Amer. J. Bot. 72(1): 99–108. 1985.

DEVELOPMENT OF ENDOPHYTIC FRANKIA SPORANGIA IN FIELD- AND LABORATORY-GROWN NODULES OF COMPTONIA PEREGRINA AND MYRICA GALE[1]

KATHRYN A. VANDENBOSCH[2] AND JOHN G. TORREY[3]

Department of Botany, University of Massachusetts, Amherst, Massachusetts, 01003 and [3]Cabot Foundation, Harvard University, Petersham, Massachusetts 01366

ABSTRACT

Field-collected nodules of *Comptonia peregrina* (L.) Coult. and *Myrica gale* L. (Myricaceae), infected by the nitrogen-fixing actinomycete *Frankia* sp., were of two types: those that lacked sporangia entirely, designated spore(−), and those that showed extensive sporangial development, designated spore(+). In spore(+) nodules of *C. peregrina,* sporangia began to develop after the differentiation of endophytic vesicles and the concomitant onset of nitrogenase activity. At the onset of sporangial differentiation, infected host cells appeared healthy. However, endophytic vesicles and host cell cytoplasm and nuclei began to senesce rapidly as sporangia developed. Staining of sectioned material with the fluorescent stain Calcofluor White suggested that vesicles, hyphae and young sporangia were enclosed within a host-derived encapsulation layer, but mature sporangia were no longer encapsulated. In both *C. peregrina* and *M. gale,* vesicles were more short-lived in spore(+) than in spore(−) nodules. Field-collected spore(+) *M. gale* nodules exhibited a pronounced seasonality of sporangial formation. Sporangia began to differentiate in June, after the formation of vesicles and became more prominent in late summer. Inter- and intraspecific cross-inoculations suggest that the ability to form sporangia in the symbiotic state is controlled by endophytic strain type rather than host genotype or host/endophyte combination. The host may, however, influence the number and seasonal appearance of sporangia formed.

MEMBERS of the genus *Frankia,* soil-inhabiting actinomycetes, are capable of infecting the roots of woody plants belonging to eight dicotyledonous families, and of inducing the formation of nodules (Bond, 1976; Torrey, 1978). These nodules are specialized symbiotic organs in which the endophyte, *Frankia,* fixes atmospheric nitrogen which is then available to the host in reduced form.

That *Frankia* is a pleomorphic organism, capable of differentiating both vesicles and sporangia from its hyphae, was known long before it was first successfully isolated from nodules (Callaham, Del Tredici and Torrey, 1978) or characterized as an actinomycete. Schaede (1933) first noticed that nodules obtained from *Alnus glutinosa* fell into two distinct categories: those in which the endophyte formed only hyphae and vesicles and those which also showed the development of a spore-like stage. This observation has been confirmed more recently by others using modern microscopic methods (Becking, deBoer and Houwink, 1964; Suetin, Pariiskaya and Kalakoutskii, 1979, 1981; van Dijk and Merkus, 1976, and van Dijk, 1978). Van Dijk (1978) coined the terms spore(+) for *A. glutinosa* nodules that showed extensive sporangial development and spore(−) for those in which sporangia were rare or absent.

The presence of sporangia has also been demonstrated conclusively in other species of *Alnus* and in *Myrica gale,* but not in many other host genera. Whether the remaining genera possess only spore(−) nodules or whether this is due to limited investigation remains to be seen.

Vesicle morphogenesis and function have received a great deal of attention, since the vesicle is the usual site for nitrogen fixation, both in cultured frankiae (Tjepkema, Ormerod and Torrey, 1980, 1981) and in the nodule (Mian and Bond, 1978, and Huss-Danell et al., 1982). By contrast, the role of sporangia in the life history of the endophyte, the control of spo-

[1] Received 18 January 1984; revision accepted 23 September 1984.

This research was supported by NSF Grant DEB-8106952, USDA Grant 83-CRCR-1-1285 and by the Cabot Foundation for Botanical Research. The authors thank S. Lancelle for helpful discussion and assistance with photography, R. Lundquist for technical assistance, and F. O'Brien for secretarial assistance. We thank S. Zitzer for seeds of *Myrica cerifera,* B. Rottink, of the Crown-Zellerbach Corporation, for *Alnus rubra* seeds, and A. Berry for a collection of *Comptonia peregrina* nodules.

[2] Author to whom correspondence should be addressed. Present address: Department of Botany, University of Wisconsin, Madison, WI 53706.

rangium morphogenesis and the consequences of sporangium differentiation for nodule function are not well understood. We address some of these problems. Most of the research to date on sporulation of *Frankia* has been carried out on nodules from *Alnus* spp. (Betulaceae); this paper extends these observations by examination of sporangium formation in the host family Myricaceae. Light microscopic observations on the ontogeny of the endophyte were made on spore(+) nodules from both laboratory- and field-grown plants. In addition, contrasts in host/endophyte interactions were made between spore(+) and spore(−) nodules from plants grown in water culture.

MATERIALS AND METHODS—*Culture of the symbionts*—Fruits of *Myrica gale* L. and *Comptonia peregrina* L. (Coult.) were collected near the Harvard Forest, and the seeds were germinated as described previously (VandenBosch and Torrey, 1983). Seeds of *Alnus rubra* Bong. from Oregon, and of *A. incana* ssp. *rugosa* (Du Roi) Clausen, collected in Pelham, Massachusetts, were soaked overnight in distilled water prior to sowing (Berry, 1983). Germination of *M. cerifera* seeds was effected by removal of the waxy exocarp and soaking the seeds overnight in 500 ppm gibberellic acid.

Two to four weeks after germination, seedlings were transferred to water culture and inoculated with a suspension of crushed nodules or with a cultured isolate of *Frankia* sp. (VandenBosch and Torrey, 1983). Spore(+) nodules to be used as inoculum were collected in the field or obtained from greenhouse stock plants. Two strains of *Frankia* were used as inocula: HFPCpII, originally isolated from *C. peregrina* (Callaham et al., 1978) and cultured on a modified *Frankia* broth (Baker and Torrey, 1979), and MgPlOi, obtained from *M. gale* (Nesme and Lalonde, unpubl.) and cultured on liquid QMOD medium (Lalonde and Calvert, 1979).

Microscopy—Nodule lobes were split longitudinally and fixed for 2 hr at room temperature in 3% glutaraldehyde in 0.025 M sodium phosphate buffer, pH 7.0. Specimens were rinsed in buffer, dehydrated in acetone and embedded flat in Spurr's (1969) low viscosity resin. Sections, 1 μm thick, were cut on a Sorval Porter-Blum ultramicrotome using glass knives, removed to gelatin-coated microscope slides (Berlyn and Miksche, 1976) and dried on a slide warming tray at 55 C. Sections to be examined with bright-field optics were stained at 55 C in 0.05% Toluidine Blue O in 1% sodium tetraborate.

For fluorescence microscopy, plastic was removed from sections attached to gelatin-coated slides by soaking the slides in sodium methoxide for 45 to 60 min (Peterson, Hersey and Brisson, 1978). Specimens were stained for host cell wall components with 0.1% Calcofluor White (American Cyanamid) for 30–60 sec (O'Brien and McCully, 1981). The periodic acid-Schiff's (PAS) procedure, which stains total polysaccharides, was carried out using 0.01% bis-(4-aminophenyl)-1,3,4-oxadiazol (BAO) (Fluka) as a fluorescent Schiff's reagent (Callaham, 1979). Specimens to be examined for autofluorescence were left unstained and mounted in low fluorescence immersion oil.

Cultured frankiae were fixed in 3% glutaraldehyde in sodium phosphate buffer before staining. The hyphae were stained in centrifuge tubes, washed in distilled water by centrifugation and mounted in distilled water. Specimens were examined with a Leitz Ortholux epifluorescence microscope equipped with an Osram HBO 200 w mercury vapor lamp. Exciter filters UG1 and BG38 were used in combination with a Leitz K430 barrier filter. Under these conditions, autofluorescence appeared a dull yellow-brown, PAS-positive material stained a bright gold and Calcofluor-stained material was bright blue.

RESULTS—*Occurrence of spore(+) nodules in nature*—*Comptonia peregrina* nodules from eight sites in Massachusetts and two in Michigan ($n \geq 10$ per site) were sectioned and examined for sporangia. Only one site, in Edgartown, Martha's Vineyard, Massachusetts, sampled in June, 1981, and March, 1982, yielded spore(+) nodules. On both occasions, 30% of the nodules collected contained sporangia or mature spores.

An extensive survey of *M. gale* nodule spore types was not made. However, *M. gale* populations bearing almost exclusively spore(+) or spore(−) nodules (Schwintzer, Berry and Disney, 1982), as well as mixed populations (S. Lancelle, pers. comm.) exist near the Harvard Forest. Spore(+) *M. gale* nodules used for the nodule phenology study were obtained from the pond-side site in Petersham, Massachusetts, described by Schwintzer (1979). Attempts to isolate a strain of *Frankia* from spore(+) nodules of either *C. peregrina* or *M. gale* have thus far been unsuccessful.

Ontogeny of the endophyte and infected host cells in nodules of Comptonia peregrina—Both spore(+) and spore(−) inoculated seedlings developed nodules within 2 wk of inoculation. Vesicles differentiated in both by 3 wk after

inoculation. Young sporangia were first discernible at 4 wk and mature sporangia were present at 6 wk. No spore(−) nodules were observed to differentiate sporangia.

Tissue organization of nodules, sampled 8 wk after inoculation, resulting from both inocula was similar. The structure of spore(−) *C. peregrina* nodules has been described (Newcomb et al., 1978). Nodules represent highly modified lateral roots. Near the nodule apex of each lobe is a meristem which gives rise to all cell types of the nodule. The youngest infected cells lie proximal to the nodule meristem, with progressively more basal cells exhibiting an ontogenetic series in host/endophyte interactions. In the youngest infected cells, invasive hyphae invaded cell to cell, became branched and these branched hyphae ramified to fill the center portions of the host cytoplasm. Septate, clavate, terminal vesicles differentiated around the perimeter of the host cell cytoplasm. The central hyphae internal to the vesicles remained undifferentiated at this stage (Fig. 2). The invasive hyphae, about 1 μm in diameter, were widely septate, at intervals of 3 to 5 μm. Spore(+) and spore(−) nodules are identical at this stage.

In spore(+) nodules, additional segmentation of the invasive hyphae (Fig. 3, 4) signaled sporangial development, initiated about 0.5 mm from the youngest infected host cells. Cleavage first occurred transversely and then parallel to the long axis of the hyphae, giving rise to a three-dimensional configuration of bacterial cells. Host cell nuclei at this stage remained intact, and other cellular features were normal, similar to those of infected cells containing mature vesicle clusters without sporangia. Sporangia differentiated primarily from the central hyphae internal to the vesicles and possibly from invasive hyphae (Fig. 3, 4). As sporangia enlarged, they became more readily distinguishable from the hyphal stage.

The host cell rapidly senesced following the onset of sporangial differentiation (Fig. 4, 5). A distinction between cytoplasm and vacuoles could no longer be made, nuclei disappeared and the cells appeared to lose turgor. Endophytic vesicles also succumbed, leaving lightly staining "ghosts" inside the periphery of the host cell wall. Endophytic sporangia continued to enlarge until the mature sporangia filled the dead host cell (Fig. 6, 7). Invasive hyphae often persist in cells containing mature sporangia. Their role in the differentiation of sporangia is not clear. They may give rise to sporangia in the internal portion of the host cell; they may also provide communication between maturing sporangia and the endophyte in other host cells.

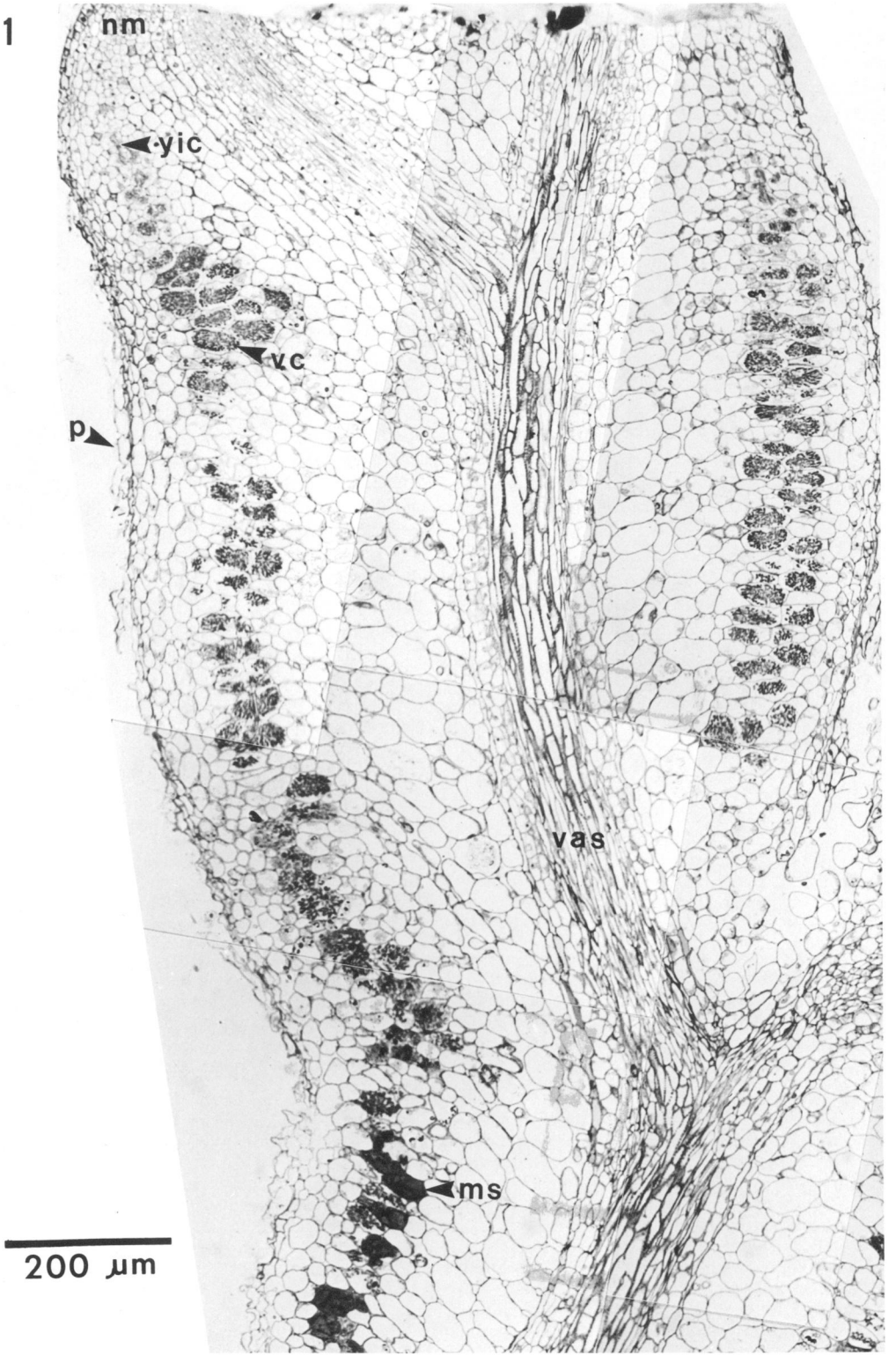
The endophyte is surrounded by host-derived polysaccharide encapsulation, within the

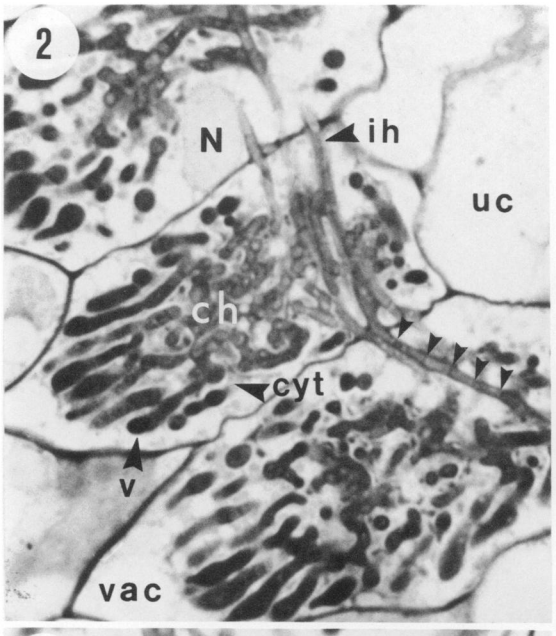
→

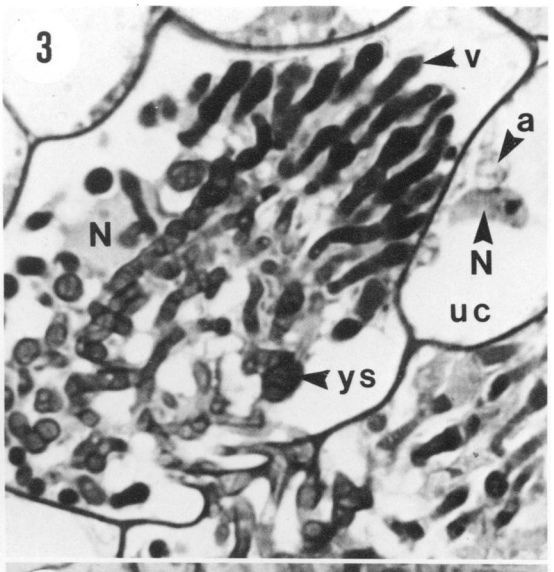
KEY TO LABELING: a, amyloplast, ch, central hyphae, cv, collapsed vesicle, cyt, host cytoplasm, hcw, host cell wall, ih, invasive hyphae, ms, mature sporangium, N, nucleus, nm, nodule meristem, nu, nucleolus, p, periderm, sp, sporangium, st, starch, sv, senescent vesicle, uc, uninfected cell, v, vesicle, vc, vesicle cluster, vac, vacuole, vas, vascular cylinder, yic, youngest infected cell, ys, young sporangium.

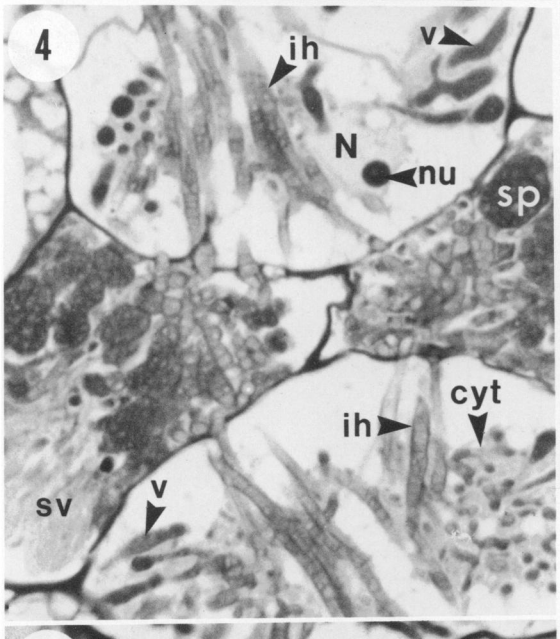
Fig. 1. Photomontage of a near-median longitudinal section through a nodule lobe from a 6-wk-old *Comptonia peregrina* nodule. The apex of the lobe is located at the top of the micrograph. Apical nodule roots are not shown. The nodule meristem is visible at the tip of the young branch lobe at the top left. Another nodule lobe, visible as a branch of the vascular cylinder, was connected to the one pictured at the bottom right. The infected host cells lie in the mid-cortex, in a cylinder around the vascular tissue. The youngest infected cells, near the nodule apex, contain endophytic filaments. In older, more basal, cells, sporangia and vesicles have differentiated.

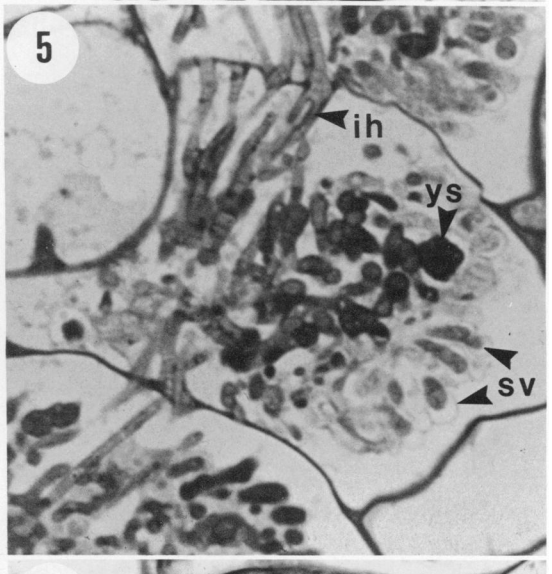
Fig. 2–7. Light micrographs of endophyte development in a 6-wk-old *C. peregrina* spore(+) nodule. **2.** Vesicle clusters in infected host cells. Invasive hyphae run from cell-to-cell. Arrowheads indicate the widely spaced septations in the invasive hypha. Septate hyphae branch off the invasive hyphae and terminate in vesicles around the host cell periphery. Host cell nuclei, cytoplasm and vacuoles are visible at this stage. ×1,280. **3.** An older vesicle cluster in a cell containing young sporangia. Sporangia are forming from the hyphae in the central region of the cell. This and later stages are absent from spore(−) nodules. Amyloplasts and a nucleus are visible in an adjacent uninfected cell. ×1,380. **4.** Various stages of sporangial development. In the cells at the top and the bottom of the micrograph, the invasive hyphae are highly septate. Host cell nuclei and cytoplasm are visible. The cells in the middle show a later stage of sporangial development in which the vesicles appear collapsed and the sporangia have undergone repeated septation and growth to become globose bodies. ×1,130. **5.** A stage of sporangial development following that shown in Fig. 3. Young sporangia are visible in the region of the cell between the invasive hyphae and the vesicles. The vesicles stain less darkly and appear to be shrinking from the encapsulation. Host cell constituents are no longer visible. ×1,280. **6.** Sporangia are the most prominent stage of the endophyte. The collapsed vesicles have been pushed against the host cell wall. Invasive hyphae persist. ×1,130. **7.** The sporangia have proliferated to fill the dead host cell. The limits of individual sporangia within the host cells are no longer visible. Individual spores (arrowheads) have separated from the mature sporangia. ×1,130.

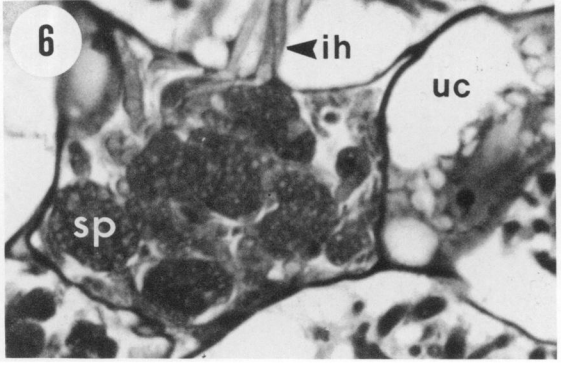

1
nm
yic
vc
p
vas
ms
200 µm

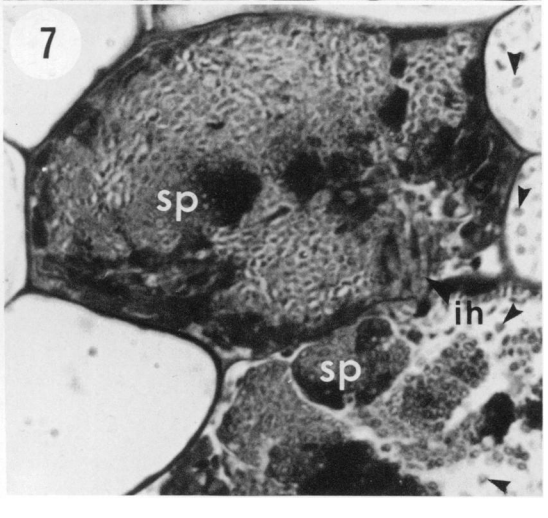

2
N
ih
uc
ch
cyt
v
vac


3
v
a
N
N
uc
ys


4
ih
v
N
nu
sp
cyt
ih
sv
v


5
ih
ys
sv


6
ih
uc
sp


7
sp
ih
sp

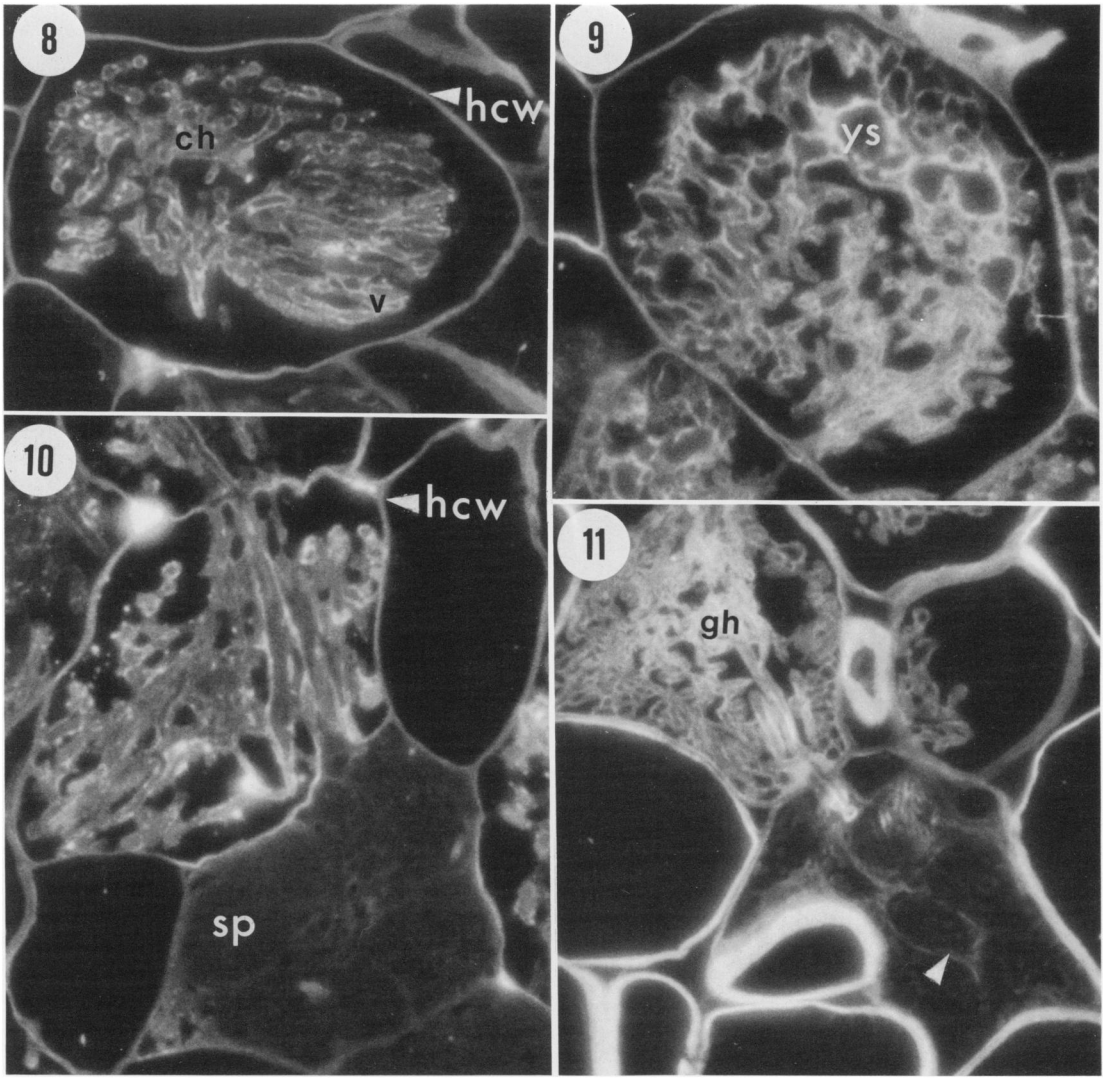


Fig. 8–11. Calcofluor White-stained sections of nodules of *C. peregrina* (8–10) and *M. gale* (11). Calcofluor stains the host cell wall and the host-produced encapsulation around the endophyte. *Frankia* itself does not fluoresce when treated with Calcofluor **8.** The host cell wall and the encapsulation around the hyphae and vesicles are brightly stained. ×1,020. **9.** Young sporangia are also encapsulated. ×1,038. **10.** The encapsulation is seen to be continuous with the host cell wall. The cell at the top contains a vesicle cluster; the one below contains mature sporangia, which appear to be no longer encapsulated. ×1,020. **11.** Infected cells from the basal portion of an *M. gale* spore(+) nodule lobe from the field. The encapsulation of the "ghost hyphae" stains brightly. Sporangia in the adjacent cell are not completely encapsulated, although remnants remain (arrowhead). ×1,020.

host cell. It is difficult to detect this capsule or to distinguish it from the bacterial structures, using general stains and bright-field optics, except when the endophyte is dead. Fluorescence microscopy and diagnostic staining were therefore used to study the encapsulation.

Host cells showed slight autofluorescence, probably due to glutaraldehyde fixation, after removal of the plastic from sections. The cell walls of infected cells were an exception. They autofluoresced more brightly than uninfected cells, similarly to xylem elements, probably indicating the presence of lignin- or suberin-like compounds (Schaede, 1933; Berg, 1983).

Frankia sp. in culture also exhibited dim autofluorescence. Hyphae, vesicles and sporangia all stained brightly with the PAS procedure, which stains all polysaccharides. The PAS procedure, therefore, is not adequate for distinguishing the encapsulation around the

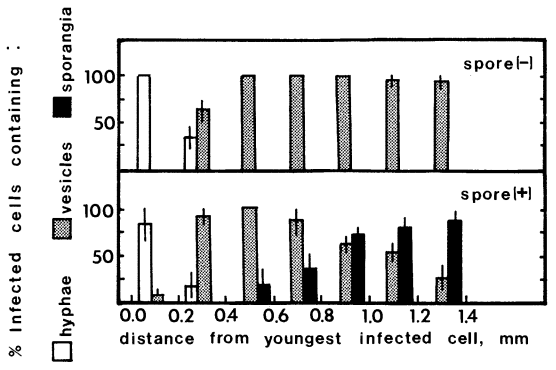


Fig. 12. Ontogeny of the endophyte in *C. peregrina* nodules harvested eight weeks after inoculation. Near-median sections of nodule lobes were chosen from three nodules of both the spore(−) (inoculum = *Frankia* sp., HFPCpII) and the spore(+) (inoculum = crushed nodules) types. Nodule lobes were divided into 0.2-mm regions, each with about 15 infected host cells. Data for the youngest region of the lobe, adjacent to the nodule apex, are illustrated toward the left end of the x-axis, with progressively older tissue towards the right. The proportion of infected host cells which contained the various endophytic forms, undifferentiated hyphae, vesicles or sporangia, was calculated for each region. Vertical bars show standard error.

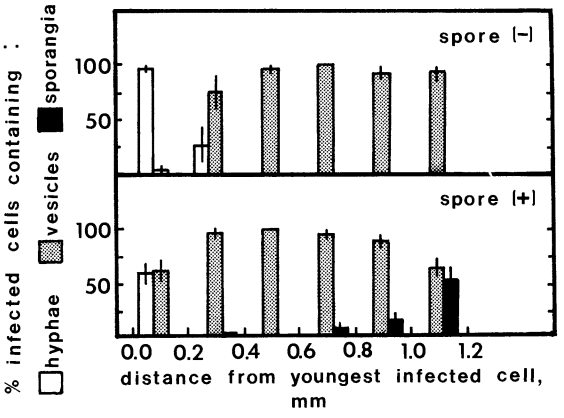


Fig. 13. Ontogeny of the endophyte in nodules of *M. gale*. Spore(+) nodules were induced using a crushed nodule inoculum; spore(−) nodules were induced with *Frankia* sp., MgPlOi. Analysis of the endophyte development as in Fig. 12. Each count represents data from approximately 25 infected host cells; Vertical bars show standard error.

endophyte in the infected host cells since it also stains the actinomycete walls. Calcofluor White, thought to stain primarily β-linked glucans, did not stain cultured *Frankia* whose walls are made up of meso-diaminopimelic acid and other amino acids and amino sugars (Lechevalier, Horrière and Lechevalier, 1979). Calcofluor is therefore more useful in visualizing the encapsulation in the nodule.

Calcofluor stained only host cell walls and the encapsulation surrounding the endophyte. The youngest hyphal stages of the endophyte were always encapsulated, as were vesicles (Fig. 8) and young sporangia (Fig. 9). Following host cell death, during enlargement of sporangia, the capsule appeared to lose Calcofluor-induced fluorescence, and to be stretched by the growing sporangia. Although remnants of the capsule sometimes remained around individual sporangia, many mature sporangia appeared not to be encapsulated (Fig. 10, 11). Thus while the host cell is alive, the endophyte is not in contact with host cytoplasm. Following host cell death, the encapsulation may be burst or perhaps enzymatically digested by the developing sporangia. In host cells where sporangial differentiation does not occur, but the endophyte and host cell simply senesce, the encapsulation remains intact and retains its staining properties (Fig. 11).

Figure 12 contrasts the ontogenetic changes of the endophyte in spore(+) and spore(−) *C. peregrina* nodules from plants grown in the laboratory. The younger portions of the nodule lobes from both nodule types showed similar high numbers of infected cells containing vesicle clusters. The two nodule types showed marked differences in the composition of the endophyte towards the base of nodules lobes. In spore(−) nodules, vesicle clusters persisted to the base of the lobe, whereas in spore(+) nodules, the vesicles senesced as the sporangia developed. This comparison indicates that differentiation of sporangia within the nodule is not merely an alternate path to senescence, but may reduce vesicle longevity and hasten the death of the host cell. *Myrica gale* nodules (Fig. 13) showed a similar pattern, but sporangia occurred in a smaller proportion of cells.

Seasonal behavior of the endophyte in spore(+) Myrica gale nodules—Mature sporangia were consistently present in the previous year's tissue (Fig. 14) in *M. gale* nodules collected mid-month from May through October, 1983, from a spore(+) field site. Vesicles had begun to differentiate in May, but the most prevalent stage was young, undifferentiated hyphae towards the apex of the nodule lobe. Vesicles continued to differentiate through June and July, and were present in progressively older tissue. Sporangial differentiation followed the formation of vescicles, as in laboratory-grown nodules. Developing sporangia were first seen in the current season's tissue in low numbers in June, became more prevalent in July and August and occurred in greatest numbers near the base of the nodule lobe. Spo-

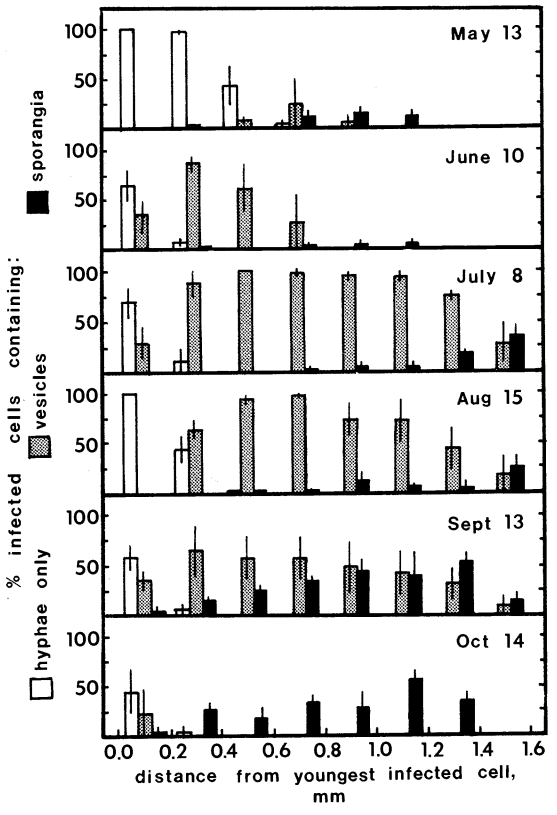


Fig. 14. Phenology of the endophyte in spore(+) *M. gale* nodules collected in the field May through October 1983. Sections from three nodules were examined from each date. Analysis of the endophyte as in Fig. 12. Each count represents data from approximately 25 cells; Vertical bars show standard error.

rangia predominated through September and October, extending into the youngest infected tissue near the lobe apex.

Interspecific cross-inoculations—Spore(+) *C. peregrina* and *M. gale* nodule suspensions and cultures of *Frankia* sp. HFPCpII were used to inoculate seedlings of *C. peregrina, M. gale, M. cerifera, Alnus rubra* and *A. incana* ssp. *rugosa* grown in water culture. The resulting nodules were examined for spores after 8 wk. Neither crushed nodule inoculum was capable of inducing nodulation on either species of *Alnus*. *Comptonia peregrina* spore(+) nodules produced spore(+) nodules on both *Myrica* species. Likewise, the crushed *M. gale* nodules induced the formation of spore-containing nodules on both *C. peregrina* and *M. cerifera,* although these nodules were ineffective and lacked vesicles (VandenBosch and Torrey, 1983). HFPCpII nodulated all of the potential host species tested and all of the resulting nodules were of the spore(−) type. These results support van Dijk's (1978) hypothesis that the expression of spores within the nodule is determined by the endophytic strain.

DISCUSSION—The differentiation in actinorhizal nodules of endophytic spores, also variously called bacteroids and granules, has been observed in field-collected nodules of *Alnus glutinosa* (Schaede, 1933; Käppel and Wartenberg, 1958; Becking et al., 1964; van Dijk and Merkus, 1976; Gardner, 1976; Suetin et al., 1979), *A. incana* (Huss-Danell et al., 1982), *A. crispa* and *A. rugosa* (Normand and Lalonde, 1982) and *Myrica gale* (Schaede, 1938; Fletcher and Gardner, 1974; Gardner, 1976; Schwintzer et al., 1982). Baker, Newcomb and Torrey (1980) depicted sporangia in ineffective nodules of *Elaeagnus umbellata* induced by *Frankia* sp. EuII in the laboratory, suggesting that sporangia may occur in field nodules of *Elaeagnus* spp. Casual reports of spores or sporangia have been made for *Casuarina cunninghamiana, Ceanothus velutinus* and *Hippophäe rhamnoides* (Gardner, 1976), but these reports require further substantiation.

It is unclear how widespread the phenomenon of sporangial differentiation is within actinorhizae. It is not surprising that most citations should be made for *Alnus* because it has been most commonly studied. The presence of sporangia in other genera have probably been overlooked, especially where limited collections have been made and structural observations have been few. Our findings indicate that spore(−) nodules are more common than spore(+) for *Comptonia peregrina* in the field.

The origin of sporangia in nodules of *Alnus* spp. has been subject to controversy. In spore(+) nodules of *Comptonia peregrina,* endophytic sporangia developed from undifferentiated central hyphae in infected cells containing mature vesicles, an observation in agreement with studies of *Alnus glutinosa* nodules (Käppel and Wartenberg, 1958; Suetin et al., 1979, 1981; van Dijk and Merkus, 1976). The views that sporangia develop from fragmentation of tightly interlaced hyphae (Becking et al., 1964) or from the segmentation of vesicles (Gardner, 1965, 1976; Schaede, 1933) were not borne out by our study.

Differentiation of sporangia may have a pronounced effect on the fate of the infected cell. Becking and co-workers (1964) never observed sporangia in cells containing endophytic vesicles and concluded that sporangia only formed in dead or dying cells or in intercellular spaces, a view in agreement with Schaede (1933). Suetin et al. (1979) depicted young sporangia in

live vesicle-containing cells and also noted that the maturation of sporangia was associated with the eventual disorganization of the host cell cytoplasm. This latter sequence is similar to that of *Comptonia peregrina,* in which young sporangia occur in healthy host cells with prominent nuclei, numerous mitochondria and endophytic vesicles. However, as sporangia develop, host cell structural features are lost, the cells lose turgor and endophytic vesicles first senesce and then flatten against the host cell walls. Whether differentiation of sporangia triggers some events leading to host cell death or whether cellular events in the host, not visible at the level of light microscopy, might presage sporangium development is not known.

All structural studies of root nodules induced by *Frankia* have revealed a polysaccharide encapsulation layer that surrounds the endophyte, is continuous with the host cell wall and is believed to be of host origin. In nodules containing only the hyphal and vesicular forms, the endophyte is always separated from the host cell cytoplasm by the encapsulation layer (Newcomb et al., 1978; Lalonde and Knowles, 1975). In spore(+) *C. peregrina* and *M. gale* nodules, young sporangia are encapsulated as long as the host cell is alive. Following host cell death, mature sporangia often appeared to be only partially encapsulated, or to lack a capsule altogether. Encapsulation material may be ruptured by the expanding sporangia, or perhaps be digested enzymatically.

Nearly all *Frankia* strains so far isolated form sporangia in culture (e.g., Baker, 1982, Normand and Lalonde, 1983, Zhang, Lopez and Torrey, 1984), but not all do so in the nodule. On the basis of cross-inoculation experiments, van Dijk and Merkus (1978) concluded that the ability to form sporangia within nodules is under genetic control of the endophyte. That viewpoint is supported by our study; the generalization may be extended to cross-inoculations between species within a family. Normand and Lalonde (1982) also adopted a two-strain-type concept based on comparisons of isolates made from two species of *Alnus.* These two strain types, that is spore(−) and spore(+), differed not only in morphology in the nodule, but also in behavior in culture. Normand and Lalonde (1982) suggested that sporulation serve as a basis for a systematic division of *Frankia* spp.

Because almost all strains of isolated frankiae are capable of forming sporangia in culture, although most do not do so within the nodule, the host must be capable of suppressing sporangium formation by the so-called spore(−) strains (e.g., HFPCpII). In spore(+) nodules of both *C. peregrina* and *M. gale* in the laboratory, and *M. gale* nodules in the field, sporangial differentiation does not occur in all infected cells. Some infected cells retain their vesicle clusters until senescence of the endophyte and host cell occurs at the base of the nodule lobe, as in spore(−) nodules. The proportion of infected cells containing sporangia may differ according to endophytic strain or to host.

Differentiation of sporangia in spore(+) nodules occurs seasonally in the field. Preliminary evidence for *M. gale* (Schwintzer et al., 1982) suggested that sporangia begin to differentiate in mid-summer and that spores persist all year in the previous year's tissue. Our study substantiates this pattern. Sporangia began to develop in June and increased in number, forming in progressively younger tissue as the season progressed. This suggests that the host may suppress sporangium formation for the most active part of the growing season. The mechanism for host control of endophytic sporulation is unknown.

The production of sporangia by the endophyte may improve the survival of *Frankia* after the death of the host nodule tissue, but it may also have a detrimental effect on the infected host cell and on the symbiosis. The differentiation of sporangia may precipitate host cell death. In spore(+) nodules of *C. peregrina* and *M. gale,* vesicle longevity is less than in spore(−) nodules. Because vesicles are believed to be the site of nitrogenase activity, sporangia may develop at the expense of nitrogen fixation.

LITERATURE CITED

BAKER, D. 1982. A cumulative listing of isolated frankiae, the symbiotic, nitrogen-fixing actinomycetes. The Actinomycetes 17: 35–42.

———, W. NEWCOMB, AND J. G. TORREY. 1980. Characterization of an ineffective actinorhizal microsymbiont, *Frankia* sp., EuII (Actinomycetales). Can. J. Microbiol. 26: 1072–1089.

———, AND J. G. TORREY. 1979. The isolation and cultivation of actinomycetous root nodule endophytes. *In* J. C. Gordon, C. T. Wheeler, and D. A. Perry [eds.], Symbiotic nitrogen fixation in the management of temperate forests, pp. 38–56. Forest Research Laboratory, Oregon State University, Corvallis, Oregon.

BECKING, J. H., W. E. DEBOER, AND A. L. HOUWINK. 1964. Electron microscopy of the endophyte of *Alnus glutinosa.* Antonie van Leeuwenhoek 30: 343–376.

BENSON, D. R., AND D. E. EVELEIGH. 1979. Ultrastructure of the nitrogen-fixing symbiont of *Myrica pensylvanica* L. (Bayberry) root nodules. Bot. Gaz. 140(Suppl.): S15–S21.

BERG, R. H. 1983. Preliminary evidence for the involvement of suberization in infection of *Casuarina.* Can. J. Bot. 61: 2910–2918.

BERLYN, G. P., AND J. P. MIKSCHE. 1976. Botanical mi-

crotechnique and cytochemistry. Iowa State University Press, Ames, Iowa.

BERRY, A. M. 1983. The development of the actinorhizal association between *Frankia* and *Alnus rubra* Bong: life history and cultural methods for the symbionts and a structural interpretation of the infection process. Ph.D. thesis. University of Massachusetts, Amherst.

BOND, G. 1976. The results of the IBP survey of root-nodule formation in non-leguminous angiosperms. *In* P. S. Nutman [ed.], Symbiotic nitrogen fixation in plants, pp. 443–474. Cambridge University Press, Cambridge, England.

CALLAHAM, D. 1979. A structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium trifolii*. M.S. thesis. University of Massachusetts, Amherst.

———, P. DEL TREDICI, AND J. G. TORREY. 1978. Isolation and cultivation *in vitro* of an actinomycete causing root nodulation in *Comptonia*. Science 199: 899–902.

VAN DIJK, C. 1978. Spore formation and endophyte diversity in root nodules of *Alnus glutinosa* (L.) Vill. New Phytol. 81: 601–615.

———, AND E. MERKUS. 1976. A microscopic study of the development of a spore-like stage in the life cycle of the root nodule endophyte of *Alnus glutinosa* (L.). Gaertn. New Phytol. 77: 73–90.

FLETCHER, W. W., AND I. C. GARDNER. 1974. The endophyte of *Myrica gale* nodules. Ann. Microbiol. (Milan) 24: 159–172.

GARDNER, I. C. 1965. Observation on the fine structure of the endophyte of the root nodules of *Alnus glutinosa* (L.) Gaertn. Arch. Mikrobiol. 51: 365–83.

———. 1976. Ultrastructural studies of non-leguminous root nodules. *In* P. S. Nutman [ed.], Symbiotic nitrogen fixation in plants, pp. 485–495. Cambridge University Press, London.

HUSS-DANELL, K., A. SELLSTEDT, A. FLOWER-ELLIS, AND M. SJÖSTRÖM. 1982. Ammonium effects on function and structure of nitrogen-fixing root nodules of *Alnus incana* (L.) Moench. Planta 156: 332–340.

KÄPPEL, M., AND H. WARTENBERG. 1958. Der Formenwechsel des *Actinomyces alni* Peklo in den Wurzeln von *Alnus glutinosa* Gaertn. Arch. Mikrobiol. 30: 46–63.

LALONDE, M., AND H. E. CALVERT. 1979. Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. *In* J. C. Gordon, C. T. Wheeler, and D. A. Perry [eds.], pp. 95–40. Symbiotic nitrogen fixation in the managements of temperate forests. Oregon State University, Corvallis, Oregon.

———, AND R. KNOWLES. 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.

LECHEVALIER, M. P., F. HORRIÈRE, AND H. A. LECHEVALIER. 1979. The biology of *Frankia* and related organisms. Dev. Indust. Microbiol. 23: 511–60.

MIAN, S., AND G. BOND. 1978. The onset of nitrogen fixation in young alder plants and its relation to differentiation in the nodular endophyte. New Phytol. 80: 187–192.

NEWCOMB, W., R. L. PETERSON, D. CALLAHAM, AND J. G. TORREY. 1978. Structure and host-actinomycete interactions in developing root nodules of *Comptonia peregrina*. Can. J. Bot. 56: 502–531.

NORMAND, P., AND M. LALONDE. 1982. Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. Can. J. Microbiol. 28: 1133–1142.

O'BRIEN, T. P., AND M. E. MCCULLY. 1981. The study of plant structure: principles and selected methods. Termarcarpi Pty. Ltd., Melbourne, Australia.

PETERSON, R. L., R. E. HERSEY, AND J. D. BRISSON. 1978. Embedding softened herbarium material in Spurr's resin for histological studies. Stain Technol. 53: 1–9.

SCHAEDE, R. 1933. Über die Symbionten in den Knöllchen der Erle und des Sandorns und die cytologischen Verhältnisse in ihnen. Planta 19: 389–419.

———. 1938. Die Actinomyceten-Symbiose von *Myrica gale* Planta 29: 32–46.

SCHWINTZER, C. R. 1979. Nitrogen fixation by *Myrica gale* root nodules in a Massachusetts wetland. Oecologia 43: 283–294.

SCHWINTZER, C. R., A. M. BERRY, AND L. D. DISNEY. 1982. Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity and shoot development in *Myrica gale*. Can. J. Bot. 60: 746–757.

SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastr. Res. 26: 31–43.

SUETIN, S. O., A. N. PARIISKAYA, AND L. V. KALAKOUTSKII. 1979. Structural aspects of endosymbiont development and interaction with host cytoplasm in *Alnus glutinosa* (L.) Gaertn. root nodules. Microbios Lett. 12: 83–92.

———, ———, AND ———. 1981. Electron microscopic study of the growth cycle of an actinomycete endosymbiont in nitrogen-fixing nodules of *Alnus glutinosa* roots. Microbiology 49: 524–528.

TJEPKEMA, J. D., W. D. ORMEROD, AND J. G. TORREY. 1980. Vesicle formation and acetylene reduction activity in *Frankia* sp. CpII cultured in defined media. Nature (London) 287: 633–635.

———, ———, AND ———. 1981. Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpII. Can. J. Microbiol. 27: 815–823.

TORREY, J. G. 1978. Nitrogen fixation by actinomycete-nodulated angiosperms. Bioscience 28: 586–592.

VANDENBOSCH, K. A., AND J. G. TORREY. 1983. Host-endophyte interactions in effective and ineffective nodules induced by the endophyte of *Myrica gale*. Can. J. Bot. 61: 2898–2909.

ZHANG, Z., M. F. LOPEZ, AND J. G. TORREY. 1984. Comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. Plant Soil 78: 79–90.