Protoplasma 131, 82–91 (1986)

PROTOPLASMA
© by Springer-Verlag 1986

Nodulation of Actinorhizal Plants by *Frankia* Strains Capable of Both Root Hair Infection and Intercellular Penetration[1]

I. M. MILLER * and D. D. BAKER

Battelle-Kettering Laboratory, Yellow Springs, Ohio

Received August 7, 1985
Accepted November 19, 1985

Summary

A morphological analysis of the initiation and development of root nodules of *Elaeagnus angustifolia* and *Myrica cerifera* inoculated with pure-cultured *Frankia* strains DDB 011610 or DDB 020110 was undertaken. From ultrastructural observations it was determined that both of these *Frankia* strains can infect *Elaeagnus* by an intercellular penetration mechanism and *Myrica* by the root hair infection mechanism. This indicates that both of these strains have the ability to infect host plant roots by either of two mechanisms. The reverse, that *Elaeagnus* or *Myrica* could be infected by both mechanisms, was not observed. The infection and nodule development processes of these two plants in combination with these strains were similar to observations made in previous studies (MILLER and BAKER 1985, TORREY and CALLAHAM 1979). However, one exception was identified in the development of the prenodule of *Myrica* when infected with strain 011610, in that endophytic hyphae developed vesicles within the cells of the prenodule. This event has not been described before for any of the actinorhizal genera and may be an indication of less than optimal compatibility between the host plant and the symbiont.

Keywords: Actinorhizae; *Elaeagnus; Frankia;* Infection processes; *Myrica;* Nodule development.

1. Introduction

The nodulation of actinorhizal plants can occur by either one of two infection mechanisms. The first mechanism, root hair infection (RHI), was considered until recently the ubquitous mode of entry for *Frankia* and has been shown to occur in the plant genera, *Alnus, Casuarina, Comptonia* and *Myrica* (CALLAHAM *et al.* 1979, BERRY 1984). In this developmental process, *Frankia* colonizes the root hairs and causes their distortion in the form of branching or curling. A *Frankia* hypha penetrates the root hair cell wall and the bacterium becomes an intracellular endophyte. In response to this invasion the plant encapsulates the advancing hypha as it progresses toward the root

[1] Contribution no. 876 of the Battelle-Kettering Laboratory.

* Correspondence and Reprints: Battelle-Kettering Laboratory, 150 East South College Street, Yellow Springs, OH 45387-0268, U.S.A.

Plate 1. All figures are from *Elaeagnus* infected with *Frankia* strain DDB 020110.

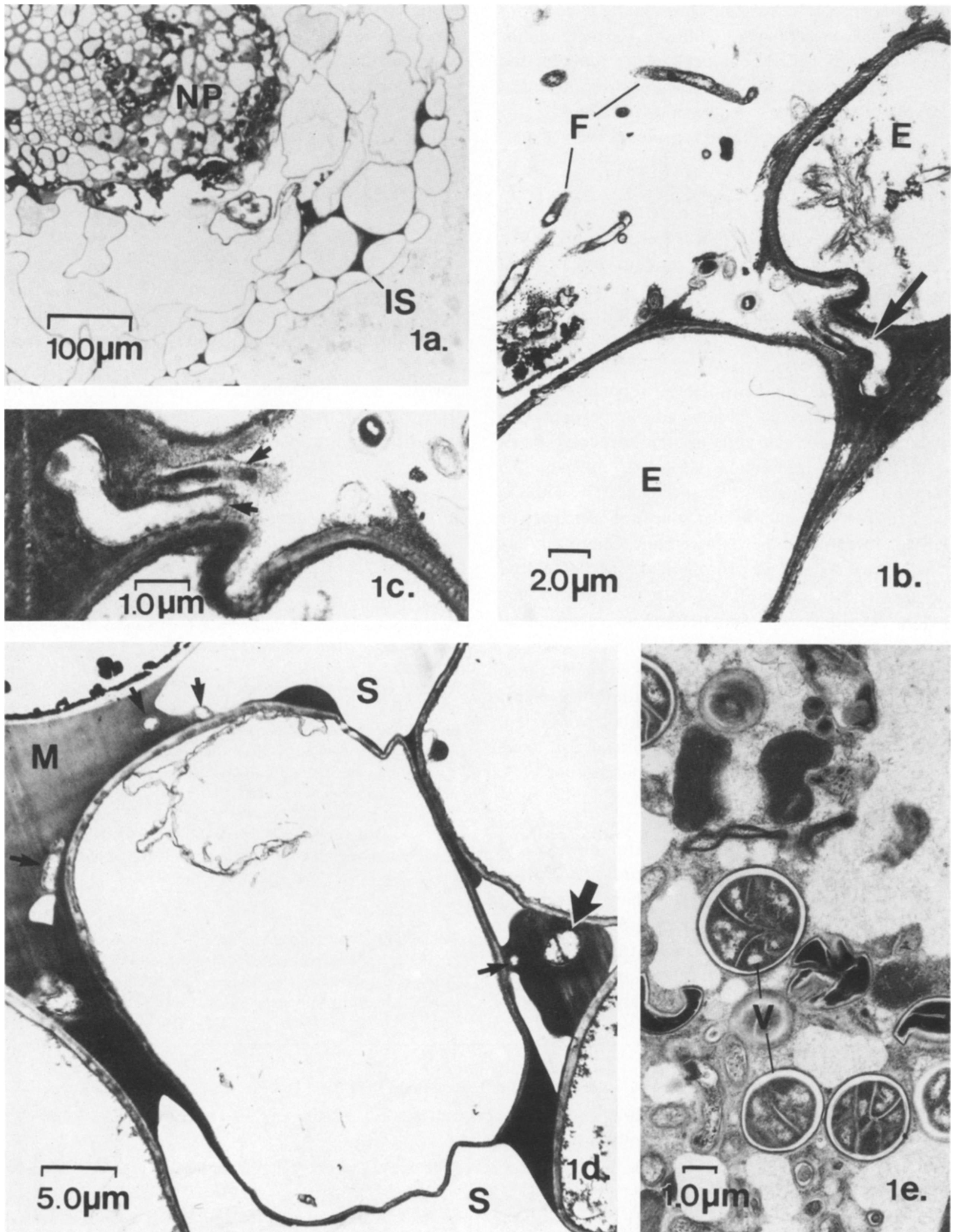
Fig. 1 *a*. TS of a root showing an infection site (*IS*) and a young nodule primordium (*NP*). × 154

Fig. 1 *b*. *Frankia* hyphae (arrow) penetrate the middle lamella between two epidermal cells (*E*). Root colonizing *Frankia* (*F*) can be seen on the root surface. × 3,600

Fig. 1 *c*. Detail of the invading *Frankia* in the middle lamella between two epidermal cells. In this instance there appears to be two hyphae entering through this single middle lamellar region (arrows). × 9,900

Fig. 1 *d*. A densely staining material (*M*) secreted from the cortical cells partially fills the intercellular spaces (*S*) of the root cortex. *Frankia* profiles (small arrows) can be seen embedded in this material. Often one or two vesicle-like structures (large arrow) can also be seen. × 2,780

Fig. 1 *e*. In mature nodule cells the *Frankia* differentiates nitrogen-fixing vesicles (*V*), in this case spherical structures scattered randomly throughout the cytoplasm. × 6,135



Figs. 1 *a–e*

cortex. A series of cellular divisions in the hypodermis leads to the formation of a swelling termed a prenodule. A nodule primordium is induced in the pericycle and the *Frankia* filaments advance toward this by means of intracellular growth. The actinomycete colonizes the cortical cells of the nodule and continuous to infect new cells as the nodule enlarges.

Only lately has the second mechanism, intercellular penetration (IP), been identified (MILLER and BAKER 1985), and so far this has been observed only in the *Elaeagnaceae*. This process differs considerably from RHI in the fact that the advance of the bacterium is almost entirely intercellular. Root hairs are few on the surface of the roots of *Elaeagnus* and are not involved in the infection process. *Frankia* cells colonize the root surface and penetrate the cuticle of the epidermal cells. Hyphae penetrate the middle lamella between two epidermal cells and gain entry to the intercellular spaces of the root. No prenodule is formed, but a nodule primordium is induced in the pericycle. The *Frankia* filaments colonize intercellular spaces of the cortex as they advance toward the young nodule. Only after they have crossed the nodule protoperiderm and colonized the intercellular spaces of the nodule cortex do they invade individual cells and become an encapsulated endophyte.

Numerous studies have been undertaken to determine cross-inoculation abilities among actinorhizal plants and some studies have indicated the ability of certain endophytes to nodulate genera of both of the above listed groups (RODRÍGUEZ-BARRUECO and MIQUEL 1979, RODRIQUEZ-BARRUECO *et al.* 1981). These studies have been criticized however, because they employed crushed nodule suspensions or soils for inocula and were not performed using pure-cultured *Frankia*. More recent studies using isolated strains have shown that indeed nodulation of two quite unrelated genera could result from inoculations of a single strain (GAUTHIER *et al.* 1984, SAINT-LAURENT and LALONDE 1984). From studies in our own laboratory we observed that nodulation could occur from a single strain in two plant genera known to have different infection mechanisms. Such phenomena could occur under several hypothetical circumstances. Firstly, the plant could have the ability to be nodulated via both RHI and IP mechanisms but each *Frankia* strain would be restricted to only one mode of entry. Secondly, the reverse could also account for the observations, in that *Frankia* strains could be capable of both RHI and IP modes of entry but that a single plant could be nodulated by only one mechanism. Lastly, both the plant and the actinomycete could be capable of both RHI and IP mechanisms. In this study we have undertaken nodulation and morphological studies of *Elaeagnus* and *Myrica* specifically to determine which hypothesis is correct. Two pure-cultured *Frankia* strains, DDB 011610 and DDB 020110, which are known to nodulate both of these host plants were used as inocula.

2. Materials and Methods

2.1. Frankia Cultures

Two pure-cultured *Frankia* strains were used as inocula. *Frankia* strain DDB 020110 was originally isolated from a specimen of *Casuarina equisetifolia* (*Casuarinaceae*) from Kahana, Hawaii. This non-pigmented strain is infective on *Elaeagnus* and *Myrica* but non-infective on *Casuarina*. It is only slightly effective on *Elaeagnus*, reducing only minimal amounts of acetylene. Its effectiveness on *Myrica* has not been assayed. *Frankia* strain DDB 011610 was originally isolated from a specimen of *Alnus rhombifolia* (*Betulaceae*) from the San Bernadino National Forest near Idyllwild, California. This red-pigmented strain is both infective and effective on both *Myrica* and *Elaeagnus*; it is non-infective on *Casuarina*. Both strains were propagated on DPM broth (BAKER and O'KEEFE 1984). Cells were harvested by centrifugation, washed with sterile distilled water and homogenized in a Potter-Elvehjem tissue grinder just before use.

2.2. Plant Material

Seedlings of *Elaeagnus angustifolia* L. (*Elaeagnaceae*) and *Myrica cerifera* L. (*Myricaceae*) germinated on sand, were transferred to hydroponic cultures jars containing a modified Crone's solution (BOND 1951). Six plants were inoculated with each *Frankia* strain

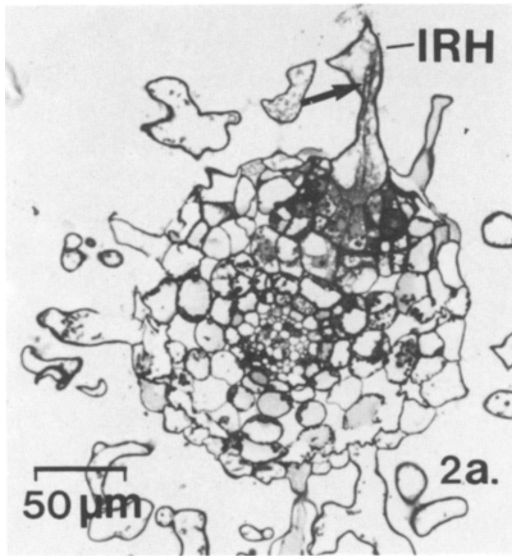
Plate 2. All figures are micrographs of *Myrica cerifera* infected with *Frankia* strain DDB 020110

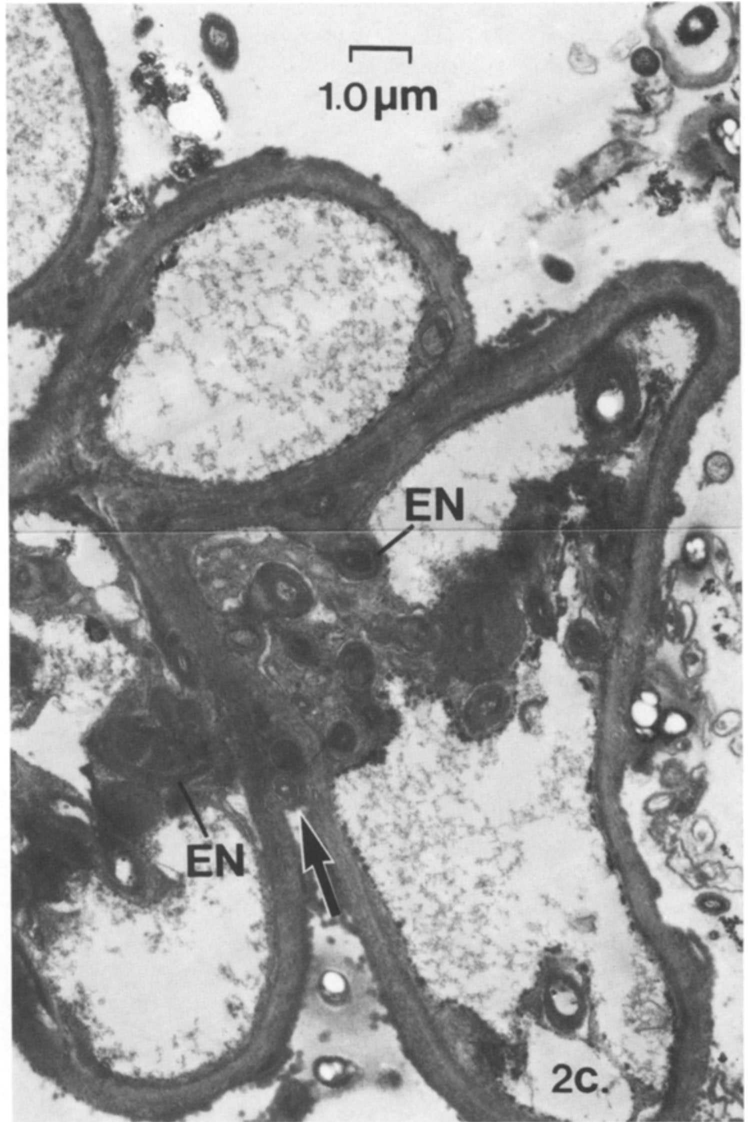
Fig. 2 *a*. TS of a root in the plane of section of an infected root hair (*IRH*). All the root hairs projecting from the root surface are branched or deformed. Note the *Frankia* hyphae in the hair (arrow). × 253

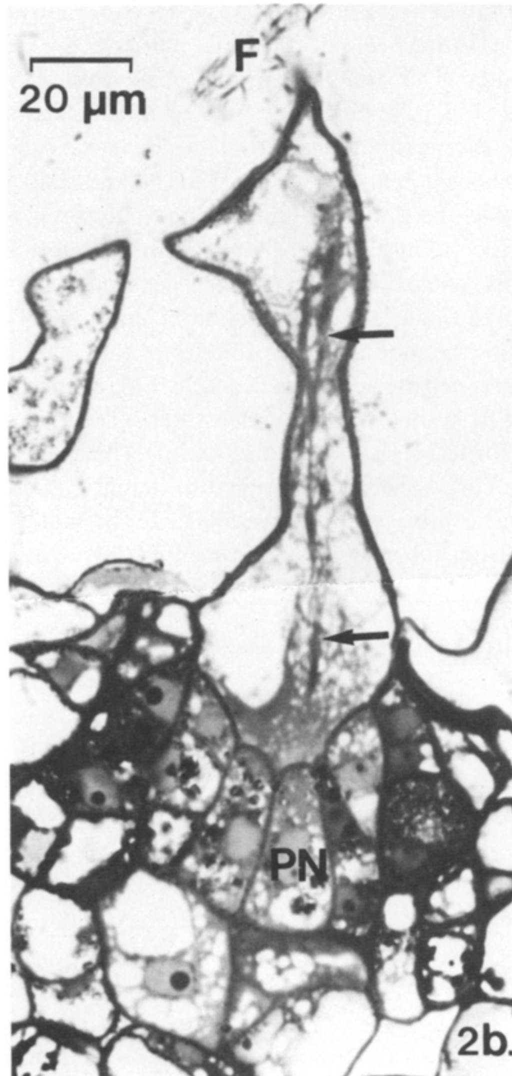
Fig. 2 *b*. Detail of the infected root hair and underlying region. The root colonizing *Frankia* (*F*) as well as the invading hyphae (arrows) can be seen. Just below the root hair is the developing prenodule (*PN*). × 658

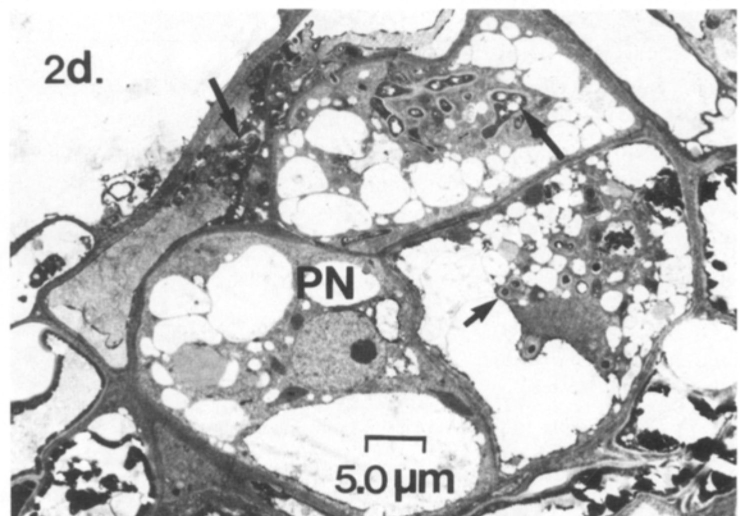
Fig. 2 *c*. Detail of the distal portion of an infected root hair. Some hyphal strands have become enclosed by the highly branched, deformed walls of the root hair (arrow). The hyphae which have entered the root hair cell have become encapsulated by a cell wall-like material (*EN*). × 8,410

Fig. 2 *d*. Electron micrograph of a young prenodule. Encapsulated strands of *Frankia* (arrows) can be seen in the cells of the prenodule (*PN*). × 1,600









Figs. 2 *a–d*

after one week in water culture and observed for nodulation after six to ten days. Six plants were left uninoculated to test for contaminating *Frankia*. None of these control plants subsequently developed root nodules.

2.3. TEM

Sections of root containing nodules at early developmental stages were excised from the plants and fixed for 4 to 6 hours at 20 °C in 3% glutaraldehyde buffered at pH 6.8 with 0.1 M phosphate buffer. After a brief wash in distilled water, the tissue was postfixed in 2% buffered osmium tetroxide for 2 to 4 hours and then dehydrated in a graded water/ethanol series. To ensure proper infiltration of the epoxy resin, the ethanol was replaced with 1,2-epoxypropane. After 15 minutes an equal volume of Polybed 812 epoxy resin was added to the solvent and the mixture left overnight. The tissue was then subjected to 4 changes of fresh resin approximately every 8 hours. Individual pieces of tissue were placed in fresh resin in BEEM capsules and polymerized at 60 °C for 36 hours. Thin sections were cut using an LKB Ultratome III, stained with 3% uranyl acetate in 50% methanol for 15–20 minutes followed by alkaline lead citrate for 2 minutes, and viewed in a Philips 200 TEM.

2.4. Light Microscopy

For light microscopy 2 µm semi-thin sections were cut on the Ultratome from blocks prepared for TEM. The sections were heat dried onto glass slides and stained with either 1% methylene blue or 1% toluidine blue in 1% sodium tetraborate. Observations were recorded using a Leitz Ortholux II photomicroscope.

3. Results

3.1. Infection of Elaeagnus with Frankia Strain DDB 020110

The mode of infection of roots of *Elaeagnus* by *Frankia* strain DDB 020110 is by intercellular penetration and colonization of the root cortex (Figs. 1 *a* and *b*). Root hairs were rarely found in those regions of the root where infection took place. The infection site in the host plant root is readily visible as the epidermal and cortical cells of the root are stimulated to secrete a material into the intercellular spaces which stains intensely with both routine light and electron microscope stains (Figs. 1 *a*, *b*, and *d*). In this mode of infection *Frankia*, which has colonized the root surface, penetrates the middle lamella between two epidermal cells by enzymatic digestion (Figs. 1 *b* and *c*). In some cases there appear to be two hyphal strands entering the root between the same two epidermal cells (Fig. 1 *c*).

At an early stage in the infection process a nodule primordium is initiated from the pericycle of the vascular bundle (Fig. 1 *a*). No prenodule or other cellular division was observed in the root cortical cells. As the infection proceeds, the *Frankia* grows through and colonizes the root cortex in an amorphous material which partially fills the intercellular spaces of the cortex (Fig. 1 *d*). During colonization of the cortex, the *Frankia* often gives rise to one or two small vesicle-like structures (Fig. 1 *d*). The next stage of infection and nodule development in this novel mechanism is the same as has been reported earlier (MILLER and BAKER 1985) in that the *Frankia* filaments continue to grow towards the nodule primordium, embedded in the amorphous matrix. The hyphae then penetrate intercellularly through the protoperiderm and ramify throughout the nodule primordium in the middle lamellae between the young cortical cells. Side branches then arise from the *Frankia* hyphae and penetrate and enter the cortical cells of the young nodule. Once inside the nodule cortical cells the endophyte differentiates the characteristic nitrogen-fixing vesicle cells. In mature nodule cells of the association between *Elaeagnus* and

Plate 3. Fig. 3 *a* is a micrograph of *Myrica cerifera* infected with *Frankia* strain DDB 020110. All others are of *Elaeagnus angustifolia* infected with *Frankia* strain DDB 011610

Fig. 3 *a*. TS of a root in the plane of section of an infected root hair (*IRH*) and a young nodule primordium (*NP*). The prenodule (*PN*) is visible in the cortex below the infected root hair. To infect the nodule primordium, the *Frankia* will travel from the prenodule through the cortex (*C*) towards the primordium. × 224

Fig. 3 *b*. TS through part of a young root showing two infection sites at the epidermis (arrows). The infection sites can be identified by the presence of densely staining material secreted into the intercellular spaces. × 164

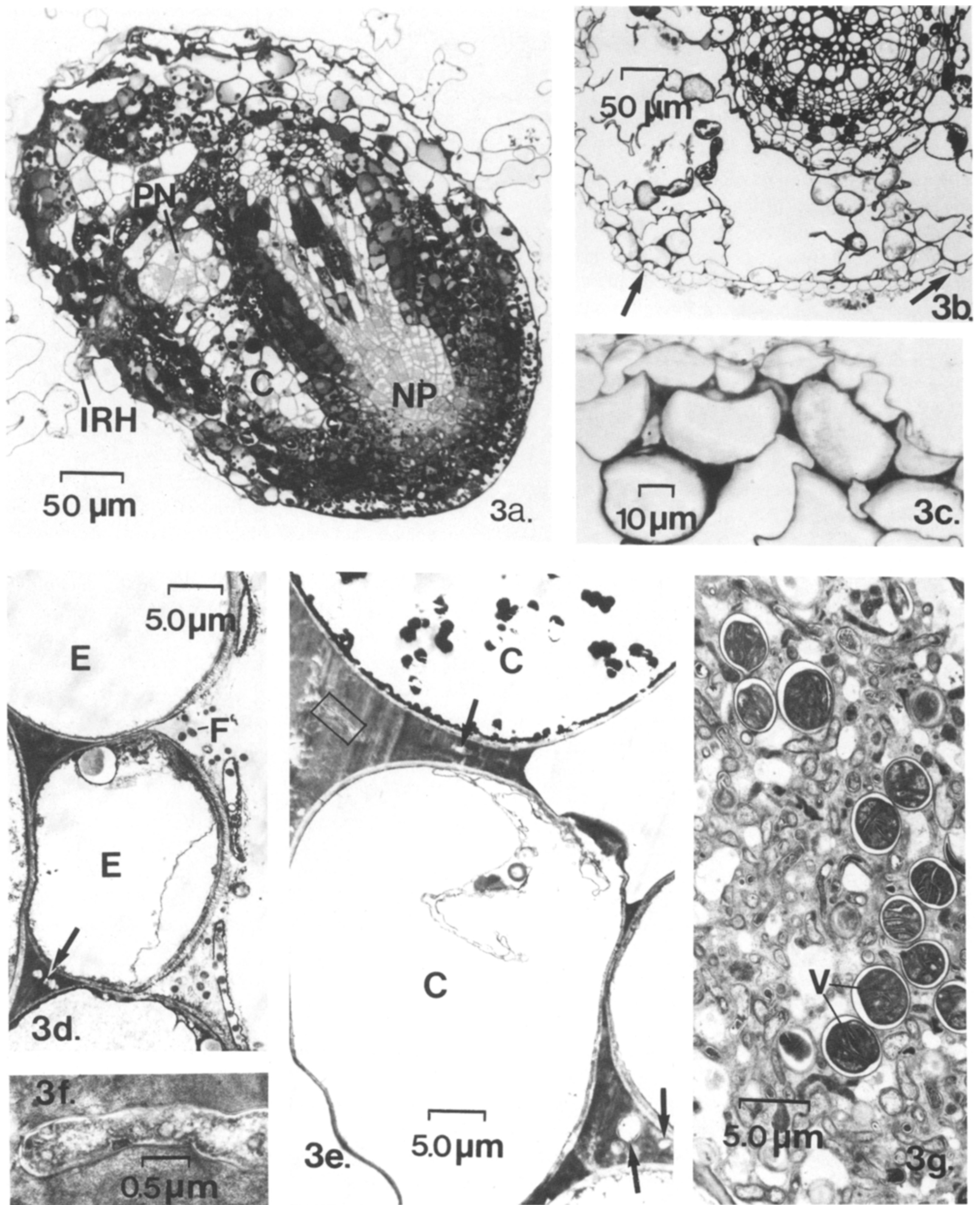
Fig. 3 *c*. Detail of one of these infection sites showing the dense material partially filling the intercellular spaces. × 625

Fig. 3 *d*. Electron micrograph of an infection site showing root colonizing *Frankia* (*F*) and a hyphal strand (arrow) in the intercellular material below the middle lamella of two epidermal cells (*E*). × 1,950

Fig. 3 *e*. *Frankia* hyphae (arrows) are found embedded in the densely staining matrix material which partially fills the spaces between cortical cells (*C*). × 2,030

Fig. 3 *f*. Detail of the boxed part of the previous figure showing a hyphal strand embedded in the matrix. The similarity of staining reaction shown by both the matrix material and the *Frankia* often make the endophyte difficult to detect, even by electron microscopy. × 17,500

Fig. 3 *g*. In mature infected nodule cells the nitrogen-fixing vesicles (*V*) are in the form of spheres randomly distributed throughout the cell. × 2,540



Figs. 3 *a–g*

Frankia strain DDB 020110 these vesicles are spherical and are found scattered randomly throughout the cell (Fig. 1 *e*).

3.2. Infection of Myrica with Frankia Strain DDB 020110

The mode of infection of roots of *Myrica* by *Frankia* strain DDB 020110 is by root hair infection (Figs. 2 *a* and *b*). In this infection process *Frankia* hyphae colonize the root surface, causing the root hairs to branch and become deformed (Figs. 2 *a* and *b*). Some of the hyphae become enclosed in a crypt formed by the junction of a number of parts of the wall of the highly branched, deformed root hair (Fig. 2 *c*). Somewhere in this crypt region actual infection takes place by *Frankia* hyphae penetrating the root hair. In response to this penetration the hyphae become encapsulated by a host-derived cell wall-like material (Fig. 2 *c*). The *Frankia* grows down through the root hair and enters the cells of the root cortex (Fig. 2 *b*). The cortical cells are stimulated to divide giving rise to a small local swelling, the prenodule (Figs. 2 *b* and *d*). *Frankia* hyphae then disseminate throughout the cells of the prenodule (Fig. 2 *d*). Once the prenodule is established, one or more nodule primordia are initiated from the pericycle. Each nodule primordium becomes infected by invasion of the primordial cortex with *Frankia* from the prenodule. One such nodule primordium and its relationship to the prenodule can be seen in the same plane as an infected root hair in Fig. 3 *a*. Microscopic examination of a mature *Myrica* nodule infected with this strain revealed that the nitrogen-fixing vesicles in the cells of the nodule cortex were club-shaped and were preferentially located around the cell periphery (not illustrated) as previously described by BENSON and EVELEIGH (1979).

3.3. Infection of Elaeagnus with Frankia Strain DDB 011610

Examination of roots of *Elaeagnus* inoculated with *Frankia* strain DDB 011610 showed that, in common with the previous strain, infection occurred by intercellular penetration of the virtually root hair-free roots and the subsequent colonization of the cortical apoplast (Figs. 3 *b–f*). Infection sites and paths in the cortex are clearly visible by the dense staining reaction of the amorphous intercellular material (Figs. 3 *b* and *c*). In contrast to *Myrica*, where one root hair infection event can lead to the initiation of several nodule primordia, more than one infection event is usually found associated with each developing nodule (Fig. 3 *b*). Once again, rhizoplane colonizing *Frankia* break through the cuticle and penetrate between epidermal cells. (Fig. 3 *d*). As with the previous strain, an amorphous matrix material through which the *Frankia* grows is present in the intercellular spaces of the cortex (Figs. 3 *c–f*). Often both the intercellular matrix and the cells of the endophyte stain with a similar electron density, making visualization of the actinomycete filaments difficult at low magnifications. The presence of the endophyte however, can be detected easily at higher magnification (Fig. 3 *f*). In infected cortical cells of the mature nodule the nitrogen-fixing vesicles are spherical and randomly dsitributed throughout the cell (Fig. 3 *g*).

3.4. Infection of Myrica with Frankia Strain DDB 011610

Infection of roots of *Myrica* with *Frankia* strain DDB 011610 was by root hair infection (Figs. 4 *a* and *b*). In common with the previous strain, the root hairs become branched and deformed; the hyphae penetrate the hair and move down through it into the root cortex

Plate 4. All figures are of *Myrica cerifera* infected with *Frankia* strain DDB 011610

Fig. 4 *a*. TS of a root in the plane of section of an infected root hair (*IRH*). An enlarged prenodule is visible (arrow) as well as part of a young nodule primordium (*NP*). Heavy contrast is due to excessive tanninization which has occurred in the cortex and epidermis of the root. × 160

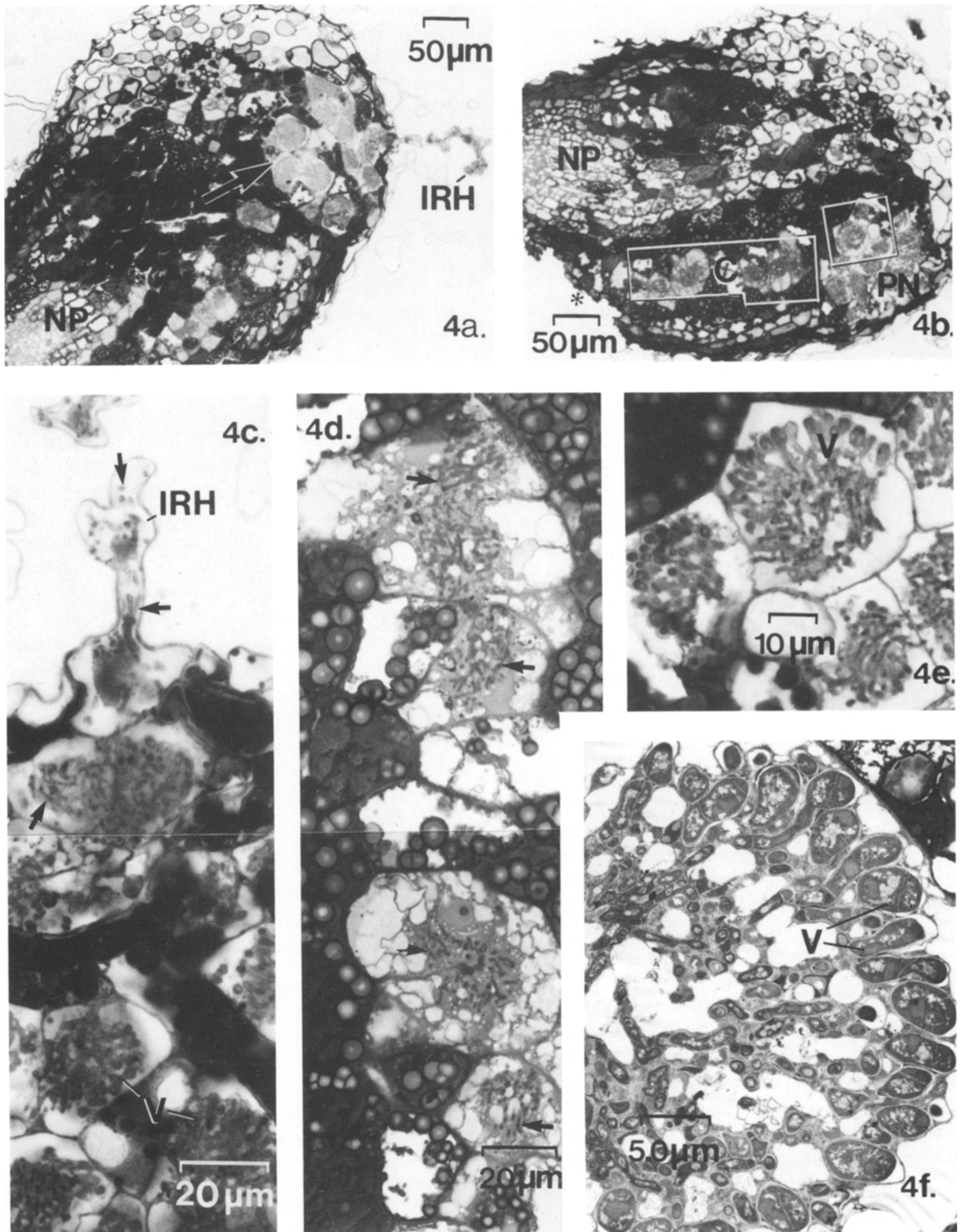
Fig. 4 *b*. A nodule primordium (*NP*), enlarged prenodule (*PN*) and infected cortical cells (*C*) can all be seen in this section. The areas delimited by the large and small enclosures are Figs. 4 *d* and 4 *e* respectively. Part of the brittle, heavily tanninized cortex and epidermis have fallen out during sectioning for electron microscopy (asterisks). × 162

Fig. 4 *c*. Detail of an infected root hair (*IRH*) and underlying prenodule. Hyphal strands are visible in the root hair and prenodule cells (arrows). Many of the prenodule cells have become enlarged and contain endophytic vesicles (*V*). × 800

Fig. 4 *d*. Detail of the root cortex between the prenodule and the nodule primordium showing undifferentiated *Frankia* hyphae (arrows) in the cortical cells. The hyphae make their way through this region to the nodule primordium where infection of the nodule cortex takes place. × 685

Fig. 4 *e*. Part of the prenodule enclosed by the box in Fig. 4 *c* showing vesiculation (*V*) of the endophyte. × 850

Fig. 4 *f*. Electron micrograph of a prenodule cell. Club-shaped vesicles (*V*) distributed around the cell periphery with hyphal elements more centrally located is a morphology usually only observed in mature infected nodule cells. × 2,500



Figs. 4 *a–f*

(Fig. 4 *b*). Once in the cortical cells of the root however a radical departure occurs from the typical process of nodule formation as described by TORREY and CALLAHAM (1979), (Figs. 4 *a* and *c*). The first infected cortical cells below the root hair become more hypertrophied than normal and the *Frankia* differentiates vesicles (Figs. 4 *b*, *e*, and *f*). The vesicles formed are club-shaped and arranged around the cell periphery (Figs. 4 *e* and *f*). While this morphology is typical of mature infected nodule cortex cells it is highly atypical prenodule morphology. It does not, however, appear to affect the function of the prenodule; hyphal strands leading from the prenodule through the root cortex towards the developing nodule primordium can be seen (Figs. 4 *c* and *d*).

4. Discussion

The nodulation of two rather unrelated actinorhizal genera can indeed occur from the infection of a single pure-cultured *Frankia* strain. What is more significant however, is that the nodulation process may be different in the two host plants, a point that we have demonstrated in this study. From our results it is apparent that the host plant has the capability for only one mode of symbiotic infection, whereas the bacterial symbiont may have the capability for more than one mechanism. Therefore in cross-inoculations the developmental sequence of nodulation is determined by the host plant.

Developmentally "flexible" strains such as those which we have reported here may well be common as they would appear to have ecological advantages over "restrictive" strains. However, although they are symbiotic with more plant genera they perhaps are less compatible with each host plant than a "restrictive" strain might be. This is a hypothesis which we are currently investigating. Early events in the infection process by the intercellular penetration mode (IP) differ from events in the root hair infection mechanism (RHI) in two major ways. Firstly, in RHI the actinomycete penetrates through the cell wall of the root hair while in IP the *Frankia* enters the plant by penetrating the middle lamella between two epidermal cells. This method of entry into the plant is not unlike the way in which some pathogenic fungi enter plant tissue. It may be that, like these fungi, the *Frankia* secrete cell wall-dissolving or pectinolytic enzymes to gain entry to the root. The second main difference is that in RHI the infection process is intracellular while in IP it is extracellular. In RHI it has been shown (LALONDE and KNOWLES, 1975) that the host plant cells secrete a pectinaceaous encapsulation material around the *Frankia* which acts as a nutritive substrate for the growth of the endophyte. It may well be that in IP the intercellular matrix material in which the *Frankia* is found is also a host plant cell secretion serving an analagous function to the encapsulation material in RHI. The presence of endophytic vesicles in the prenodular cells of *Myrica* inoculated with DDB 011610 is the first such report of its kind. This observation suggests that interactions between the host and the bacterial symbiont are dynamic and not predetermined by either symbiont. Therefore we might expect to see other differences in nodule development between hosts and *Frankia* strains in other combinations.

It should be noted that the symbiotic morphology of *Frankia* is determined by the host plant. RODRIQUEZ-BARRUECO and MIGUEL (1979) reported that vesicle morphology always was the same in individuals of the same genus even if the inoculum came from crushed nodules of another genus having a different vesicle morphology. LALONDE (1979) demonstrated the same phenomenon with inoculations of *Alnus* (forming spherical vesicles) using the pure-cultured *Frankia* strain CpI1 which produces club-shaped vesicles in *Comptonia*. Each of the strains in this study formed spherical vesicles in *Elaeagnus*, but formed club-shaped vesicles in *Myrica*. Vesicle morphology in these flexible strains is similarly modified by the host during the development of the symbiosis.

The induction of symbiotic root nodules in actinorhizal plants is a complex interaction between the host plant and *Frankia*. Developmental pathways will differ among host-endophyte combinations and cannot necessarily be classified into simple categories. More studies of nodule induction, morphology and physiology will be required to fully understand the range of symbiotic compatibility within the actinorhizal symbioses.

References

BAKER, D., O'KEEFE, D., 1984: A modified sucrose fractionation procedure for the isolation of *Frankiae* from actinorhizal root nodules and soil samples. Plant Soil **78**, 23–28.

BENSON, D. R., EVELEIGH, D. E., 1979: Ultrastructure of the nitrogen-fixing symbiont of *Myrica pensylvanica* L. (Bayberry) root nodules. Bot. Gaz. **140 (S)**, 15–21.

BERRY, A. M., 1984: The actinorhizal infection process: review of recent research. In: Current Perspectives in Microbial Ecology (KLUG, M., REDDY, C., eds.), pp. 222–229. Washington: Amer. Soc. Microbiol.

BOND, G., 1951: The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. Ann. Bot. **15**, 447–459.

CALLAHAM, D., NEWCOMB, W., TORREY, J. G., PETERSON, R. L., 1979: Root hair infection in actinomycete-induced root nodule initiation in *Casuarina, Myrica,* and *Comptonia.* Bot. Gaz. **140 (S)**, 1–9.

— TORREY, J. G., 1977: Prenodule formation and primary nodule development in roots of *Comptonia (Myricaceae).* Can. J. Bot. **55**, 2306–2318.

GAUTHIER, D., FRIONI, L., DIEM, H. G., DOMMERGUES, Y., 1984: The *Colletia spinosissima-Frankia* symbiosis. Acta Oecologica/Oecologia Plantarum **5**, 231–239.

LALONDE, M., 1979: Immunological and ultrastructural demonstration of nodulation of the European *Alnus glutinosa* (L.) Gaertn. host plant by an actinomycetal isolate from the North America *Comptonia peregrina* (L.) Coult. root nodule. Bot. Gaz. **140 (S)**, 35–43.

— KNOWLES, R., 1975: Ultrastructure, composition, and biogenesis of the encapsulation material surrounding the endophyte in *Alnus crispa* var. *mollis* root nodules. Can. J. Bot. **53**, 1951–1971.

MILLER, I. M., BAKER, D. D., 1985: The initiation, development and structure of root nodules in *Elaeagnus angustifolia* L. (*Elaeagnaceae*). Protoplasma **128**, 107–119.

RODRIQUES-BARRUECO, C., MIGUEL, C., 1979: Host plant-endophyte specificity in actinomycete-nodulated plants. In: Symbiotic Nitrogen Fixation in the Management of Temperate Forests (GORDON, J., WHEELER, C., PERRY, D., eds.), pp. 143–159. Corvallis: Oregon State University Forest Research Laboratory.

— — CAÑIZO, A., Host plant endophyte specificity in actinorhizal plants. In: Current Perspectives in Nitrogen Fixation (GIBSON, A., NEWTON, W., eds.), pp. 476. Canberra: Aust. Acad. Sci.

SAINT-LAURENT, L., LALONDE, M., 1984: Isolation and characterization of *Frankia* strains from *Myrica gale* L. Paper presented at Montmorency Forest, Laval University, August 1984.

TORREY, J. G., CALLAHAM, D., 1979: Early nodule development in *Myrica gale.* Bot. Gaz. **140 (S)**, 10–14.