Host–endophyte interactions in effective and ineffective nodules induced by the endophyte of *Myrica gale*

KATHRYN A. VANDENBOSCH
Department of Botany, University of Massachusetts, Amherst, MA, U.S.A. 01003
AND
JOHN G. TORREY
Cabot Foundation, Harvard University, Petersham, MA, U.S.A. 01366
Received December 1, 1982

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.

Suspensions of crushed root nodules of *Myrica gale* containing the actinomycete *Frankia* induced nodule formation on roots of seedlings of *M. gale* and *Comptonia peregrina* grown in nutrient water culture. Nodules formed on *M. gale* were normal in structure and exhibited nitrogenase activity (measured as acetylene reduction) and provided the necessary nitrogen for seedling development. These effective nodules showed typical external and internal structure with the endophyte developing both vesicles and sporangia within cortical cells of the host tissue. Small nodules formed on *C. peregrina* representing the primary nodule stage. They lacked nitrogenase activity and were termed ineffective. Vesicles failed to develop within these ineffective nodules. However, sporangia were formed in infected cells and within intercellular spaces of the nodule cortical tissue. In addition, prominent amyloplasts occurred in infected cells of the ineffective nodules, a feature lacking in effective nodules. Exogenously supplied combined nitrogen increased seedling growth but did not improve nodule development or endophyte morphogenesis in the ineffective nodules.

VANDENBOSCH, K. A., et 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.

Des suspensions de nodules racinaires écrasés de *Myrica gale*, contenant l'actinomycète *Frankia*, induisent la formation de nodules sur les racines de plantules de *M. gale* et de *Comptonia peregrina* cultivées en milieu hydroponique. Les nodules formés sur le *M. gale* ont une structure normale, ils présentent une activité nitrogénasique (mesurée par la réduction de l'acétylène) et ils fournissent l'azote nécessaire au développement de la plantule. Ces nodules efficaces présentent une structure externe et interne typique, l'endophyte développant des vésicules et des sporanges à l'intérieur des cellules corticales de l'hôte. Sur le *C. peregrina*, il se forme de petits nodules représentant le stade de nodule primaire. Ces nodules ne manifestent pas d'activité nitrogénasique et sont qualifiés d'inefficaces. Aucune vésicule ne se développe dans ces nodules inefficaces. Cependant, il se forme des sporanges dans les cellules infectées et dans les espaces intercellulaires du tissu cortical du nodule. De plus, des amyloplastes très évidents se rencontrent dans les cellules infectées des nodules inefficaces, mais non dans les nodules efficaces. Lorsqu'on fournit au système des composés azotés exogènes, l'azote augmente la croissance de la plantule, mais n'améliore pas le développement du nodule, ni la morphogénèse de l'endophyte dans les nodules inefficaies.

[Traduit par le journal]

Introduction

Establishment of an effective dinitrogen-fixing symbiotic association between a soil microorganism and a host plant leading to the formation of root nodules involves a complex sequence of events. The complexity of the interactions has been most closely studied in legume–*Rhizobium* associations (Dart 1975, 1977; Vincent 1980; Newcomb 1980; Bauer 1981; Dazzo and Hubbell 1982). Many of these same sequential events, differing in structural and physiological detail, have been reported in actinorhizal–*Frankia* associations (Callaham and Torrey 1977; Lalonde 1977; Newcomb *et al.* 1978; Berry and Torrey 1983). Early events include host – soil microorganism recognition, root hair deformation, root hair invasion, bacterial filament growth, root cortical cell proliferation, prenodule formation, nodule lobe initiation, and endophyte proliferation and differentiation within the cortical cells. Effective nodules are formed when all these events are consumated and terminal vesicles, the site of nitrogen fixation in actinorhizae (Mian and Bond 1978; Tjepkema *et al.* 1980, 1981; Torrey *et al.* 1981; Schwintzer *et al.* 1982) are differentiated within nodule cortical cells.

As in legume–*Rhizobium* symbioses, different combinations of host and bacterium meet with differing degrees of success in actinorhizae. If failure occurs in early events, such as root hair deformation or the invasion of root hairs by *Frankia*, the symbiosis is not initiated and the microorganism is termed noninfective. In the case of successful infection, the symbiosis also may achieve differential success, reflecting differences in compatibility between the symbionts of the influence of environmental factors. Should the root cortical tissue fail to proliferate or should the endophyte fail to differentiate within the host cells, the result will be

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

limited nitrogenase activity or its complete absence. In the latter case, the nodules are termed ineffective.

With a diverse array of woody dicotyledonous plants known to be susceptible to *Frankia* infections, including in excess of 170 species in 19 genera distributed among 8 families, there probably exist a number of distinct *Frankia* strains which produce effective nodulation. To date, at least two large cross-inoculation groups have been well characterized by inoculating host seedlings with cultured isolates of *Frankia* or crushed nodule suspensions (Quispel and Burggraaf 1981; Lalonde *et al.* 1981). Within these cross-inoculation groups endophytes from one host generally can infect another host in the same group successfully. One of these groups is made up of host plants belonging to the Elaeagnaceae (Baker *et al.* 1980; Baker *et al.* 1981). The second group is more complex, consisting of members of the genus *Alnus* in the family Betulaceae together with genera in the family Myricaceae. Within this group there exists an interesting range of host–endophyte responses.

In Table 1 are summarized reports on nodulation patterns within the *Alnus–Myrica* cross-inoculation group. The table is separated into two parts, one based on inoculation with cultured isolates, the other with crushed-nodule suspensions or field-soil inocula. While the latter studies are prone to complications owing to methodology and are therefore subject to more scrutiny, they are helpful nevertheless in discerning trends in host range and symbiotic performance. The use of pure cultures of *Frankia* strains has greatly aided the determination of host ranges and will be the ultimate basis for such determination. In general, it is apparent that endophytes from any host within the group can nodulate other host species or genera within the group. With the possible exception of AvsI2, *Frankia* isolates are highly successful in effectively nodulating all tested host species in the group. Results of crosses involving crushed-nodule suspensions or soil inocula are more variable. There exist a few instances in which the endophyte is noninfective. In other combinations cross inoculation leads to ineffective nodules. In particular, nodule suspensions prepared from *Myrica gale* L. have been reported repeatedly (see references in Table 1) to produce nodules which are ineffective.

In the present study we have chosen to examine closely one such ineffective combination not previously reported, i.e., the endophyte from *M. gale* applied as crushed-nodule suspension to seedling roots of *Comptonia peregrina* (L.) Coult. The resulting ineffective nodules were examined physiologically and structurally and compared with effective nodules of *M. gale*. The intention was to learn more about the nature of the host–endophyte interaction and the causes of the failure to establish a successful symbiosis.

Materials and methods

Plant material

Seeds of *Comptonia peregrina* were collected prior to abscission, dried, and stored at 4°C. To induce germination, they were scarified, soaked for 24 h in gibberellic acid (GA_3), and sown in moist sand, following the findings of Del Tredici and Torrey (1976). Locally collected *Myrica gale* seeds were soaked 24 h in 500 ppm GA_3 and cold-stratified prior to sowing them in moist sand. Both types of seed were maintained in a growth chamber with a 16 h light : 8 h dark cycle, a daytime temperature of 24°C, and a nighttime temperature of 19°C. Seedlings were fertilized twice weekly with $\frac{1}{4}$-strength Hoagland's solution (Hoagland and Arnon 1950).

Approximately 3 weeks after germination, seedlings were transferred to 450-mL water culture jars, three per jar, containing $\frac{1}{4}$-strength Hoagland's solution, pH 6.5, minus nitrogen. Water cultures were maintained in a growth chamber at the above regime.

In a single experiment, ineffectively nodulated seedlings were supplied with graduated levels of nitrate 3 weeks after inoculation, approximately 1 week after nodule initiation. Nitrate was supplied as KNO_3 at 0, 5, 10, and 50 ppm N, with two seedlings per treatment.

Inoculum preparation and inoculation procedures

Nodules of *M. gale* from the field or from greenhouse plants were sectioned by hand and examined with phase optics for the presence of spores or were cleared and stained with lactophenol cotton blue (Nicholson 1959) and examined with bright-field optics. Those found to be spore positive were used as an inoculum. Living nodule tissue was dissected into lobes and surface sterilized for 20 min in 30% hydrogen peroxide. Nodule pieces were thoroughly rinsed in distilled water, then ground in a sterile mortar and pestle. The ground material was resuspended in distilled water and filtered through cheesecloth to remove large debris. Individual seedlings of *M. gale* and *C. peregrina* were inoculated with 0.03–0.05 g fresh weight of ground nodule tissue suspended in 1 mL of distilled water.

Frankia sp. CpI1, originally isolated from *C. peregrina* (Callaham *et al.* 1978), was used to produce effective nodules on both host species. CpI1 was grown in *Frankia* broth (Baker and Torrey 1979) modified to include vitamins, chelated iron, and major inorganic salts. Four-week-old cultures were harvested by centrifugation at 2000 rpm and washed once in sterile distilled water. Seedlings were each inoculated with an equivalent of 0.005–0.01 mL packed cell volume of CpI1 suspended in 1 mL of distilled water.

Following nodulation, which occurred 8 days to 2 weeks after inoculation, water culture solutions were changed weekly and pH was adjusted with additions of hydrochloric acid or sodium hydroxide as necessary. Three or more seedlings of each host species were left uninoculated as controls for contamination during each experiment. No uninoculated controls developed nodules during the course of these experiments.

Analysis of nodules: gas chromatography

Assays for nitrogenase activity were carried out 6–10 weeks after inoculation, using the acetylene-reduction method

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

TABLE 1. Results of cross inoculations between hosts in the *Alnus–Myrica* group and *Frankia* endophytes from members of the group (E, effective symbiosis; I, ineffective symbiosis; N, noninfective)

	Endophyte source										
	Frankia isolates					Crushed-nodule suspension or field-soil inocula*					
Host species	*Alnus rubra* ArI3 and ArI5	*A. viridis* ssp. *crispa* AvcI1	*A. viridis* ssp. *sinuata* AvsI2	*Comptonia peregrina* CpI1 and CpI4	*Myrica pensylvanica* MpI1	*A. glutinosa*	*A. jorulensis*	*M. cerifera*	*M. cordifolia*	*M. gale*	*M. pilulifera*
Betulaceae											
Alnus glutinosa	E(3)†	E(1)		E(8,15)		E(9,12)	I(9)			N(12)	
A. incana ssp. *rugosa*	E(3,6)	E(1,6)	N(6)	E(6,8, 15,16)	E(6)	E(9)	E(9)			I(16)	
A. jorulensis							I(9,13)				
A. multinervis							E(9)	E(9)			
A. rubra	E(3,6)	E(1,6)	E(6)	E(6,8, 15,16)	E(6)	E(9) I(2)	E(9)			E(14)	
A. sieboldiana						E(9)	I(9)				
A. viridis ssp. *crispa*	E(3,6)	E(1,6)	E(6)	E(6,8, 15)	E(6)						
A. viridis ssp. *sinuata*	E(3)	E(1,6)									
Myricaceae											
Comptonia peregrina	E(3,6)	E(1,6)	E(6)	E(6,5, 16)	E(6)					I(16)	
Myrica cerifera		E(1)		E(6,15, 16)	E(16)			E(9)*		I(4,7, 16)	
M. cordifolia								I(9)*	E(9)*	I(9)	I(9)
M. faya						E(14)		E(10)*	E(10)*	I(10) E(14)	E(10)
M. gale	E(3,6)	E(1,6)	E(6)	E(6,15, 16)	E(6)	E(11,12)		E(9)*	N(9)*	E(9, 16)	N(9)
M. pensylvanica				E(16)	E(16)						

*Indicates use of field-soil inoculum; others in this category not so designated were inoculated with crushed-nodule suspensions.

†Numbers denote literature references for data reported as follows: 1, Baker and Torrey, 1979; 2, Becking 1966; 3, Berry and Torrey 1979; 4, Bond, 1967; 5, Callaham *et al.* 1978; 6, Dillon and Baker 1982; 7, Gardner and Bond 1966; 8, Lalonde and Calvert 1979; 9, MacIntosh and Bond 1970; 10, Mian *et al.* 1976; 11, Quispel and Burggraaf 1981; 12, Rodriguez-Barrueco and Bond 1976; 13, Rodriguez-Barrueco and Rodriguez-Barrueco 1966; 14, Rodriguez-Barrueco and Miguel 1979; 15, Torrey *et al.* 1980; 16, K. VandenBosch, unpublished data.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

TABLE 2. Infectivity and effectivity of nodules resulting from cross inoculations between *Comptonia peregrina* and *Myrica gale* and their endophytes

Assay date after inoculation	Host	Inoculum	No. plants	Nodules/plant, $\bar{x}$	Acetylene reduction (μmol $C_2H_4 \cdot plant^{-1} \cdot h^{-1}$, $\bar{x} \pm$ SD)
6 weeks	*M. gale*	CpI1	6	16.7	0.63±0.41
		M. gale nodules	12	26.2	0.37±0.28
	C. peregrina	CpI1	5	14.2	0.07±0.08
		M. gale nodules	11	33.6	0.00±0.00
10 weeks	*C. peregrina*	CpI1	5		0.55±0.76
		*M. gale** nodules	10		0.00±0.00

*Plants assayed after having received 5 ppm N as NO_3^- for 4 weeks. See text.

(Burris 1974). In an initial experiment, whole seedlings were removed from water culture and placed in 245-mL incubation jars. In subsequent experiments, individual nodulated root systems were excised and placed in the incubation jars. To each jar was added 1 mL of distilled water to maintain a humid atmosphere. The roots were incubated in 10% acetylene (v/v; generated from CaC_2) for 1 h at 24°C. At the end of the incubation period, 1-mL gas samples were withdrawn from the incubation jars and injected into a Carle 9700 gas chromatograph equipped with a flame ionization detector for the detection of ethylene produced by nodules. Jars incubated without plants and with unnodulated seedlings were used as controls.

Preparation of material for light microscopy

Nodule lobes were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.025 *M* sodium phosphate buffer, pH 7.0. After rinsing five times in buffer, the tissue was dehydrated in acetone by increasing acetone concentration by 10% increments. Specimens were infiltrated with Spurr's low-viscosity resin (Spurr 1969) and embedded flat. Sections 1–1.5 μm in thickness were cut on a Sorval Porter–Blum ultramicrotome. Staining of sections for examination in the light microscope was with 0.05% toluidine blue in 1% sodium tetraborate over an alcohol lamp or on a slide warming tray maintained at 55°C.

Results

Infectivity of strains and effectivity of nodules

Results of an initial cross-inoculation experiment are summarized in Table 2. Both *Myrica gale* and *Comptonia peregrina* seedlings developed nodules when inoculated with either *Frankia* sp. CpI1 or *M. gale* crushed-nodule preparations. All test seedlings became nodulated. Prenodules were first observed 8–10 days following inoculation with CpI1 and 12–14 days following inoculation with *M. gale* crushed nodules.

Both inocula produced abundant nodules on each host species. However, those *C. peregrina* nodules induced by the *M. gale* endophyte remained small and the host plants became chlorotic in appearance, similar to the uninoculated controls. Six weeks following inoculation, the seedlings were assayed for nitrogenase activity. Both types of *M. gale* nodules and nodules of the *C. peregrina* – CpI1 cross had nitrogenase activity, whereas such activity was absent in *C. peregrina* nodules containing the *M. gale* endophyte.

Comptonia peregrina seedlings were returned to water culture and ineffectively nodulated and control seedlings were given a nutrient regime of $\frac{1}{4}$-strength Hoagland's solution plus 5 ppm N as nitrate, pH 6.5. After four additional weeks of culture, the seedlings were again assayed for nitrogenase activity. *Comptonia peregrina* – CpI1 showed increased activity per seedling, but nodules of *C. peregrina* containing the *M. gale* endophyte remained ineffective. In two subsequent experiments, seedlings were given 5 ppm N as nitrate following nodulation to sustain seedling development. In both experiments, the results were similar to those tabulated above.

Structure of nodules and ontogeny of infected cells

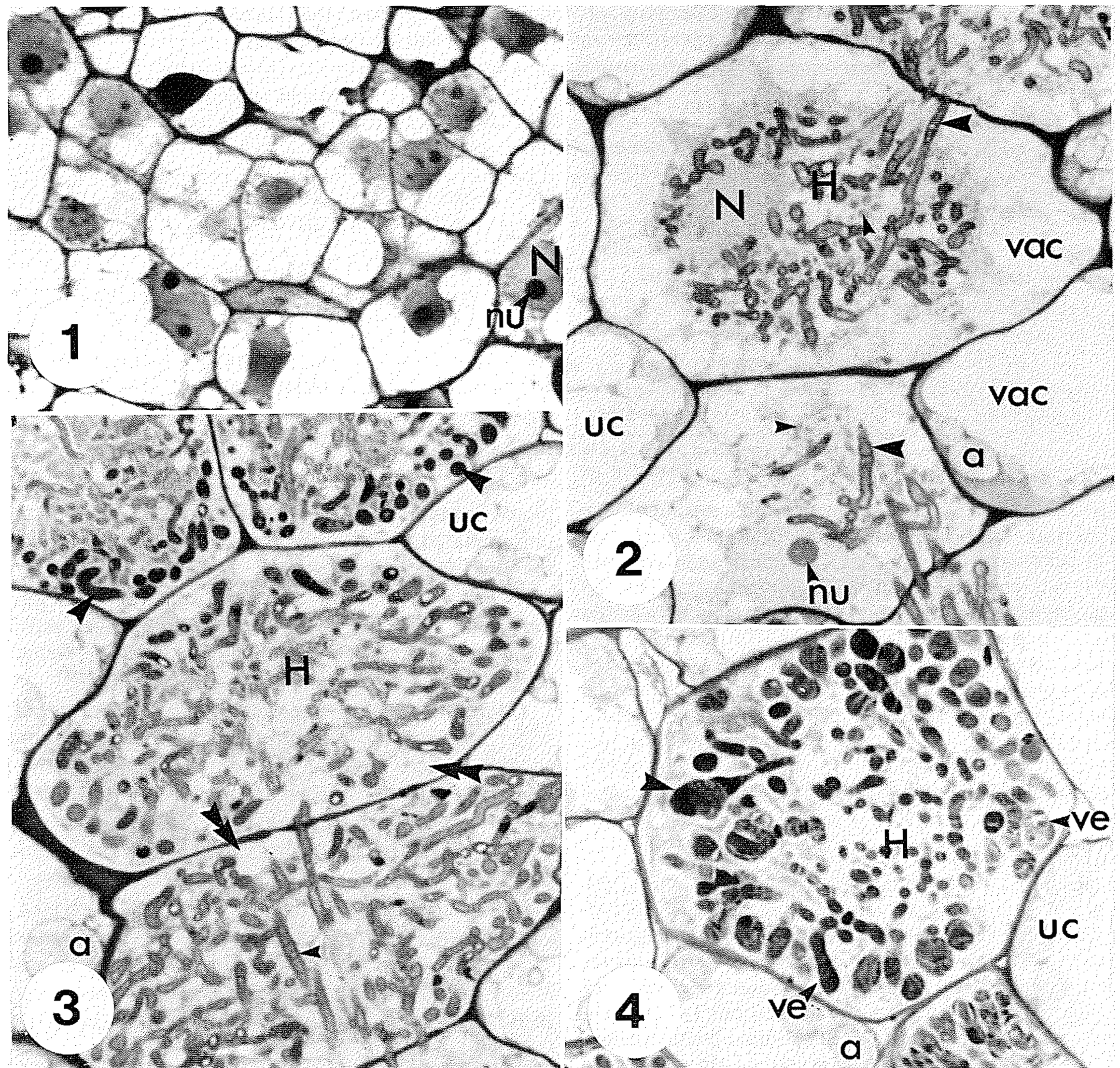
Effective *M. gale* nodules containing the *M. gale* endophyte were contrasted with ineffective *C. peregrina* nodules produced by the same inoculum. Both types of nodules showed complex structure, with multiple nodule lobes and nodule roots produced apically on the lobes. *Myrica gale* nodules showed repeated branching of lobes and attained up to 1 cm in size. *Comptonia peregrina* nodules remained small, with generally two to four lobes, representing the primary nodule condition (Callaham and Torrey 1977). Occasionally, some nodule lobes exhibited limited branching.

Both types of nodules showed tissue organization like that described for effective *C. peregrina* nodules

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

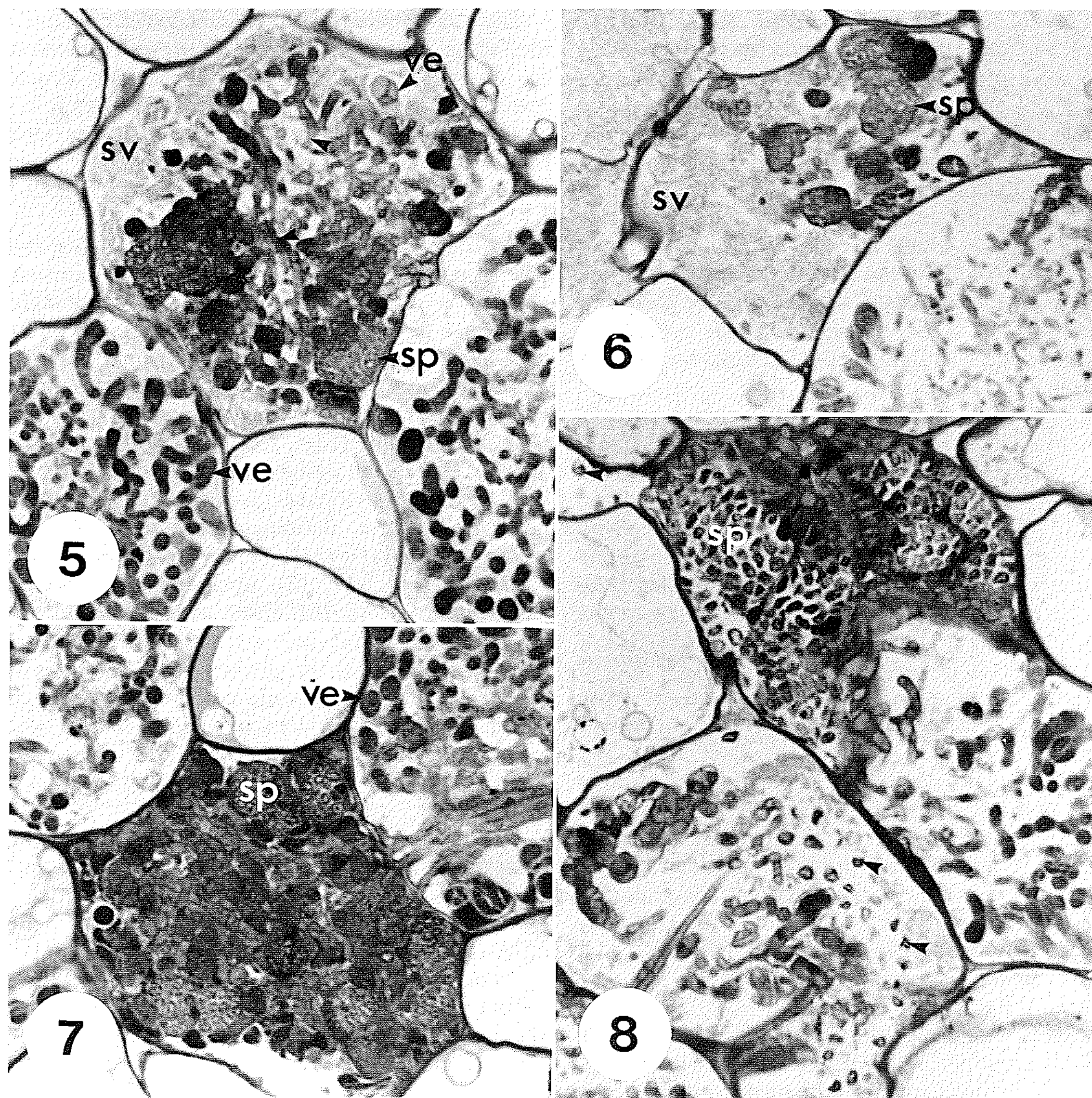


FIGS. 1–4. Stages in the development of effective *Myrica gale* nodules. ×1200. Fig. 1. Light micrograph showing uninfected meristematic cells of a *Myrica gale* nodule. Cells are small with large nuclei (N) and prominent nucleoli (*nu*). Fig. 2. Early infection stage of *M. gale*. Numerous small vacuoles (*vac*) surround the central cytoplasm of the enlarged cells. Colonizing hyphae of *Frankia* (large arrowheads) enter the cells perpendicular to the host cortical cells. Hyphae (H) proliferate to fill central portions of cytoplasm and surround the central nucleus (N). Adjacent uninfected cells (*uc*) have large central vacuoles and peripheral cytoplasm containing amyloplasts (*a*). Fig. 3. The transition from the hyphal to vesicular stage of the endophyte in *M. gale*. Hyphae (H) have filled the host cytoplasm and have begun to differentiate vesicles, visible as terminal swellings (large arrowheads) in the upper cells. Numerous small vacuoles (double arrowheads) are still visible in the infected host cells. Note septations (small arrowhead) in central hyphae. Fig. 4. Infected *M. gale* cell with mature vesicles (*ve*). Highly septate vesicles are oriented around periphery of host cell. Hyphae (H) occupy the center of the cells. Large arrow indicates a possible young sporangium. Uninfected cells (*uc*) remain highly vacuolate with prominent amyloplasts (*a*).

(Newcomb *et al.* 1978) and for *M. gale* nodules (Fletcher and Gardner 1974; Schaede 1938; Schwintzer *et al.* 1982). The nodule lobe has a meristem at its apex which gives rise proximally to primary tissues, similar to those produced during primary root growth. The endophyte is contained within the middle cortical cells and does not penetrate other tissues. The meristem, likewise, remains uninfected. The most newly infected cortical cells are found closest to the nodule meristem, while a progression of development of infected cells is found toward the proximal portion of the lobe.

The meristematic cells of *M. gale* nodules are

RIGHTSLINK



FIGS. 5–8. Further stages in the development of effective *M. gale* nodules. ×1200. Fig. 5. A view of young sporangia in an infected *M. gale* cell. Sporangia (*sp*) appear as polyhedral bodies with numerous divisions. The cell containing sporangia still contains a few vesicles (*ve*) and some senescent vesicles (*sv*). Hyphae (small arrowheads) are visible in the center of the cell. Adjacent infected cells still possess viable vesicles (*ve*). No host cytoplasmic detail is visible at this stage. Fig. 6. Another cell with young sporangia (*sp*). Note abundance of senescent vesicles (*sv*) and filaments. Fig. 7. A more advanced stage of sporangium formation in *M. gale*. Sporangia (*sp*) fill the entire cell. Adjacent infected cell has viable vesicles (*ve*) and no sporangia. Fig. 8. Nodule cells containing mature sporangia. Sporangia (*sp*) appear as loose packets of spores. Some spores (small arrowheads) are being released from broken cells into intercellular spaces.

typically about 15 μm in size and roughly isodiametric (Fig. 1). Nuclei are prominent and possess a large, single nucleolus. The cytoplasm possesses many small organelles, barely visible at the magnification afforded by the light microscope. Uninfected cortical cells are similar in appearance to meristematic cells but are more vacuolate and larger in size.

At the youngest stages of the infection (Fig. 2) the cytoplasm primarily occupies the center portion of the cell. Numerous small vacuoles are visible around the periphery of the cell. Nuclei are central and enlarged. Colonizing hyphae (Schwintzer *et al.* 1982) enter the host cell, passing through the center of the cytoplasm and running longitudinally through a file of cells. The

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

hyphae branch and proliferate to fill the cytoplasm (Figs. 2, 3) and surround the nucleus. Numerous mitochondria can be seen in the central portions of the cytoplasm, between hyphae. Uninfected cells at this stage are highly vacuolate. Their cytoplasm is oriented around the periphery of the cells and contains numerous amyloplasts with multiple starch grains.

When the endophyte has filled the cytoplasm, vesicles begin to develop which are first visible as terminal hyphal swellings. Fully developed vesicles are clavate in shape, septate, and distributed around the periphery of the cell (Figs. 4, 5). Branched hyphae occupy the center of vesicle-containing cells. Vacuoles may still be seen around the cell periphery.

Sporangia begin to develop in vesicle-containing cells and may mature when adjacent infected cells still contain vesicles. Young sporangia are difficult to distinguish from septate vesicles (Fig. 4) but undergo continued cleavage to become globose bodies with numerous polyhedral subdivisions (Figs. 4, 5, 6). As development of the sporangia progresses, vesicles in the same cells senesce and cytoplasmic detail is not longer discernable. Hyphae may persist in these cells. Eventually, sporangia fill the entire cell (Figs. 7, 8). At maturity, the sporangia appear as packets of loose spores. If the cells are broken, the spores may escape into intercellular spaces and other cells (Fig. 8). The endophyte does not develop synchronously in adjacent infected cells and sporangia do not develop in all cells.

The early stages of infection in ineffective nodules of *C. peregrina* approximate those of *M. gale* nodules described above. Cells of the meristem are similar in size and appearance to those of *M. gale* (Fig. 9). Newly infected cells have numerous vacuoles (Figs. 10, 11). Filaments first invade the center of the cell and then ramify to fill the cell.

In cells containing proliferating hyphae, starch commonly accumulates in prominent amyloplasts (Figs. 12, 13, 14), in direct contrast with mature infected cells of *M. gale*, where amyloplasts are never seen. Filaments are also occasionally seen in intercellular spaces between infected cells (Fig. 13).

The most obvious difference between effective and ineffective nodules containing the *M. gale* endophyte is the absence of vesicles in the latter. No structures resembling *Comptonia*-type vesicles were seen in any ineffective nodules sectioned. The hyphal stage does not represent the endpoint of development in these nodules, however, as sporangia do develop in many cells and intercellularly as well (Fig. 16).

Contrary to the situation in effective *M. gale* nodules, where sporangia develop distant from the meristem in the region of mature vesicle clusters, sporangia in ineffective *C. peregrina* nodules may begin to differentiate in newly infected cells in close proximity to the meristem (Fig. 15). When sporangial development commences, the host cytoplasm maintains a normal, healthy appearance (Figs. 13, 15), although amyloplasts appear only in the earliest stages. During sporangial development, amyloplasts disappear and the host cell begins to senesce. Sporangia appear first within the center of the cell (Figs. 13, 14, 15) and progress to fill the entire cell (Figs. 17, 18). Sporangia may also occur intercellularly (Fig. 16). Often as sporangial development progresses, the host cell collapses and loses its integrity (Fig. 17). In extreme cases, when walls between infected host cells collapse, the endophyte appears to proliferate extensively in intercellular spaces. Indeed, at that point the endophyte is occupying apoplastic space within the nodule, although it was derived from former host cells.

When mature, sporangia appear similar to those in *M. gale*. Also similar to *M. gale*, several different stages of endophyte may exist in adjacent host cells.

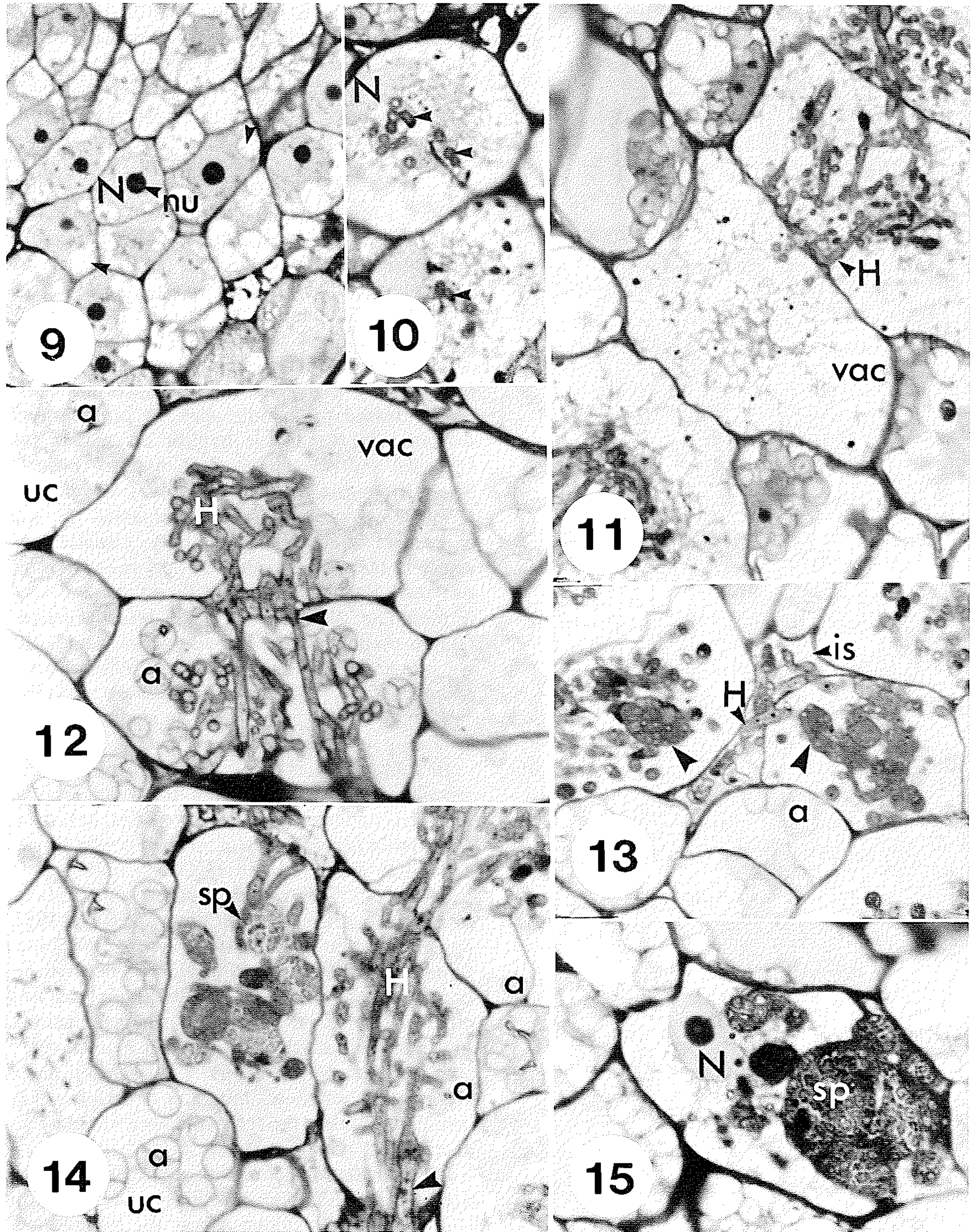
Effects of externally supplied nitrate on host–endophyte interactions in ineffective nodules

Four weeks after treatment with graduated levels of nitrate, ineffectively nodulated *C. peregrina* seedlings showed varied amounts of growth, according to the nitrogen supplied. However, nodules of all four treatments remained similar. None progressed beyond the primary nodule stage. All treatments showed hyphal and sporangial development of the endophyte and

FIGS. 9–15. Stages in the development of ineffective *C. peregrina* nodules. ×1200. Fig. 9. Meristematic cells of an ineffective nodule of *Comptonia peregrina*. Cells have small vacuoles (small arrowheads) and large nuclei (N) with prominent nucleoli (*nu*). Fig. 10. Earliest stage of infection in ineffective *C. peregrina* nodule. Nuclei (N) are visible at the edge of the cell. Endophytic hyphae seen in transection (small arrrowheads) invade the host cytoplasm. Fig. 11. Early infection of *C. peregrina*. Invading hyphae (H) branch to fill central portions of the host cytoplasm. Infected cells have numerous small vacuoles (*vac*). Fig. 12. Hyphal proliferation stage in ineffective nodule. Colonizing hyphae (large arrowhead) penetrate host cell wall at right angles. Hyphae (H) fill center of cytoplasm. Infected cells, as well as uninfected cells (*uc*), have numerous large amyloplasts (*a*) with multiple starch grains. Fig. 13. Young sporangia (large arrowheads) differentiating in infected cells. Note also the endophytic hyphae (H) in the intercellular space (*is*) between infected host cells. Fig. 14. Young sporangia (*sp*) in an ineffective nodule. Sporangia differentiate first in the central portion of the cell. Amyloplasts (*a*) are absent from the sporangia-containing cell but are still present in uninfected cells (*uc*) and nearby infected cells containing undifferentiated hyphae. Note extremely large hypha penetrating host cell wall (large arrowhead). Fig. 15. An infected cell in close proximity to the nodule meristem. A large sporangium (*sp*) is differentiating. Note the prominent nucleus (N) and cytoplasmic strands.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

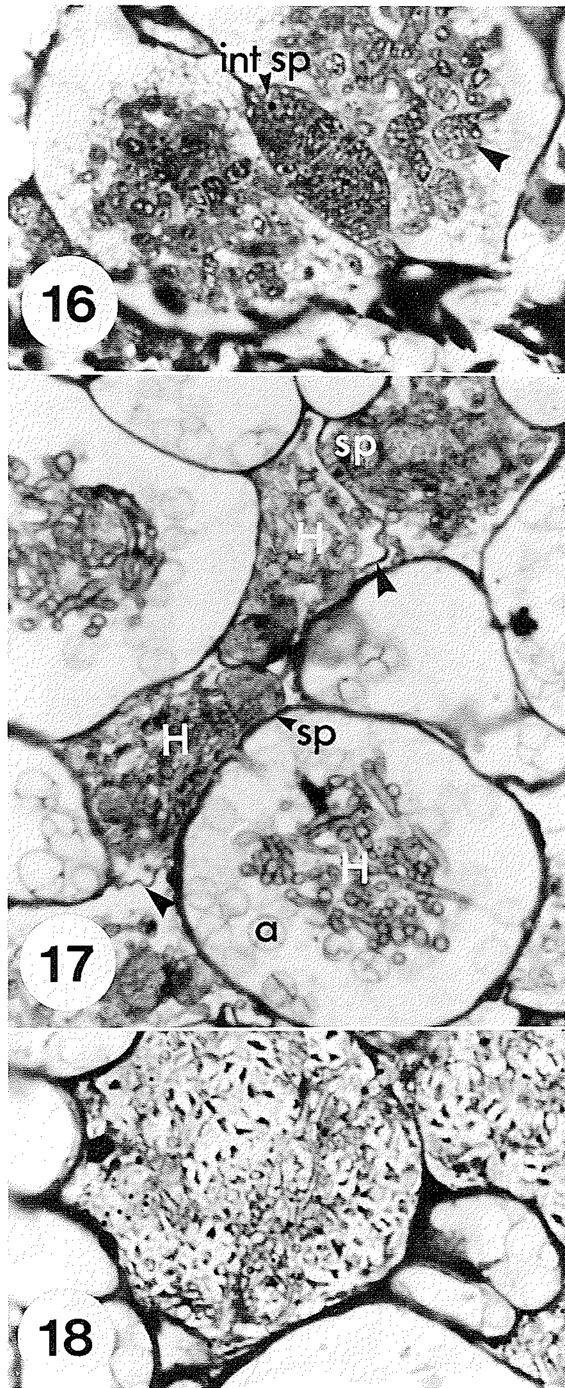
RIGHTSLINK



Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.



FIGS. 16–18. Late stages in the development of ineffective nodules of *C. peregrina*. ×1200. Fig. 16. Infected cells slightly proximal to the one depicted in Fig. 15. Young sporangia (large arrowhead) are forming in the cells. A large intercellular sporangium (*int sp*) occurs between the two cells. Fig. 17. A more advanced stage of sporangial formation. Sporangia (*sp*) appear as globose bodies. Hyphae (H) occupy a large proportion of the cell volume. Host cell walls (large arrowheads) between these cells are collapsing. Note that adjacent infected cells appear to be at an earlier stage of development with central hyphae, prominent amyloplasts (*a*), and no sporangia. Fig. 18. The final stage of spore formation. Sporangia appear as loose packets of polyhedral spores and fill the entire cell.

lacked vesicles. In addition, starch was present in infected cells of nodules of all treatments. These results suggest that nodule growth and endophyte proliferation in ineffective nodules are arrested early in development and are not merely limited by the unavailability of combined nitrogen.

Discussion

Once *Frankia* has achieved infection of a root hair cell in a susceptible host, the endophyte thereafter shows distinct phases of development (van Dijk and Merkus 1976; Newcomb *et al.* 1978; Suetin *et al.* 1979; Schaede 1933, 1938; Schwintzer *et al.* 1982). The invasive stage is characterized by large cordlike filamentous hyphae which penetrate successive cortical cell walls and traverse cell diameters rapidly, growing within the cortex toward the nodule apex. A proliferative stage follows with vegetative hyphae of smaller diameter (~1 μm) and branching to fill invaded cortical cells. The differentiation phase involves development of terminal vesicles and is accompanied by the first expression of nitrogenase activity. Usually at a later stage, in the same or other cortical cells and involving different filaments, terminal or intrahyphal sporangia differentiate, ultimately filling the infected cells. As the nodule cortical cells senesce, vesicles and filaments become deflated and empty, spores differentiate within sporangia forming thickened spore walls, and ultimately sporangia break open, releasing mature spores within senescing nodule cortical cells.

In actinorhizal nodules resulting from certain cross inoculations, one or more of these stages is absent. Ineffective nodules fail to form vesicles, as is the case of *Comptonia peregrina* roots infected by the *Myrica gale* endophyte described above. Other similar cases have been listed in Table 1. In these cases, sometimes referred to as incompatible crosses (Dillon and Baker 1983), it is apparent that both host and endophyte contribute to the failure in ways still to be understood, since both host and endophyte are able to form effective symbioses with other partners. Another type of incompatibility is illustrated by associations between *Frankia* sp. AvsI2 and members of the genus *Alnus* (Dillon and Baker 1983). AvsI2 exhibited low rates of nodulation and the resulting nodules, though containing vesicles, reduced acetylene at a very low rate.

Other crosses result in lack of sporangial differentiation, the so-called "spore-minus" situation. Van Dijk (1978) produced evidence that the differentiation of

RIGHTSLINK

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

sporangia and spores within developing nodules is under the genetic control of the endophyte rather than the host. Further evidence concerning this incompatibility needs to be provided.

In the *C. peregrina* – *M. gale* endophyte symbiosis described above, the first symptom of aberrancy in development is the accumulation of starch in the amyloplasts of cells containing proliferating hyphae. Starch deposition occurs both in infected cells and in noninfected cells. A concomitant occurrence is the failure of hyphae to form terminal vesicles. These events suggest that the host cells divert energy from vesicle formation and nitrogenase activity to storage as starch.

Secondly, sporangial formation by the endophyte occurs early in differentiation, whereas ordinarily in spore-plus, effective symbioses, spore formation is deferred, occurring in older infected tissues some distance from the nodule meristem (van Dijk and Merkus 1976; Suetin *et al.* 1979). During sporangial formation the host cytoplasm senesces and the host cell may collapse. Therefore, in cells that differentiate sporangia soon after infection, the possibility of differentiating vesicles is lost.

A third symptom of abnormality in this incompatible cross is the development of hyphal proliferation in intercellular spaces of the host and differentiation of sporangia and spores within these spaces.

Finally, a fourth characteristic of this type of ineffectiveness is the limited development of the nodules themselves. Usually only one or two nodule lobes formed and the structure appears to be arrested at the primary nodule stage. Even provision of exogenous nitrogen to supplant the loss of endogenous N_2 fixation did not lead to improved nodule development.

Failure of root nodules to form vesicles was also characteristic of the ineffective nodules of *Elaeagnus umbellata* Thunb. inoculated with *Frankia* sp. EuI1 reported by Baker *et al.* (1980). In this situation also sporangia were formed prematurely and in intercellular spaces and nodule development was arrested early. Here the aberrancies were attributed to the genetic characteristics of the *Frankia* strain since normal nodulation occurred in *E. umbellata* inoculated with nodule suspensions from the same host.

There is clear evidence that the host may influence vesicle expression within the nodule. *Frankia* sp. CpI1 forms club-shaped vesicles in normal nodules of its own host *C. peregrina* (Newcomb *et al.* 1978). Nodules formed on *Alnus crispa* (Ait.) Pursh. when inoculated with CpI1 contain vesicles that are spherical in shape, typical of *Alnus* infected with its own endophyte (Lalonde 1979; Rodriguez-Barrueco and Miguel 1979).

The primary question posed by the incompatible cross described here concerns the nature of the control exerted on hyphal differentiation resulting in failure of vesicle formation. Both the host, *C. peregrina*, and the endophyte obtained from *M. gale* nodules were capable of forming effective symbioses with other partners. Does the host actively inhibit vesicle formation in the incompatible cross, or does it fail to provide substrates, or is there some other cause?

Evidence from studies of *Frankia* grown in pure culture may shed some light on the physiological response resulting in failure of vesicle formation. Tjepkema *et al.* (1981) showed that CpI1 grown in culture failed to form vesicles in the presence of fixed nitrogen compounds. Ammonium ion at 1 m*M* completely suppressed vesicle formation. Amino acids such as glutamate reduced vesicle formation by 50% at a concentration of 100 mg L^{-1}. In an earlier observation (Tjepkema *et al.* 1980) vesicle formation in CpI1 in culture was inhibited by 1% cellobiose. Whether these or other conditions could be involved in *in vivo* suppression of vesicle formation remains to be determined.

Another possibility is that the *M. gale* endophyte within the *C. peregrina* host fails in normal differentiation as a result of lack of substrates. Such instances are known in the legume–*Rhizobium* symbiosis (Vincent 1980). In one case reported by Truchet *et al.* (1980) a mutant strain of *R. meliloti* known to be leucine requiring in culture, produced ineffective nodules on its host. When the host was provided leucine or one of its precursors in aseptic culture, the defective *Rhizobium* was released from the infection thread, bacteroids were formed, and nitrogenase activity ensued. Whether such a substrate deficiency could be part of the explanation for the incompatibility described here remains to be explored.

Whether ineffective nodules exist in natural stands in the field is not known. Theoretically the possibility exists since diverse hosts and endophytes often occupy closely adjacent sites or grow together. It is clearly important for host plants to exclude or control potentially incompatible and therefore ineffective microorganism associates. Whether competing effective *Frankia* strains are more successful in infection and establishment of nodules is a matter needing further study. As possibilities develop to introduce *Frankia* as inoculant for nursery plantations, it is particularly important that we understand the basis for compatible and effective symbioses as contrasted with incompatible ineffective associations.

Acknowledgements

This research was supported in part by research grant DEB-8106952 from the U.S. National Science Foundation and by the Maria Moors Cabot Foundation for Botanical Research of Harvard University. The authors thank Alison Berry, Sue Lancelle, Shirley LaPointe, Ralph Lundquist, and Christa Schwintzer for advice and assistance.

RIGHTSLINK

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.

BAKER, D., W. L. PENGELLY, and J. G. TORREY. 1981. Immunological analysis of relationships among isolated frankiae (Actinomycetales). Int. J. Syst. Bacteriol. **31**: 148–151.

BAKER, D., and J. G. TORREY. 1979. The isolation and cultivation of actinomycetous root nodule endophytes. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Oregon State University, Corvallis, OR. pp. 38–56.

BAUER, W. D. 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. **32**: 407–449.

BECKING, J. H. 1966. Interactions nutritionelles plantes–actinomycetes. Rapport général. Ann. Inst. Pasteur Paris, **111**(Suppl.): 211–246.

BERRY, A. M., and J. G. TORREY. 1979. Isolation and characterization *in vivo* and *in vitro* of an actinomycetous endophyte from *Alnus rubra* Bong. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Oregon State University, Corvallis, OR. pp. 69–83.

——— 1983. Root hair deformation in the infection process of *Alnus rubra* Bong. Can. J. Bot. **61**. This issue.

BOND, G. 1967. Nitrogen fixation in some non-legume root nodules. Phyton, **24**: 57–66.

BURRIS, R. H. 1974. Methodology. *In* The biology of nitrogen fixation. *Edited by* A. Quispel. North-Holland Publishing Co., Amsterdam. pp. 9–33.

CALLAHAM, D., P. DEL TREDICI, and J. G. TORREY. 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. Science (Washington, D.C.), **199**: 899–902.

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

DART, P. J. 1975. Legume root nodule initiation and development. *In* The development and function of roots. *Edited by* J. G. Torrey and D. T. Clarkson. Academic Press, London. pp. 467–506.

——— 1977. Infection and development of leguminous nodules. *In* A treatise on dinitrogen fixation. Sect. III. Biology. *Edited by* R. W. F. Hardy and W. S. Silver. John Wiley and Sons, New York. pp. 367–472.

DAZZO, F. B., and D. H. HUBBELL. 1982. Control of root hair infection. *In* Ecology of nitrogen fixation. Vol. II. *Edited by* W. J. Broughton. Oxford University Press Inc., London. pp. 274–309.

DEL TREDICI, P., and J. G. TORREY. 1976. On the germination of seeds of *Comptonia peregrina*, the sweet fern. Bot. Gaz. (Chicago), **137**: 262–268.

DILLON, J. T., and D. BAKER. 1982. Variations in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. New Phytol. **92**: 215–219.

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

GARDNER, I. C., and G. BOND. 1966. Host plant – endophyte adaptation in *Myrica*. Naturwissenschaften, **53**: 161–162.

HOAGLAND, D. R., and D. I. ARNON. 1950. The water culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. No. 347.

LALONDE, M. 1977. Infection process of the *Alnus* root nodule symbiosis. *In* Recent developments in nitrogen fixation. *Edited by* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco. Academic Press, London. pp. 569–589.

——— 1979. Immunological and ultrastructural demonstration of nodulation of the European *Alnus glutinosa* (L.) Gaertn. host plant by an actinomycetal isolate from the North American *Comptonia peregrina* (L.) Coult. root nodule. Bot. Gaz. (Chicago), **140**: S35–S43.

LALONDE, M., and H. E. CALVERT. 1979. Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Oregon State University, Corvallis, OR. pp. 95–110.

LALONDE, M., H. E. CALVERT, and S. PINE. 1981. Isolation and use of *Frankia* strains in actinorhizae formation. *In* Current perspectives on nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Australian Academy of Science, Canberra. pp. 296–299.

MACINTOSH, A. H., and G. BOND. 1970. Diversity in the nodular endophytes of *Alnus* and *Myrica*. Phyton, **27**: 79–90.

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.

MIAN, S., G. BOND, and C. RODRIGUEZ-BARRUECO. 1976. Effective and ineffective root nodules in *Myrica faya*. Proc. R. Soc. London Ser. B, **194**: 285–293.

NEWCOMB, W. 1980. Control of morphogenesis and differentiation of pea root nodules. *In* Nitrogen fixation. Vol. II. *Edited by* W. E. Newton and W. H. Orme-Johnson. University Park Press, Baltimore, MD. pp. 87–102.

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.

NICHOLSON, T. H. 1959. Mycorrhiza in the Gramineae. I. Vesicular–arbuscular endophytes, with special reference to the external phase. Trans. Br. Mycol. Soc. **42**: 421–438.

QUISPEL, A., and A. J. BURGGRAAF. 1981. *Frankia*, the diazotrophic endophyte from actinorhiza's. *In* Current perspectives in nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Australian Academy of Science, Canberra. pp. 229–236.

RODRIGUEZ-BARRUECO, C., and G. BOND. 1976. A discussion of the results of cross-inoculation trials between *Alnus glutinosa* and *Myrica gale*. *In* Symbiotic nitrogen fixation in plants. *Edited by* P. S. Nutman. Cambridge University Press, Cambridge. pp. 561–565.

RODRIGUEZ-BARRUECO, C., and C. MIGUEL. 1979. Host plant–endophyte specificity in actinomycete-nodulated plants. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Oregon State University, Corvallis, OR. pp. 143–159.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

RODRIGUEZ-BARRUECO, H. B., and K. C. RODRIGUEZ-BARRUECO. 1966. Fixation of nitrogen in root nodules of *Alnus jorulensis*. Phyton, **23**: 103–110.

SCHAEDE, R. 1933. Über die Symbionten in den Knöllchen der Erle und des Sanddornes und die cytologischen Verhältnisse in ihnen. Planta, **19**: 389–416.

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

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. Ultrastruct. Res. **26**: 31–43.

SUETIN, S. O., A. N. PARIPKAJA, 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.

TJEPKEMA, J. D., W. ORMEROD, and J. G. TORREY. 1980. Vesicle formation and acetylene reduction activity in *Frankia* sp. CpI1 cultured in defined media. Nature (London), **287**: 633–635.

——— 1981. Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. Can. J. Microbiol. **27**: 815–823.

TORREY, J. G., D. BAKER, D. CALLAHAM, P. DEL TREDICI, W. NEWCOMB, R. L. PETERSON, and J. D. TJEPKEMA. 1980. On the nature of the endophyte causing root nodulation in *Comptonia*. *In* Nitrogen fixation. Vol. II. *Edited by* W. E. Newton and W. H. Orme-Johnson. University Park Press, Baltimore, MD. pp. 217–227.

TORREY, J. G., J. D. TJEPKEMA, G. L. TURNER, F. J. BERGERSEN, and A. H. GIBSON. 1981. Dinitrogen fixation by cultures of *Frankia* sp. CpI1 demonstrated by $^{15}N_2$ incorporation. Plant Physiol. **68**: 983–984.

TRUCHET, G., M. MICHEL, and J. DÉNARIÉ. 1980. Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically defective mutants of *Rhizobium meliloti*. Differentiation (Berlin), **16**: 163–172.

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

VAN DIJK, C., 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.

VINCENT, J. M. 1980. Factors controlling the legume–*Rhizobium* symbiosis. *In* Nitrogen fixation. Vol. II. *Edited by* W. E. Newton and W. H. Orme-Johnson. University Park Press, Baltimore, MD. pp. 103–129.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.