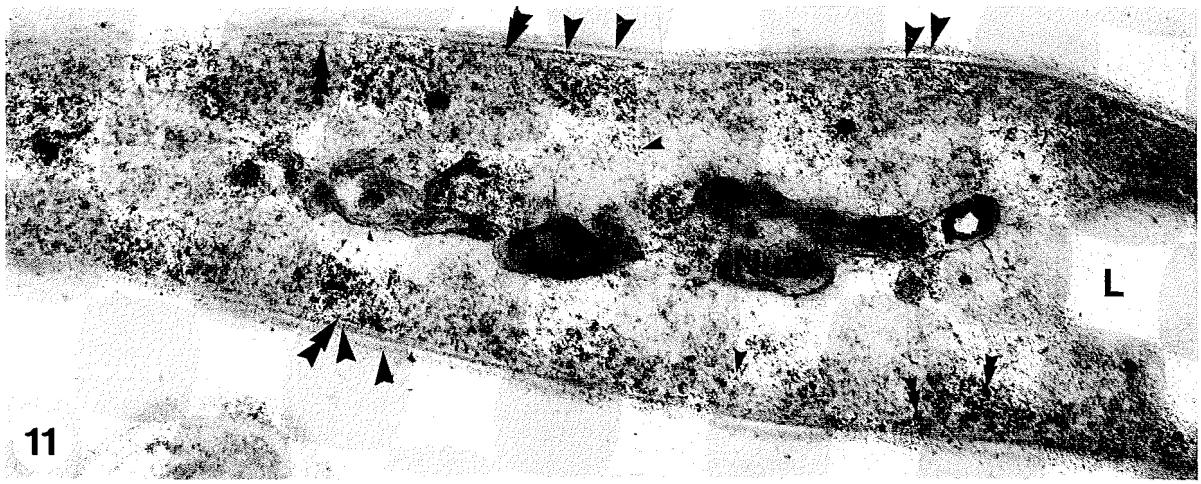
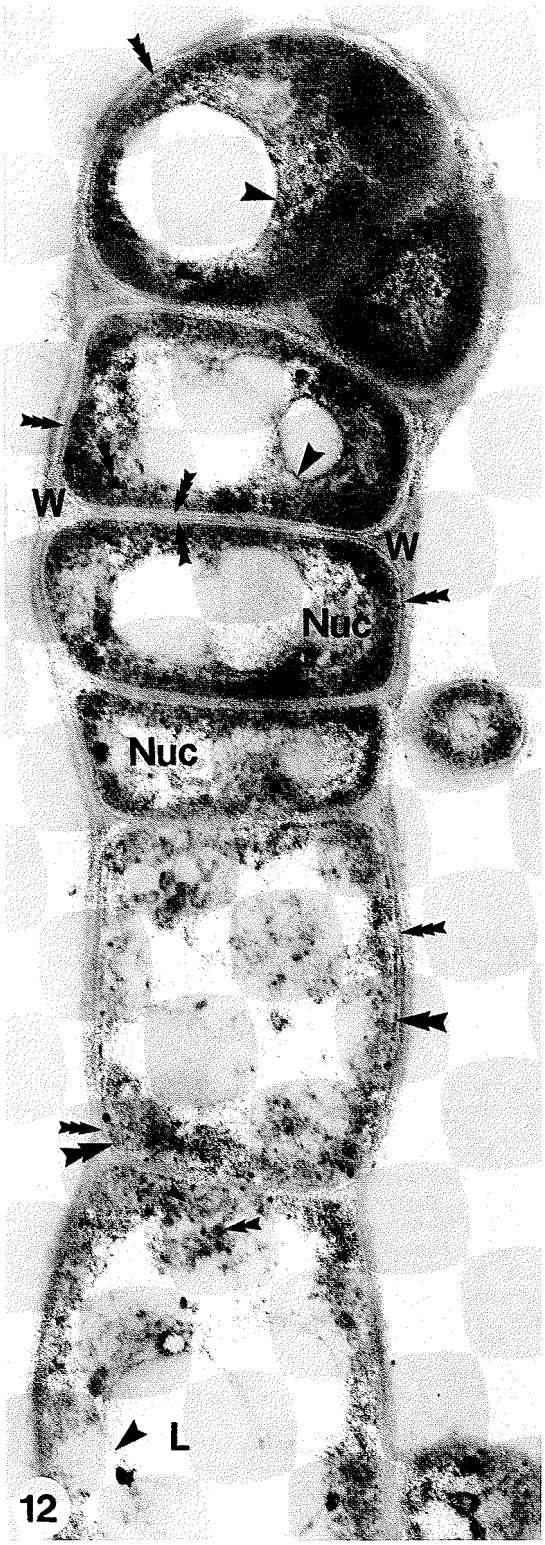


FIG. 11. TEM of a sporangium produced *in vitro*. A hypha with two electron-dense wall layers (single large arrows), plasma membrane (double large arrows), nucleoid region, lipid-like droplets, numerous ribosomes (single small arrows), and glycogen-like particles (double small arrows). × 76 270.

1979) which may appear to be partially sloughed off (Figs. 11–14 and 15*a*). Older mature spores possess more electron-dense layers and thicker translucent layers (Fig. 16) than immature spores; thus, mature spore walls are thicker than those of developing spores. Wall material is also present between developing spores and becomes more diffuse or less electron dense in older sporangia as the spores separate from one another (Figs. 14 and 15*a*).

Within the infected cells of ineffective root nodules induced by *Frankia* sp. EuI1, the endophytic actinomycete branches (Fig. 17) and ramifies (Fig. 18) throughout the cytoplasm of the host cell. Such cells contain large nuclei with prominent nucleoli, numerous proplastids, mitochondria, and small to intermediate size vacuoles, many free ribosomes and polyribosomes, and a few profiles of rough endoplasmic reticulum (Figs. 19–22). The endophyte is surrounded by a polysaccharide capsule (Figs. 20, 21*b*, and 22). The infected cells may undergo senescence when only a small endophytic mycelium is present. If the growth of the endophyte persists, a densely packed mycelium or "endophyte cluster" develops, occupying most of the nodule cell (Figs. 23 and 24) but no morphological differentiation of the endophyte (e.g., vesicle formation) occurs within these clusters, and no nitrogen fixation activity develops (Baker *et al.* 1979). Host cells containing endophytic clusters may have ring-shaped vacuoles enclosing small areas of cytoplasm (Fig. 24*b*); these areas may be sites of lysosomal activity although no direct enzymatic evidence for their role in the cell exists. These cells also contain large numbers of free ribosomes, many mitochondria and proplastids, and a few profiles of rough endoplasmic reticulum (Fig. 24). The filaments of the actinomycete also grow in association with the cell walls, being intimately appressed to the inner host cell wall surface (Figs. 22, 25, and 26) or within the middle lamella between adjacent cells (Fig. 26). The endophyte may form sporangia intercellularly within the middle lamella (Fig. 28) as well as intracellularly (Fig. 27). Sporangia persist in degenerating host cells containing many endophytic hyphae, presumably endophytic clusters (Fig. 27). The sporangia formed within nodule cells are made up of numerous spherical to ovoid spores (Fig. 27), which appear similar to those formed *in vitro* (Figs. 14 and 15*a*).

FIGS. 12 and 13. TEM of sporangia produced *in vitro*. Fig. 12. A longitudinally sectioned early sporangium. The cytoplasm contains numerous ribosomes (single small arrows), glycogen-like particles (double small arrows), lipid-like inclusions that appear to be surrounded by a membrane (single large arrows), and nucleoid regions. A plasma membrane (double large arrows) separates the cytoplasm from the wall. The wall consists of a thin inner layer separated from a thicker outer layer by an electron-dense layer (triple small arrows). Two cross sections of hyphae are also present. × 50 780. Fig. 13. A longitudinally sectioned sporangium containing maturing spores. The distal spores are larger and more spherical than the spores adjacent to the enlarged hyphal cells (cf. Figs. 1 and 12). The cytoplasm contains numerous lipid-like droplets. Note the two wall layers which are separated by a thin electron-dense layer (arrows). × 23 100.



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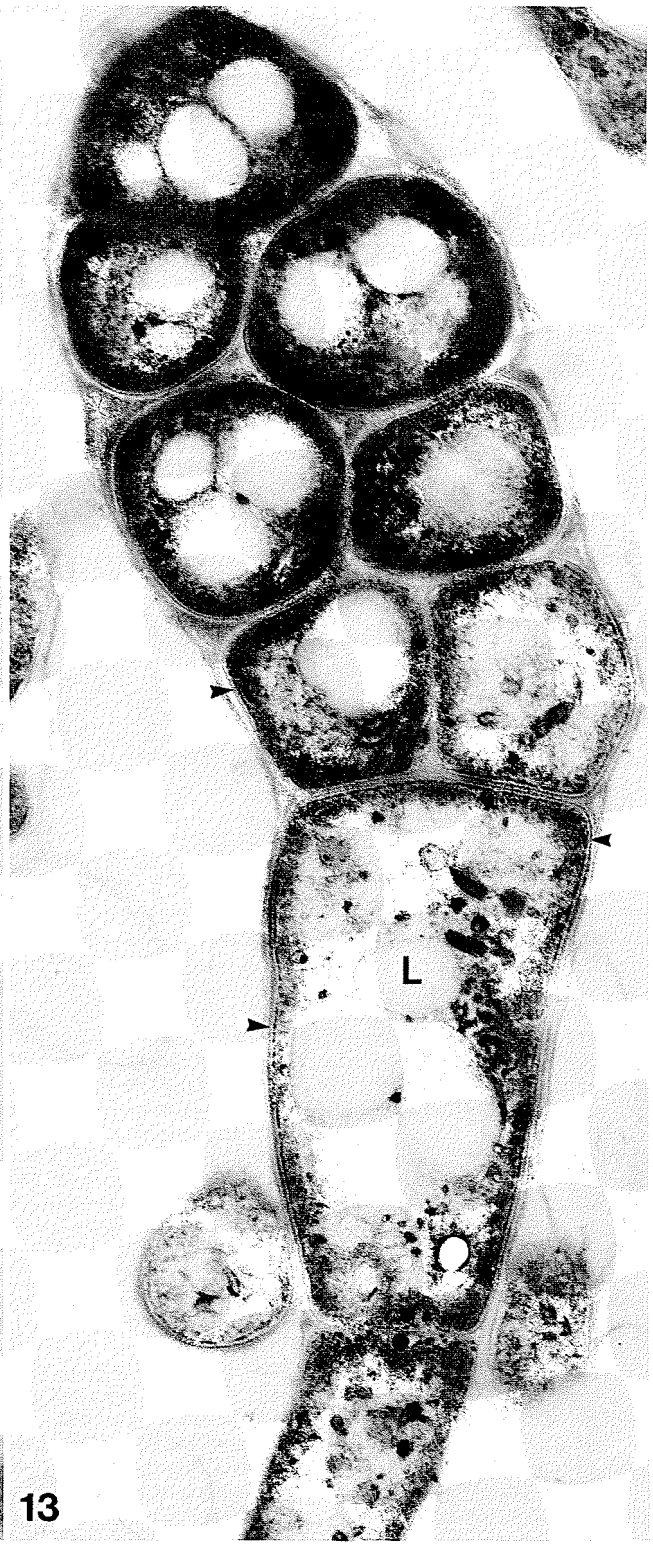

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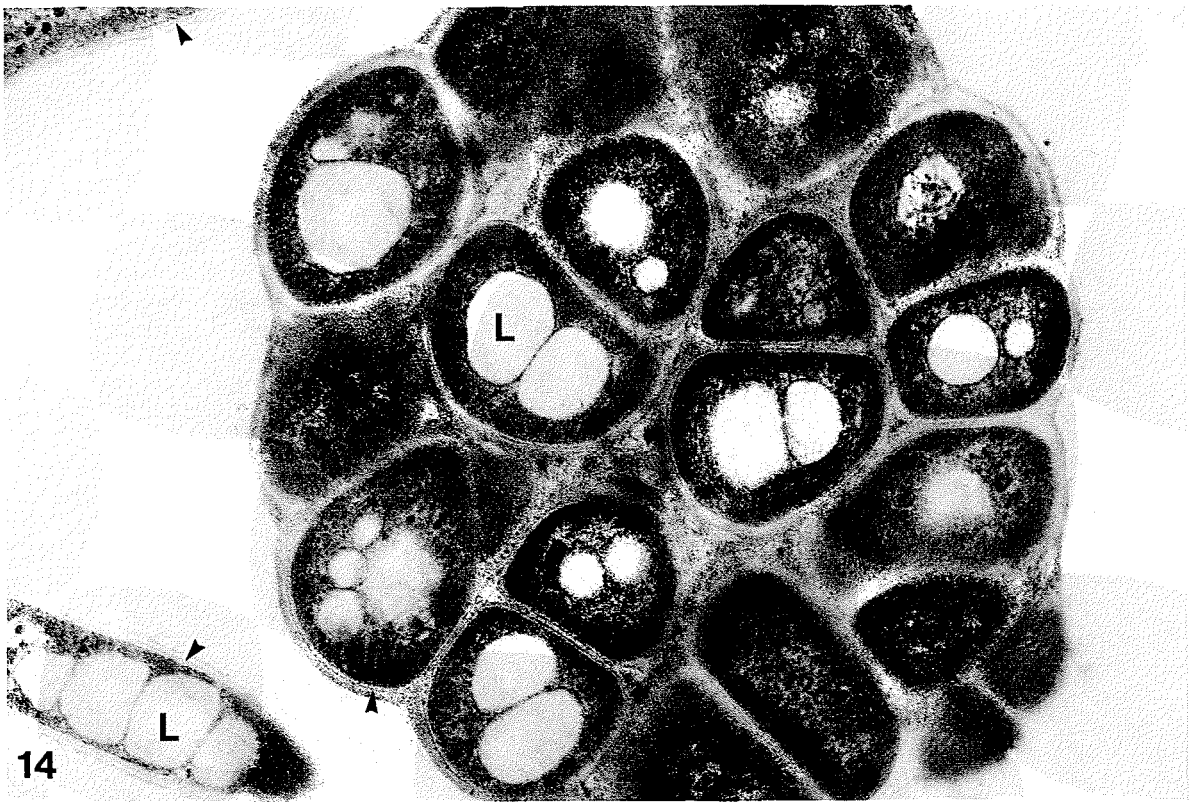


FIG. 14. TEM of a sporangium produced *in vitro*. A transversely sectioned early sporangium showing the paired arrangement of immature spores (cf. Fig. 10). Note the lipid-like inclusions and electron-dense (arrows) and translucent layers of wall surrounding both hypha and spores. × 39 900.

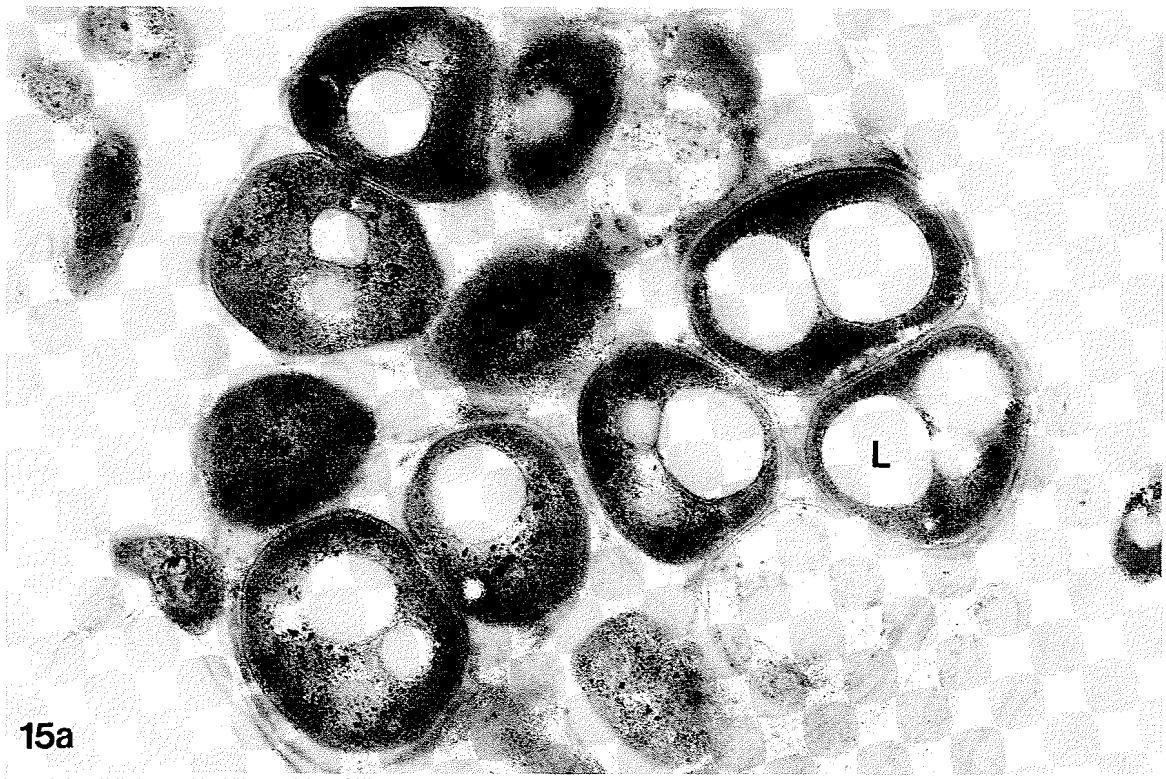
FIGS. 15 and 16. TEM of sporangia produced *in vitro*. Fig. 15*a*. A transversely sectioned sporangium which is developmentally more mature than that shown in Fig. 14. Although the spores have become more separated from one another, the paired arrangement of spores is still evident. Lipid-like inclusions and the layered walls are also illustrated. × 32 890. Fig. 15*b*. Higher magnification of a portion of Fig. 15*a* showing tubular structures (arrows) in developing spore. × 89 650. Fig. 16. Mature spore with lipid-like inclusions and multilayered wall consisting of multiple electron-dense (small arrows) and translucent layers adjacent to the plasma membrane (large arrows). × 65 780.

FIG. 17. SEM of an ineffective nodule infected cell at early invasive stage of hypha with the infection arising from one invasion site. ×5770. FIG. 18. SEM showing numerous hyphae ramifying throughout an infected cell of an ineffective nodule. ×3180. FIG. 19. LM of an ineffective nodule cell containing a large nucleus with a prominent nucleolus, numerous proplastids, several vacuoles, and hyphae. × 1470. FIG. 20. LM using epifluorescence optics of an ineffective nodule cell demonstrating the fluorescence of the capsule surrounding the endophytic hyphae and the host cell wall as well as the large amyloplasts in an adjacent unifected cell. Also shown are numerous vacuoles and the large nucleus and nucleolus. × 1380.

FIG. 21*a*. TEM of an infected cell similar to those shown in Figs. 19 and 20. Shown are the large nucleus and nucleolus, numerous proplastids, and mitochondria. Endophytic hyphae are surrounded by a capsule (arrows). × 5000. FIG. 21*b*. Higher magnification of outlined portion of Fig. 21*a* illustrating the large nucleus and nucleolus, nuclear envelope with numerous nuclear pores shown in cross section (single small arrows) and longitudinally (double small arrows), numerous polyribosomes (single large arrows), host plasma membrane (double large arrows), and the capsule which surrounds the endophytic hyphae. × 12 500.

FIG. 22. TEM of an ineffective nodule cell which contains many endophytic hyphae surrounded by a capsule (arrows), profiles of rough endoplasmic reticulum, small vacuoles, and a large lobed nucleus with a prominent nucleolus. Note the endophytic hyphae growing appressed to the cell wall. ×7010.

FIG. 23. SEM of ineffective nodule cell containing a densely packed endophytic mycelium or endophytic cluster. Shrinkage of cell contents is presumed to have occurred in preparation. × 1130. FIG. 24*a*. TEM of cell similar to that shown in Fig. 23. Numerous endophytic hyphae are shown, each of which is surrounded by a capsule. Also present are proplastids, small vacuoles, and areas of cytoplasm encircled by ring-shaped vacuoles which may be presumptive lysosomes. ×5480. FIG. 24*b*. Higher magnification of outlined portion of Fig. 24*a* showing hypha growing adjacent to host cell wall, capsule (arrows) surrounding endophytic hyphae, mitochondria, proplastids, Golgi, rough endoplasmic reticulum, and ring-shaped vacuoles containing cytoplasm. × 16 000.



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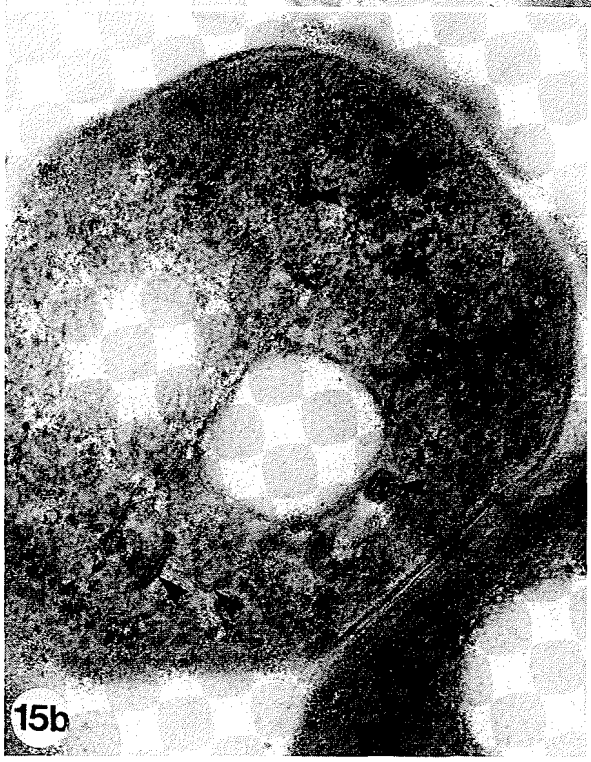

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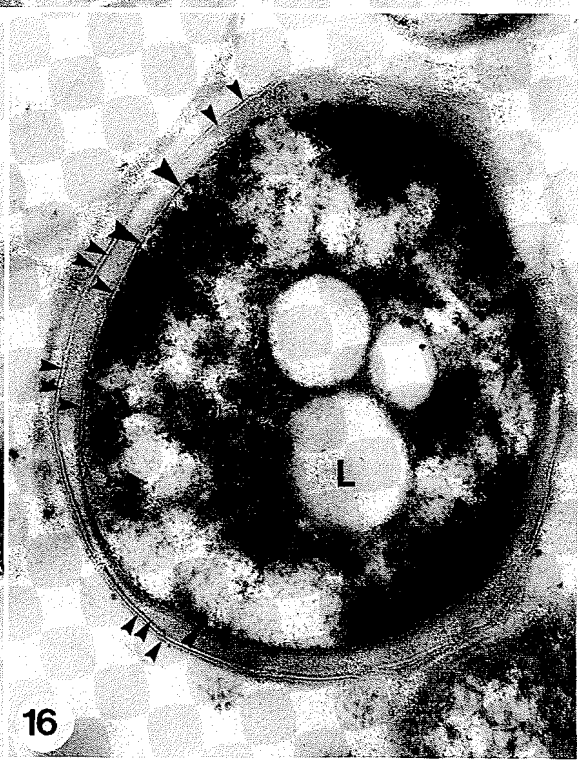

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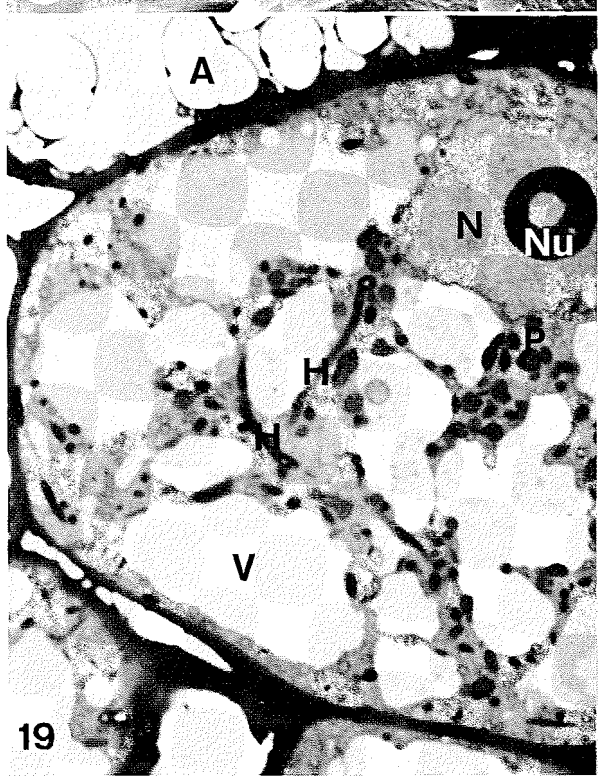

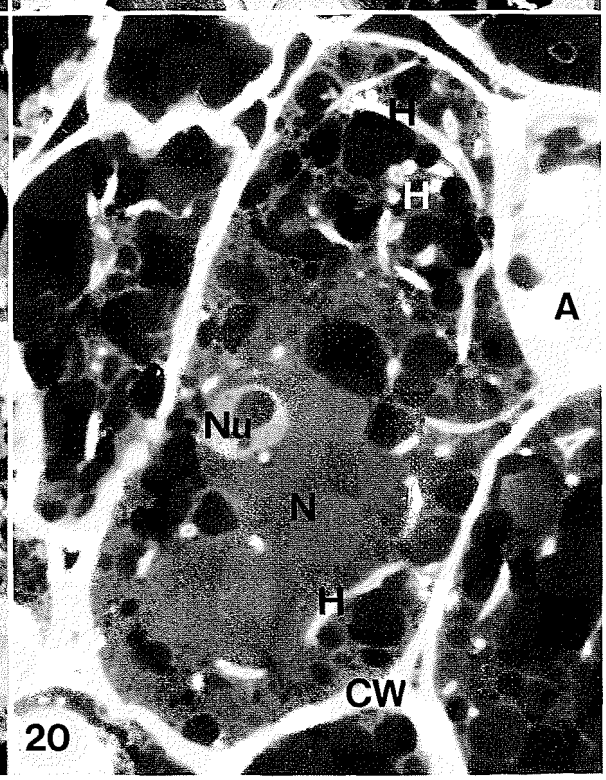

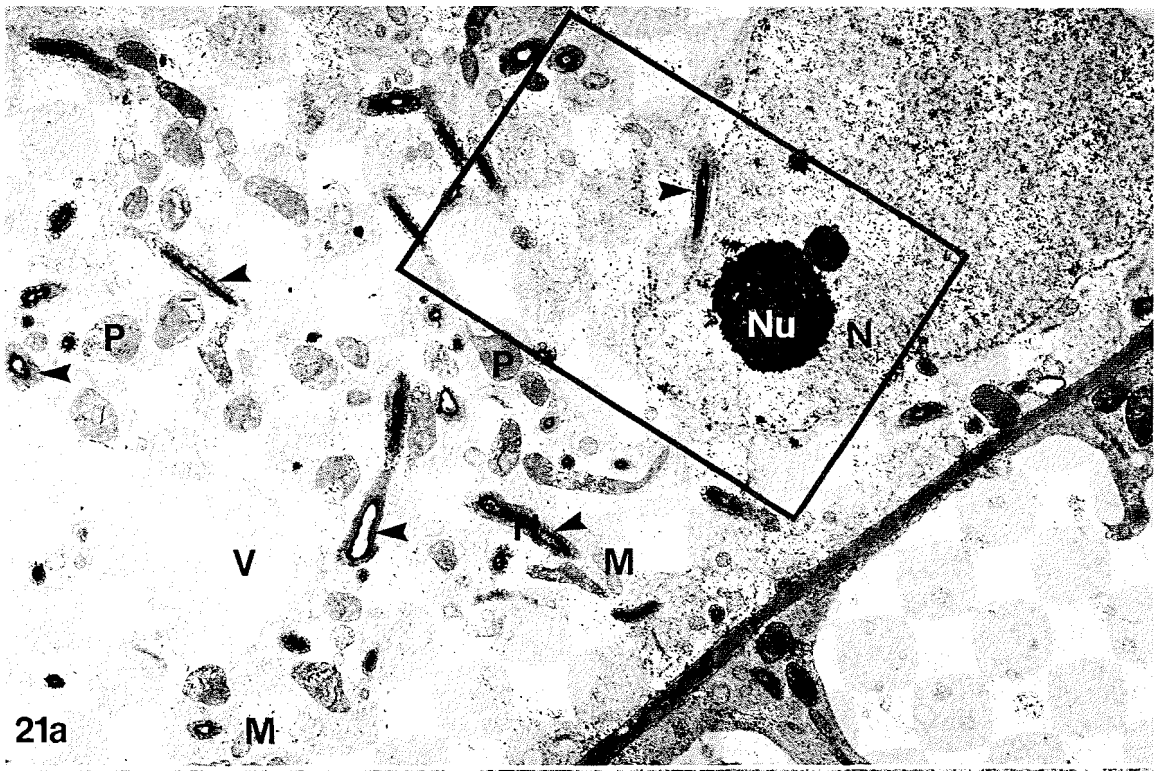

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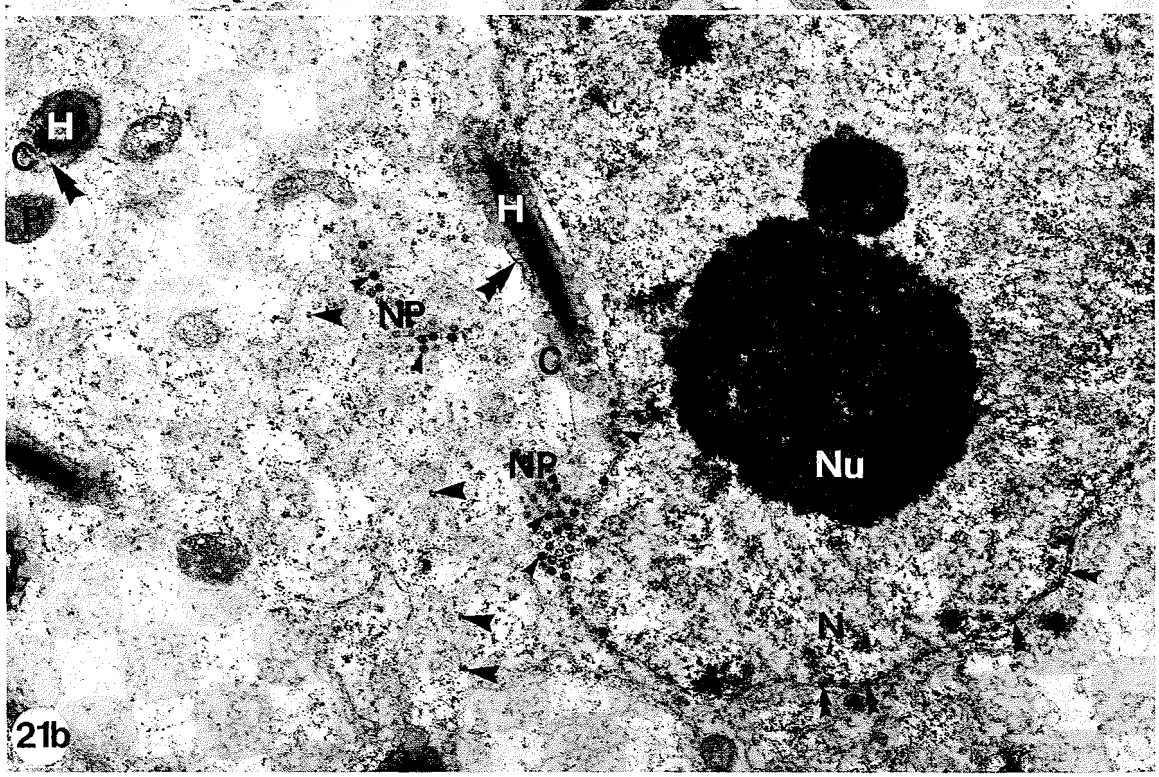

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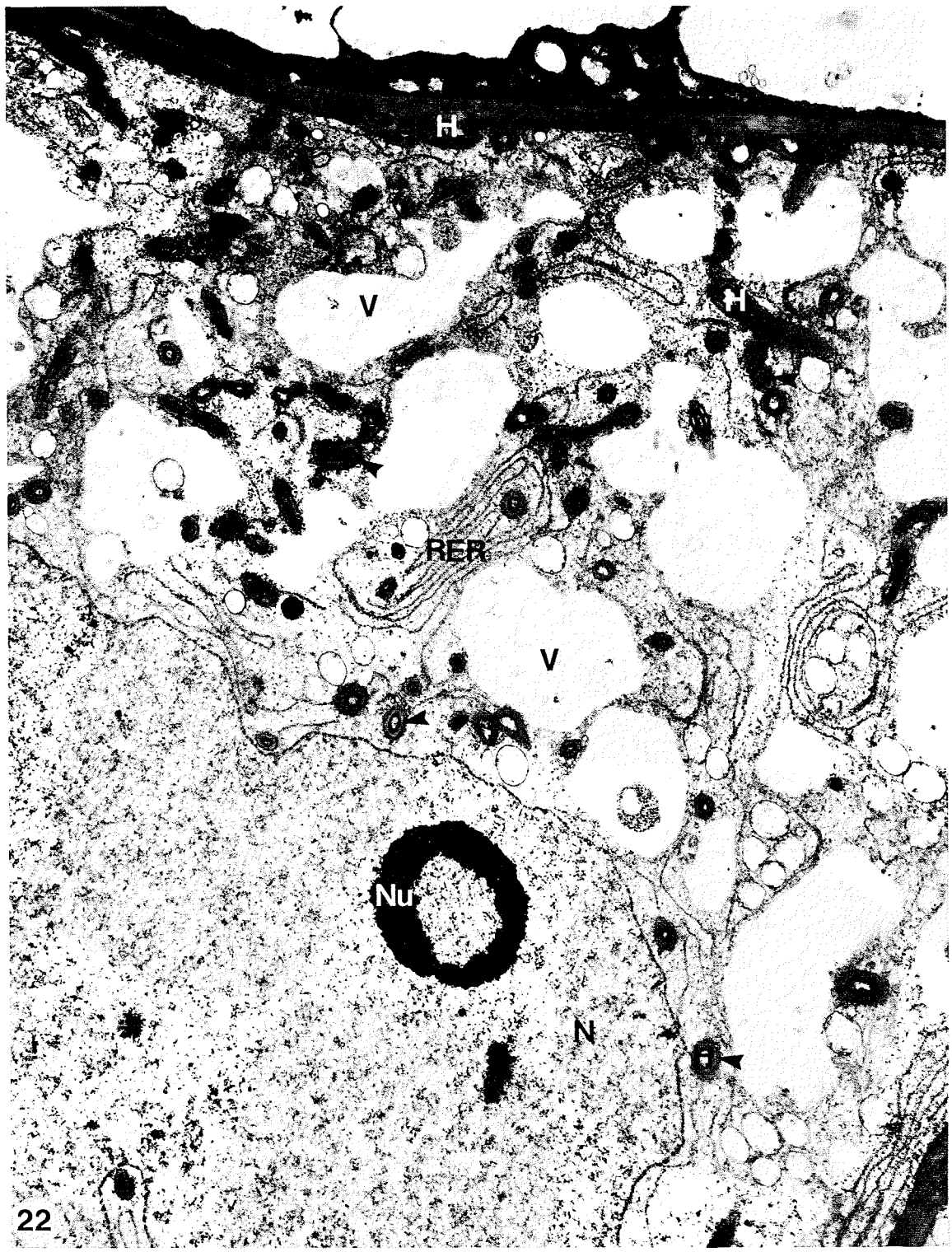

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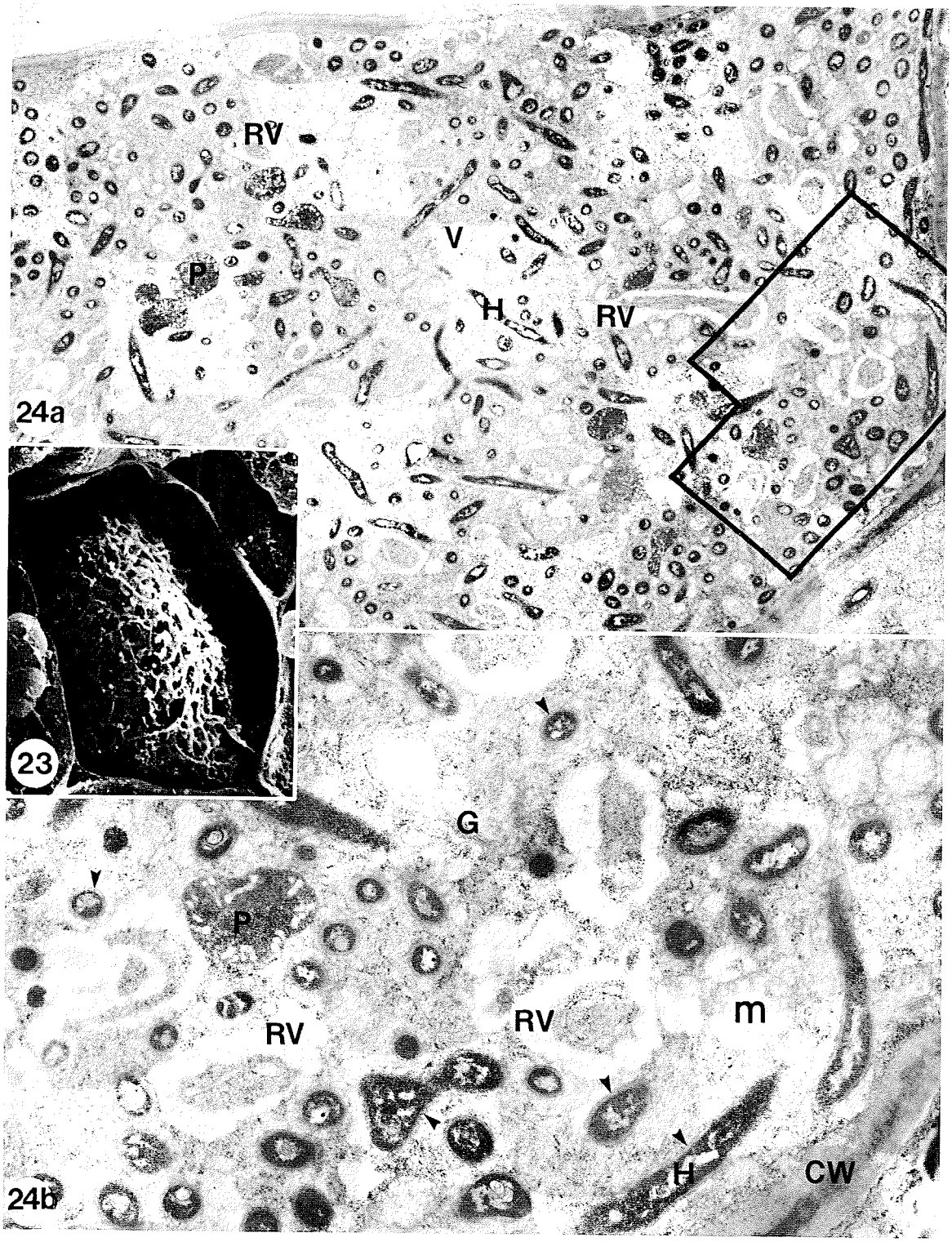


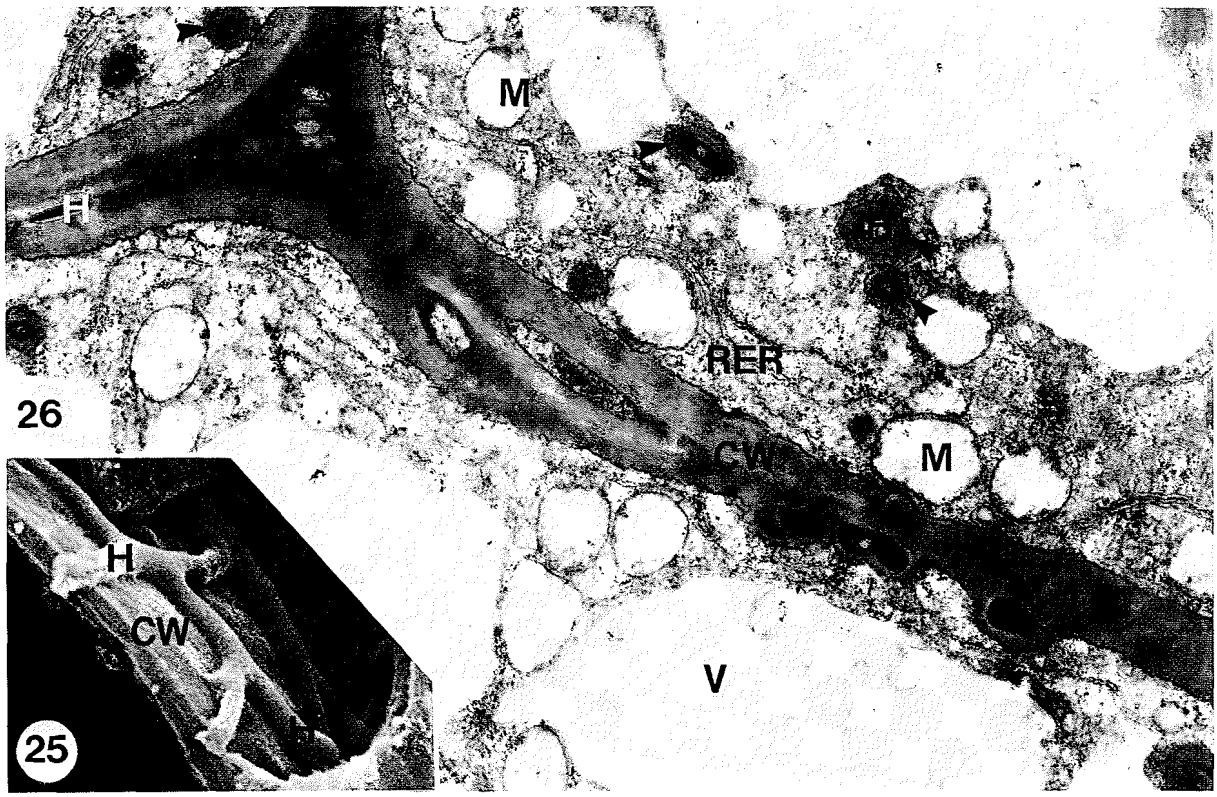


FIG. 25. SEM of endophytic hyphae growing on surface of cell wall within an ineffective nodule. × 11 090. FIG. 26. TEM of cell similar to that shown in Fig. 25. Endophytic hyphae are present within and on the surface of the cell wall and in the host cytoplasm. In all instances the hyphae are surrounded by host cell wall or capsule (arrows). × 13 180.

Effective, i.e., nitrogen-fixing, nodules from plants grown in water culture and inoculated with a suspension of crushed field-grown nodules contain many infected cells with endophytic clusters that have formed numerous small spherical or globose vesicles (Figs. 29 and 32) at the ends of branches of filaments throughout the host cytoplasm. The vesicles are formed terminally on short side branches of the vegetative mycelium (Fig. 30) and are never intercalary. The vesicles, possibly the sire of nitrogenase activity (Baker *et al.* 1979), are ca. 2–3 μm in diameter (Fig. 30) and are always surrounded by a polysaccharide capsule that envelopes the vegetative hyphae as well (Figs. 31 and 33). The vesicles are nonsepate initially but eventually form numerous septa (Figs. 32 and 33). No sporangia have been observed in effective nodules of *E. umbellata*.

Discussion

Frankia sp. EuI1 is an infective, nodule-inducing actinomycete which has striking morphological similarities to previously isolated nodule actinomycetes (Callaham *et al.* 1978; Berry and Torrey 1979). It is easily distinguished, however, on the basis of several distinctive features. *Frankia* sp EuI1 is generally more diminutive than other frankiae in terms of filament diameter, sporangial size and spore size. The elaboration of a diffusible blood-red to brown pigment is a biochemical trait which easily characterizes it *in vitro*.

Recently, isolated frankiae have been studied on the basis of cell wall composition (Lechevalier and Lechevalier 1979), a technique useful in the taxonomy of the aerobic actinomycetes (Lechevalier and Lechevalier 1970). The data on cell wall chemistry also indicate that *Frankia* sp EuI1 is easily distinguished; the *Elaeagnus* isolate shows two distinctive features: the lack of xylose in the cell wall and the presence of fucose as an unusual wall component. Further judgements on the taxonomic status of this and other isolated frankiae must await the isolation and characterization of greater number of the actinorhizal endophytes that at present.

Due to the large number of plant species known to bear root nodules (Torrey 1978), an exhaustive

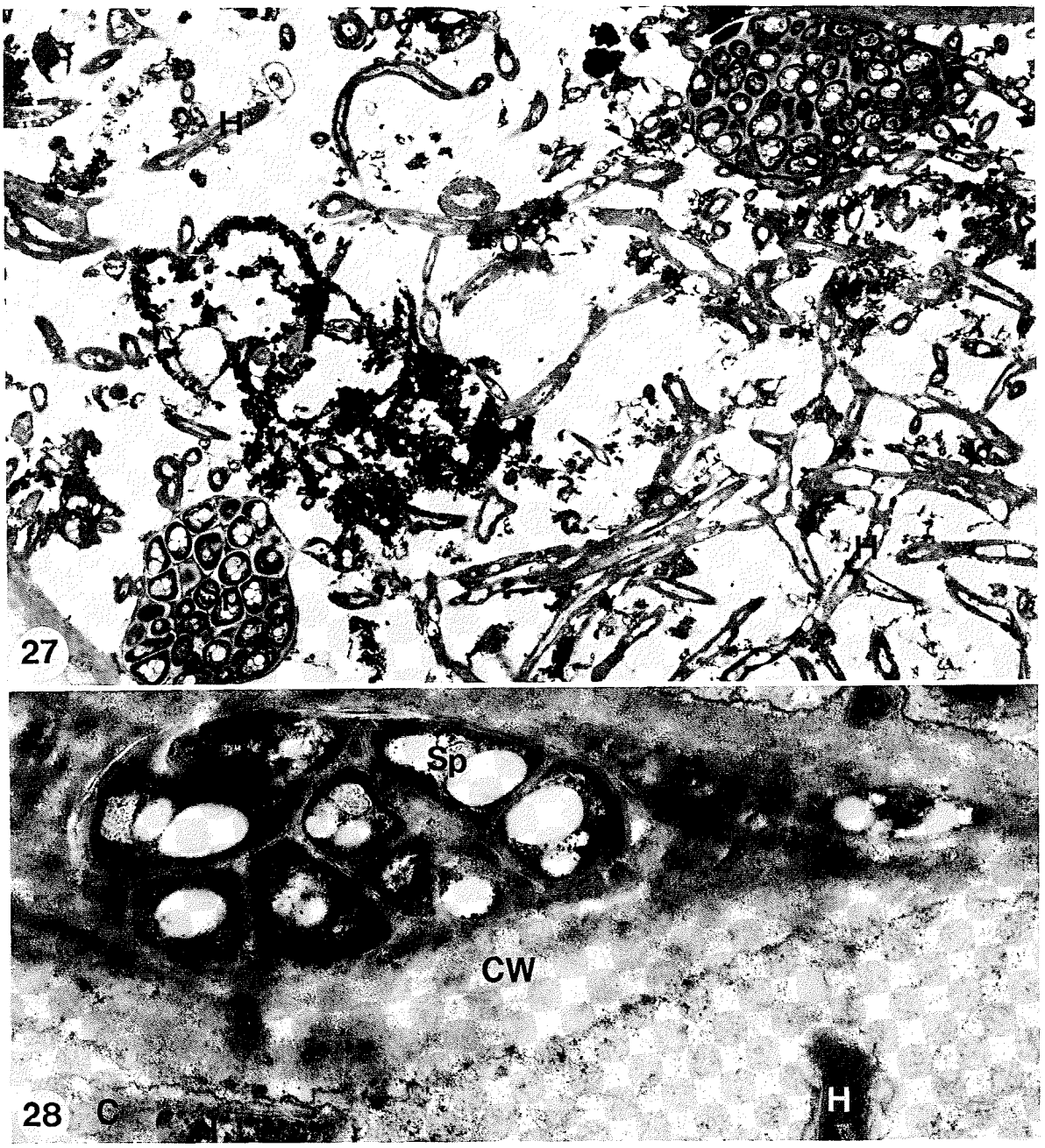
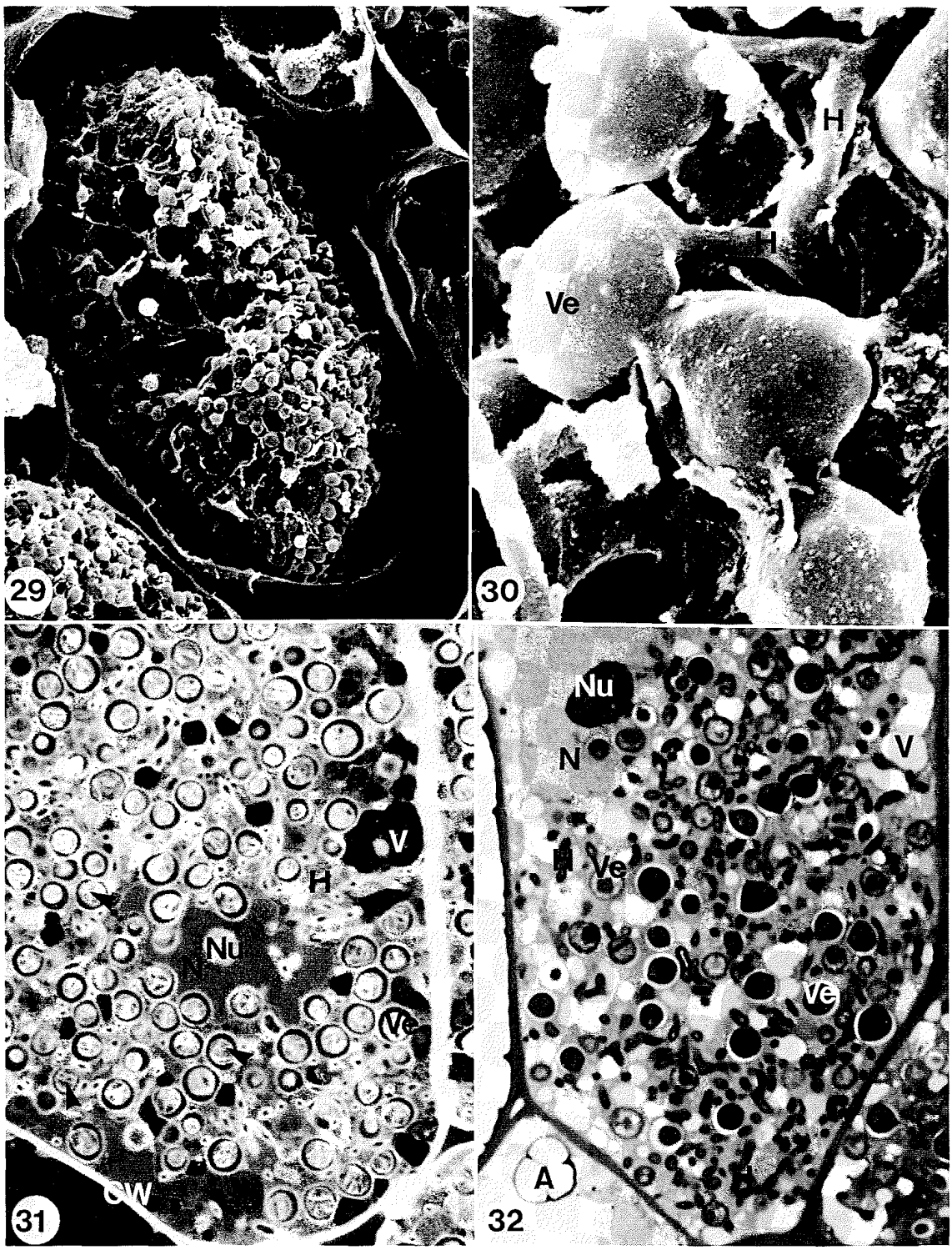


FIG. 27. TEM illustrating endophytic hyphae and sporangia within senescent infected cell of an ineffective nodule. ×6610.
FIG. 28. TEM of sporangium growing in middle lamella of host cell wall within an ineffective nodule. Note the encapsulated hyphae and healthy appearing cytoplasm in adjacent cell. × 24 880.

study to determine the host specificity of *Frankia* sp. EuI1 was not undertaken in this study. However, from the results that were obtained, it was shown that there occurs no overlap with the known host ranges of the other isolated frankiae which nodulate *Alnus* and members of the Myricaceae (Torrey *et al.* 1980; Berry and Torrey 1979; Baker and Torrey 1980). The host range of EuI1 was comparatively narrower than that of the other group of isolated actinomycetes. Studies to determine whether this narrowness is the result of the inability of the organism to develop an effective



FIGS. 29–32. Infected cells of an effective *E. umbellata* nodule. Fig. 29. SEM illustrating numerous endophytic vesicles and hyphae. × 1120. Fig. 30. SEM at higher magnification showing hyphal branching pattern and terminal position of endophytic vesicles. × 14 260. Fig. 31. LM using epifluorescence optics demonstrating PAS-positive (white in figure) substances which include host cell wall, capsule surrounding endophytic hyphae and vesicles, and endophytic cell walls and septa (arrows). × 1530. Fig. 32. LM illustrating large host nucleus and nucleolus as well as numerous endophytic hyphae and nonseptate and septate vesicles. An amyloplast in an adjacent uninfected cell is also shown. × 1490.

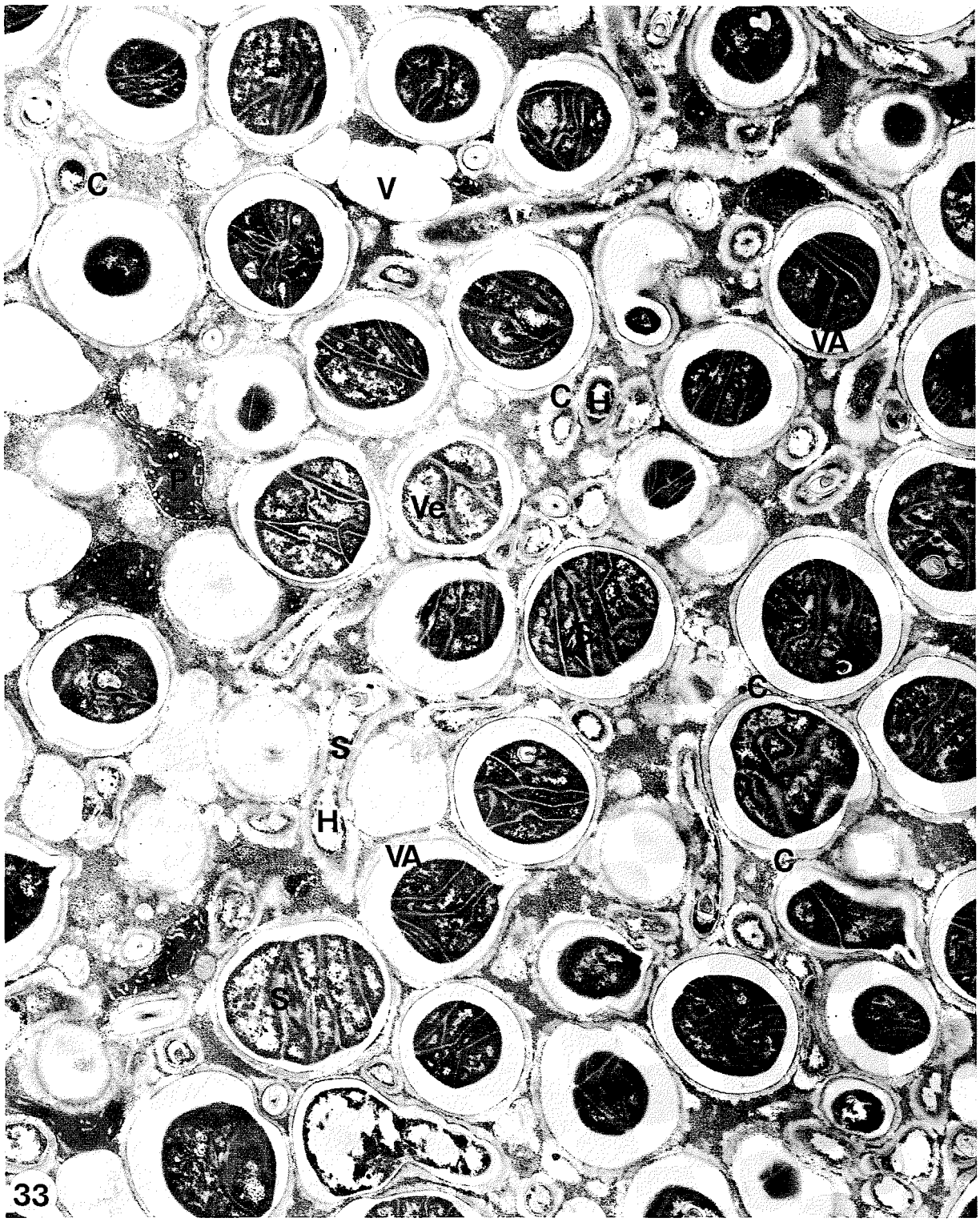


FIG. 33. TEM of a mature infected cell of an *E. umbellata* effective nodule showing endophytic vesicles and hyphae both of which contain septa and are surrounded by capsule. The electron translucent space or void area between the capsule and vesicle wall is an artefact presumably produced during specimen preparation. ×9350.

nitrogen-fixing symbiosis or an inherent trait of the strain must await the isolation of an effective endophyte. Definite relationships between host plants and EuI1 cannot be defined until further cross-inoculation studies have been performed.

The inability of this organsim to develop vesicles in symbiosis with its host and the concomitant lack of nitrogenase activity provide strong evidence for the hypothesis that the vesicle is the site of active nitrogen fixation or is intimately involved in the reduction reaction. Akkermans (1978) discussed the evidence in support of this view. Although the actual cause for the ineffectivity of EuI1 is not readily apparent, some of the early events of nodule morphogenesis appear similar in both ineffective and effective root nodules of *E. umbellata*. Within the infected cortical cells of both types of developing nodules the host nuclei and nucleoli have enlarged and the numbers of proplastids, mitochondria, and ribosomes have increased, presumably in response to the invasion of the endophytic actinomycete. In both effective and ineffective nodules the endophyte is enveloped in polysaccharide capsule believed to be synthesized and deposited by the host cytoplasm (Lalonde and Knowles 1975; Newcomb *et al.* 1978). *Frankia* sp. EuI1 grows differently than an organism which forms effective nodules in its preference for growing within the middle lamella or along the inner surface of the host cell walls. The inability of EuI1 to form vesicles represents an apparent morphogenetic block but is difficult to reconcile with its ability to form *in vivo* sporangia which have not been observed in effective *E. umbellata* nodules. Similar sporangia have been observed *in vivo* in *A. glutinosa* (van Dijk and Merkus 1976) and in *M. gale* (Torrey 1978).

Basically, *Frankia* sp. EuI1 resembles the *C. peregrina* isolate in its ultrastructure and morphogenesis (Newcomb *et al.* 1979). However, there are some minor differences. Although the walls of both isolates contain electron-dense layers, this layer is much more extensive and constant in EuI1. No electron-dense droplets are associated with the walls of the EuI1 as are with the walls of the *Comptonia* isolate (Newcomb *et al.* 1979). The spores of the *Comptonia* isolate contain large electron-dense regions which are not present in the spores of EuI1. The lipid-like inclusions of EuI1 superfically resemble the vacuole-like structures of the *Comptonia* isolate in that both appear to be membrane bound; however, these latter structures have reticulate contents whereas those of the lipid-like inclusions appear homogeneous. Older EuI1 spores contain more electron-dense wall layers than those of the *Comptonia* isolate. The premature senescence of some infected cells indicates a breakdown in the symbiosis. However, if the senescence is delayed until the endophytic clusters are present, the breakdown may function as a morphogenetic trigger for sporangial development. The role, if any, of the ringshaped vacuoles in cell breakdown is uncertain in view of the lack of data regarding the contents, i.e., enzymes characteristic of lysosomes, in these organelles. Hopefully, further studies of the biochemistry and genetics of these organisms will elucidate differences among EuI1 and effective isolates and provide a better understanding of the symbiosis in general.

Acknowledgements

This research was funded by the Cabot Foundation for Botanical Research of Harvard University, Grant No. DEB-77-02249 from the National Science Foundation and grants from the Advisory Research Council of Queen's University and the Natural Sciences and Engineering Research Council of Canada. The authors wish to thank Laurel McIntyre, Faye Murrin, and Ed Seling for their excellent technical assistance.

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