Plant Physiol. (1984) 76, 556–560
0032-0889/84/76/0556/05/$01.00/0

Consequences of Sporangial Development for Nodule Function in Root Nodules of *Comptonia peregrina* and *Myrica gale*[1]

Received for publication January 12, 1984 and in revised form July 13, 1984

KATHRYN A. VANDENBOSCH*[2] AND JOHN G. TORREY
Department of Botany, University of Massachusetts, Amherst, Massachusetts 01003, (K. A. V.); and Cabot Foundation, Harvard University, Petersham, Massachusetts 01366 (J. G. T.)

ABSTRACT

***Frankia* sp., the actinomycetous endophyte in nitrogen-fixing actinorhizal nodules, may differentiate two forms from its hyphae: vesicles and sporangia. In root nodules of *Comptonia peregrina* (L.) Coult. and *Myrica gale* L., sporangia may be either absent or present. Nitrogenase activity and symbiotic efficiency were contrasted in spore(+) and spore(−) nodules of these two host genera. Seedlings of *C. peregrina* nodulated with the spore(+) inoculum showed only 60% of the nitrogenase activity and 50% of the net size of their spore(−) counterparts after 12 weeks of culture. Measurements of acetylene reduction (*i.e.*, nitrogenase activity) were coordinated with samplings of nodules for structural studies. Significant differences in acetylene reduction rates were discernible between spore(+) and spore(−) nodules commencing 4 weeks after nodulation, concomitant with the maturation of sporangia in the nodule. Spore(+) nodules ultimately reached less than half of the rate of nitrogenase activity of spore(−) nodules. Both types of nodules evolved only small amounts of molecular hydrogen, suggesting that both were equally efficient in recycling electrons lost to the reduction of hydrogen ions by nitrogenase. Respiratory cost of nitrogen fixation, expressed as the quotient of micromole CO_2 to micromole ethylene evolved by excised nodules, was significantly greater in spore(+) than in spore(−) nodules. *M. gale* spore(−) nodules showed variable effectivity, though all had low CO_2 to ethylene evolution ratios. *M. gale* spore(+) nodules resembled *C. peregrina* spore(+), with low effectivity and high respiratory cost for nitrogen fixation.**

Members of the genus *Frankia*, a soil-inhabiting actinomycete, infect roots of some woody dicotyledonous plants and induce nodules. The root nodules are specialized symbiotic organs which fix N_2 (elemental nitrogen). *Frankia* develops within the nodule and in culture as a filamentous bacterium which differentiates two morphologically distinct forms from its hyphae: swollen, terminal vesicles and multicellular sporangia. In almost all available strains of the cultured endophyte, both sporangia and vesicles form under some cultural conditions.

In the nodule, however, sporangia are frequently absent. The occurrence of sporangia within the nodule has been studied most carefully in *Alnus* spp. (5, 6, 17, 18), where the expression of sporangial formation is thought to be determined by the endophytic strain. Van Dijk (5) first coined the terms spore(+) and spore(−) for nodules containing sporulating and nonsporulating endophytes, respectively.

The vesicle has been identified as the putative site for nitrogenase activity in the endophyte, both in the nodule (11) and in culture (20, 21). The effects of sporangial development on nodule function are not known. Studies performed with seedlings of *Alnus* spp. (8, 14) indicated that sporangial formation in the nodule adversely affected productivity of the host plant, as measured by shoot height, total biomass and nitrogen content. Little is known about what factors might be responsible for this lowered productivity.

In this study, an investigation of sporangial formation in nodules of the host family Myricaceae was made. We have tested the hypothesis that the differentiation of sporangia within nodules limits productivity of the symbiosis and decreases the amount of nitrogen fixed. Concurrent structural observations of sporangial development were correlated with the specific activity of nitrogen fixation in *Comptonia peregrina* nodules. In addition, the RE[3] of nitrogenase activity and respiratory cost for fixation were determined for the two nodule types.

MATERIALS AND METHODS

Biological Material and Inoculation Procedures. Locally collected seeds of *Comptonia peregrina* (L.) Coult. and *Myrica gale* L. were germinated in a controlled environment chamber, following cold treatment or scarification and a presoak in gibberellic acid. Approximately 3-week-old seedlings were transferred to water culture and placed in a controlled environment chamber with a 16:8h light:dark cycle, a 24°C daytime temperature and a 19°C nighttime temperature. Seedlings in water culture were inoculated with a preparation of a cultured *Frankia* strain or a crushed nodule suspension. Crushed nodules of *M. gale* or *C. peregrina*, obtained from field populations or from greenhouse stocks and found to contain spores, were used as inocula to produce spore(+) nodules on seedlings of the same species, because of the difficulties of isolating spore(+) strains of *Frankia* (K. A. VandenBosch, unpublished data; [14]). Cultured isolates which were used as inocula were HFPCpI1 (23) an isolate from *C. peregrina*, and MgP10i (X. Nesme, P. Normand, M. Lalonde, unpublished data) and R82 (J. S. Ruan and M. P. Lechevalier, unpublished data), isolates from *M. gale*. Details of germination, inoculation, and water culture procedures are outlined in VandenBosch and Torrey (24).

Another group of *C. peregrina* seedlings was inoculated with either spore(+) *C. peregrina* nodule suspensions or cultured spore(−) strain *Frankia* HFPCpI1 (CpI1), potted in sterilized sand and maintained in the greenhouse. Potted *C. peregrina* seedlings were fertilized twice weekly with one-quarter strength

[1] Supported by National Science Foundation Grant DEB-8106952, United States Department of Agriculture Grant 83-CRCR-1-1285, and The Cabot Foundation for Botanical Research, Harvard University.

[2] Present address: Department of Botany, University of Wisconsin, Madison, WI 53706.

[3] Abbreviation: RE, relative efficiency.

Hoagland solution (pH 6.5) minus nitrogen (9). This experiment was conducted in the months of May through July and no artificial lighting was used to supplement natural daylight.

In both the water culture and sand culture experiments, six or more seedlings were left uninoculated as controls for spontaneous nodulation by contaminating *Frankia* strains. No controls developed nodules during the course of these experiments.

Gas Exchange Assays. Nitrogenase activity of nodules was measured using gas chromatographic assays of acetylene reduction (4). Nodules were excised from the host plant and the nodule roots removed. The nodules were then placed in assay vials which were capped with serum stoppers. A piece of moist filter paper was placed in each vial to prevent dehydration of the nodules. The nodules were incubated in an atmosphere of 10% acetylene (v/v; generated from CaC_2) for 1 h at 24°C. Nodules were observed to maintain a linear rate of acetylene reduction for longer than 90 min after excision and nodule-specific activity was not affected by the removal of nodule roots, in agreement with published observations (19, 22).

Gas samples were withdrawn from the incubation vials with 1-ml syringes and injected into a Carle model 9500 gas chromatograph equipped with a flame ionization detector. Ethylene was resolved on a 1.22-m Porapak R (80–200 mesh) and N (50–80 mesh) column, maintained at 80°C. Vials incubated without nodules served as controls for background ethylene.

Measurements of respiration as CO_2 evolution were withdrawn simultaneously at the end of the assay period. These samples were analyzed on a Carle model 8700 gas chromatograph equipped with a thermal conductivity detector. The CO_2 was resolved on a 0.91-m Porapak column as described above. Column temperature was maintained at 80°C. The carrier gas was helium.

Hydrogen evolution was also measured with the thermal conductivity gas chromatograph, using argon as a carrier gas. Hydrogen was measured on a 1.83-m molecular sieve (MS 5A, 40–60 mesh) column. To determine RE of nitrogenase activity, excised nodules were first incubated in air to observe hydrogen evolution and subsequently incubated in an atmosphere of 10% acetylene in air for measurement of acetylene reduction, following the procedures of Schubert and Evans (15).

Assays for whole plant acetylene reduction rates were carried out in 4.5-l plastic tubs in a controlled environment chamber, maintained at 24°C. The plants, remaining in their pots, were removed from the greenhouse at noon and allowed to come to temperature equilibrium in the chamber for 1 h. The plants were then incubated in an atmosphere of 10% acetylene in air for 1 h. Gas samples were assayed as described above. Following the assay, the plants were returned to the greenhouse.

Microscopic Observations. Methods for structural observation of nodules are detailed in VandenBosch and Torrey (25).

RESULTS

Comptonia peregrina seedlings in water culture, inoculated with either *Frankia* sp. CpI1 or crushed *C. peregrina* spore(+) nodules, all developed nodules within 2 weeks of inoculation. A summary of the structural observations and the results of assays of nitrogenase activity as acetylene reduction are shown in Figure 1. Nitrogenase activity commenced about 1 week after nodulation (3 weeks after inoculation), at about the same time that vesicles appeared in the nodule. Young sporangia first appeared in spore(+) nodules 4 weeks after inoculation, with mature sporangia first discernible at 6 weeks. No sporangia were observed in nodules induced by CpI1. Rates of acetylene reduction by both nodule types increased linearly through 8 weeks after inoculation. Maximum rates attained by both spore(+) and spore(−) nodules were 3.88 and 9.40 μmol ethylene evolved g^{-1} fresh weight nodule h^{-1}, respectively. A repetition of this experiment was conducted, yielding similar results.

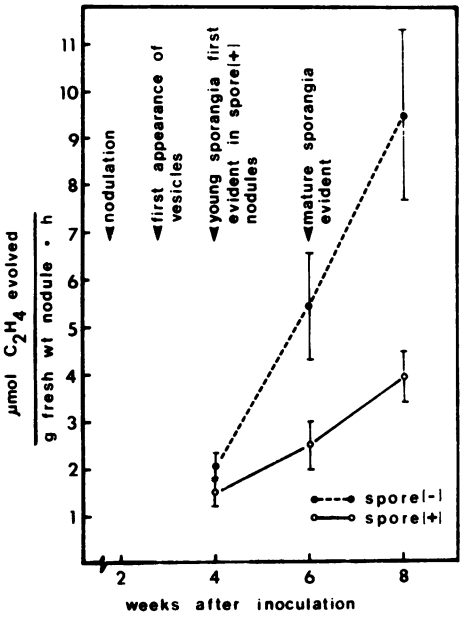

FIG. 1. Nitrogenase activity and endophyte differentiation in developing nodules of *C. peregrina*. Plants were inoculated with either *Frankia* strain CpI1 [spore(−)] or crushed *C. peregrina* spore(+) nodules [spore(+)]. Bars represent SE values for 12 nodules per treatment per assay.

Table I. *Analysis of Variance of Nitrogenase Activity in Developing C. peregrina Nodules*

Source	Degrees of Freedom	Sum of Squares	Mean Square	*F*
Treatments	5			
Nodule type	1	164.5298	164.5298	15.16***
Date of assay	2	288.6724	144.3362	13.30**
Interaction	2	76.2750	38.1375	3.51
Error	66	716.1145	10.8502	

Treatment Comparisons	Degrees of Freedom	Mean Square	*F*
Between nodule types			
At 4 weeks	1	1.7985	0.17NS[b]
At 6 weeks	1	51.6854	4.76*[c]
At 8 weeks	1	187.3209	17.26**

[a] **, Indicates a significant difference at P = 0.01.
[b] No significant difference.
[c] *, Indicates a significant difference at P = 0.05.

Rates of nitrogenase activity in the two nodule types were compared using analysis of variance. The results of the analysis are presented in Table I. Significant differences in rates were found between the two nodule types and among the assay dates. A significant interaction was also found to occur between nodule type and assay date. Single degree of freedom comparisons were made between the nodule types for each of the three assay dates. At 4 weeks after inoculation, before mature sporangia had developed in the young spore(+) nodules, no significant difference in acetylene reduction rates was discernible. By 6 weeks, when mature sporangia had differentiated in spore(+) nodules, the difference in rates between the two nodule types was significant. This difference increased, both in magnitude and in significance, at 8 weeks after inoculation.

Comptonia peregrina seedlings inoculated with crushed *C.*

Table II. *Whole Plant Comparison of the Effect of Inoculum on Nodule and Plant Development*

Each of the treatment groups and the control group were comprised of five plants. All parameters were measured 12 weeks after inoculation, except for acetylene reduction, which was assayed three times between 8 and 12 weeks, and the data were pooled.

	Inoculum		
	C. peregrina nodules spore (+)	CpI1 spore (−)	Control
No. nodules·plant^{-1}	17.0 NS[a]	12.4	0.0
g fresh wt nodules·plant^{-1}	0.12 NS[a]	0.10	0.0
μmol ethylene·plant^{-1}·h^{-1}	0.85 **[a]	1.45	0.0
Total plant fresh wt, g	1.63c[b]	3.32d	1.01c
Shoot height, cm	10.5c[b]	15.9d	8.2c

[a] Data for inoculated plants were compared using analysis of variance. Significance as in Table I.

[b] Treatment means for inoculated and control plants were compared using Duncan's new multiple range test. Any two means not followed by the same letter are significantly different for P = 0.05.

Table III. *Nitrogenase Activity and Respiration of Excised C. peregrina Nodules*

	Weeks after Inoculation	Nodule Type	
		Spore (+)	Spore (−)
μmol ethylene·g^{-1} nodule·h^{-1}	6	7.38	11.05 **[a]
	12	4.25	9.79 **
μmol CO_2·g^{-1} nodule·h^{-1}	6	64.54	78.22 NS
	12	43.27	57.36 NS
μmol CO_2/μmol ethylene	6	9.6	7.9 NS
	12	11.7	6.2 **

[a] Data for the two nodule types were compared using analysis of variance for each assay date. Levels of significance as in Table I.

peregrina nodules or *Frankia* sp. CpI1 and grown in sand in the greenhouse were used for a study of the effect of nodule type on total plant nitrogenase activity and growth. The uninoculated control group and each of the two treatment groups were comprised of five seedlings. The controls received no nitrogen and appeared quite chlorotic after 12 weeks. The data presented in Table II show that the two inocula produced similar numbers and weights of nodules on the host seedlings. Despite similarities in nodulation, the two symbiotic combinations differed significantly in productivity (growth) and effectivity (nitrogenase activity). Seedlings nodulated with the spore(+) inoculum developed only half of the total plant fresh weight and two-thirds of the shoot height of their spore(−) counterparts. Shoot height and total plant fresh weight of spore(+)-nodulated seedling could not be distinguished statistically from that of the controls. Acetylene reduction of the potted, inoculated seedlings was assayed on three dates and these data were pooled to yield the treatment means expressed in Table II. Spore(+)-nodulated seedlings showed only about 60% of the acetylene reduction capacity of the spore(−)-nodulated seedlings.

The ratio of nodule respiration to acetylene reduction, a measure of energy cost of nitrogenase activity (22), was calculated for excised *C. peregrina* nodules from sand culture. This parameter also includes cost of nodule maintenance and growth. Measurements made on two dates, 6 and 12 weeks after inoculation (Table III). As in the water culture experiment, nitrogenase activity of the nodules which lacked spores exceeded that of spore-producing nodules. Respiration of the two nodule types was similar. The ratio of these two nodular functions, CO_2 evolution/ethylene evolution, was therefore greater for spore(+) nodules than for spore(−) nodules, though this difference was significant only at the second assay date.

Table IV. *RE of Nitrogenase Activity of C. peregrina Nodules*

	Nodule Type	
	Spore (+)	Spore (−)
μmol H_2·g^{-1} nodule·h^{-1}	0.09	0.62
μmol ethylene·g^{-1} nodule·h^{-1}	3.17	16.51
$1-\frac{H_2 \text{ evolved}}{\text{ethylene evolved}}$	0.98	0.96 NS[a]

[a] Relative efficiencies of the two nodule types were compared using analysis of variance. Significance as in Table I.

Measurements of H_2 evolution and acetylene reduction were used to calculate the RE of nitrogenase, following the equation of Schubert and Evans (15):

$$RE = 1 - \frac{\text{rate of } H_2 \text{ evolution in air}}{\text{rate of acetylene reduction}}$$

RE reflects both the efficiency of electron allocation to nitrogen via nitrogenase and the presence/activity of an uptake hydrogenase. REs of the two nodule types, assayed 11 weeks after inoculation, are compared in Table IV. Both types of nodules evolved only very low amounts of molecular hydrogen when incubated in air. Both spore(+) and spore(−) nodules, therefore, had high REs of electron transfer despite the difference in their capacities to reduce acetylene.

A survey of measurements of acetylene reduction and respiration were also carried out with excised *Myrica gale* nodules induced by various inocula. The results from these experiments are summarized in Table V. The only inoculum to produce spore(+) nodules on *M. gale* was a suspension of crushed spore(+) *M. gale* nodules. Spore(+) *M. gale* nodules exhibited the lowest rates of acetylene reduction. Those nodules induced by isolates R82 and CpI1 also had low rates. MgP10i produced nodules with a higher specific activity, similar to the spore(−) nodules produced by inoculation of *C. peregrina* with CpI1. Despite dissimilarities in rates of acetylene reduction, the energy cost for nitrogen fixation was similar for all the spore(−) nodules. This value (4.7–7.6) was similar to that observed for spore(−) *C. peregrina* nodules (6.2). Nodule energy costs were consistently greater for spore(+) *M. gale* nodules, as was observed for *C. peregrina*.

DISCUSSION

After *Frankia* sp. successfully nodulates an actinorhizal host, many developmental steps must yet occur in order to attain full symbiotic effectivity. Number of nodules alone is a poor predictor of symbiotic success of particular host/endophyte combinations. Cross-inoculation surveys performed with *Frankia* isolates (7, 14) have indicated that there is a wide range of symbiotic effectivity resulting from various host/endophyte combinations within the *Alnus/Myrica* cross-inoculation group.

Few studies have specifically compared the performance of spore-producing nodules [spore(+)] and those in which spore production is rare or absent ([spore(−)]. Normand and Lalonde (14), in a survey of isolates from *Alnus crispa* and *A. rugosa*, noted that spore(+) nodules supported 30% less host plant growth than did spore(−) nodules. Hall *et al.* (8) and Maynard (10)

Table V. *Nitrogenase Activity and Respiration in Excised M. gale Nodules*

Data expressed as mean ± SE.

Inoculum	Nodule Type	Week[a]	n[b]	μmol ethylene·g^{-1} nodule·h^{-1}	μmol CO_2·g^{-1} nodule·h^{-1}	μmol Co_2/μmol ethylene
M. gale	Spore(+)	8	12	3.82 ± 1.47	40.55 ± 10.30	10.6
Spore(+) nodules		12	5	4.13 ± 0.39	41.07 ± 2.63	9.9
MgPlOi	Spore(−)	8	6	12.09 ± 3.01	56.40 ± 8.01	4.7
R82	Spore(−)	8	6	5.44 ± 1.60	41.18 ± 5.77	7.6
CpI1	Spore(−)	12	8	6.46 ± 0.38	47.05 ± 4.20	7.3

[a] Number of weeks after inoculation.
[b] Number of replicates.

similarly found spore(+) nodules to confer less benefit upon the host plant. Both studies observed that, although the spore(+) inoculum produced abundant nodulation, height growth and whole plant dry weight of *A. glutinosa* seedlings were substantially less than *A. glutinosa* seedlings nodulated with spore(−) inocula.

Little insight has been gained into the causes for the differences in productivity between seedlings with the two types of nodules. Possibilities include that sporangial differentiation within the nodule reduces the number or longevity of endophytic vesicles and hence that the rate of nitrogen fixation is lower in spore(+) nodules. Second, the RE of spore(+) nodules may be lower, Third, energy cost for nodule maintenance and nitrogenase activity may be higher per unit nitrogen fixed in spore(+) nodules.

In the current study, *C. peregrina* seedlings nodulated with a spore(+) inoculum showed only 60% of the nitrogenase activity and 50% of the net size of similar seedlings whose nodules lacked sporangial formation, despite comparable nodulation. Thus *Comptonia* follows a pattern similar to published contrasts between *Alnus* spp. spore(+) or (−) nodulated seedlings. Young spore(+) and spore(−) nodules in *Comptonia* initially exhibited no difference in acetylene reduction rates, but differences developed as the nodules developed. This divergence in rates of nitrogenase activity was correlated with the ontogenetic development of sporangia within the nodule.

In light microscopic studies of *C. peregrina* and *M. Gale* nodules, early stages of sporangial morphogenesis were observed in host cells containing normal cell constituents and mature vesicle clusters (25). As sporangium development proceeded, however, both endophytic vesicles and host cell nuclei and cytoplasm rapidly senesced and were degraded. Vesicles are more short lived in spore(+) than in spore(−) nodules, which may account for the differences in acetylene reduction rates observed in the current study. Becking *et al.* (1) and Suetin *et al.* (17, 18) have also described host cell and enophytic vesicle death concomitant with the differentiation of sporangia in *A. glutinosa* nodules.

In nitrogen-fixing organisms, ATP and electrons available for the reduction of molecular nitrogen may also be utilized by nitrogenase for reducing protons to molecular hydrogen. Schubert and Evans (15) devised an expression for the RE of nitrogenase activity, in which the proportion of electrons used to reduce protons to molecular hydrogen is subtracted from the total electrons used by nitrogenase to reduce all available substrates. In the absence of an uptake hydrogenase, RE measures the efficiency of electron transport to nitrogen by nitrogenase. In symbioses where H_2 is recovered by an efficient hydrogenase, RE values may approach 1. Among legume/*Rhizobium* symbioses, Schubert and Evans (15) found a wide range of RE, from 0.99 to 0.20, in the legume hosts surveyed. Actinorhizal plant/*Frankia* symbioses, conversely, are almost universally high in RE (12, 15), probably due to recovery of H_2 through an efficient uptake hydrogenase (2, 3).

In the contrast reported here between spore(+) and (−) *C. peregrina* nodules, neither nodule type was observed to evolve large amounts of molecular hydrogen. Spore(+) and spore(−) nodules had RE values of 0.98 and 0.96, respectively. RE of nitrogenase is therefore not accountable for the difference in plant growth observed between seedlings nodulated with the two inocula.

Another measure of nodule efficiency is the ratio of respiration to nitrogenase activity in the nodule, which gives an estimate of energy required for nitrogen fixation. The estimate of carbon cost is, thus, one for nodule maintenance and growth as well as nitrogenase activity and transport of fixed nitrogen. Tjepkema and Winship (22) surveyed a number of legume and actinorhizal nodules and noted that the ratio of CO_2 evolved to C_2H_2 reduced was remarkably similar between the two groups of symbiotic nitrogen fixing organisms, despite wide ranges of respiration and nitrogen fixation rates. The reported values varied from 2.8 to 8.7, with most values falling in the range of 3.5 to 4.5. When the respiration to nitrogen fixation ratios were examined for spore(+) and (−) nodules of *C. peregrina* and *M. gale*, a significant difference between the two nodule types was discovered. For nodules lacking spore production, the ratio was in the range of 5.0 to 7.5; that for spore-producing nodules was in the range of 10.0 to 11.7. The latter value is higher than published estimates of nodule respiratory cost of nitrogen fixation in actinorhizae, except in a seasonal study of *M. gale* nodules where the ratio was high at the beginning and end of the season when nitrogenase activity was quite low (16). Therefore, it appears that spore(+) nodules expend more host photosynthate per unit of atmospheric nitrogen fixed than do spore(−) nodules.

The lower effectivity and higher carbon cost/nitrogen gain of the spore(+) nodules studied suggest that sporangial differentiation is an expensive process which confers no obvious advantage on the host. The differentiation of sporangia within the infected host cell may precipitate host cell death and degeneration of endophytic vesicles. These observations, based on a comparison of a small number of strains of *Frankia*, suggest that sporangia represent a more parasitic stage in the life cycle of the actinomycetous endophyte.

The current study compared a small number of strains of *Frankia* sp. Further studies are required to substantiate the generality of the observed phenomena. To date, most isolates of *Frankia* sp. have been spore(−) strains, although spore(+) strains are common in nature in some host species. From our preliminary study, it appears that the sporulating character of *Frankia* may greatly affect symbiotic productivity. Renewed effort should be given to isolation and characterization of spore(+) strains of *Frankia*. Studies of actinorhizal nodule physiology should discriminate between spore(+) and spore(−) strains.

LITERATURE CITED

1. BECKING JH, WE DEBOER, AL HOUWINK 1964 Electron microscopy of the endophyte of *Alnus glutinosa*. Antonie von Leewenhoek. J Microbiol Serol

30: 343–376
2. BENSON DR, DJ ARP, RH BURRIS 1979 Cell-free nitrogenase and hydrogenase from actinorhizal root nodules. Science 205: 688–689
3. BENSON DR, DJ ARP, RH BURRIS 1980 Hydrogenase in actinorhizal root nodules and root nodule homogenates. J Bacteriol 142: 138–144
4. BURRIS RH 1974 Methodology. *In* A Quispel, ed, The Biology of Nitrogen Fixation. North Holland Publ. Co., Amsterdam, pp 9–33
5. VAN DIJK C 1978 Spore formation and endophyte diversity in root nodules of *Alnus glutinosa* (L.) Vill. New Phytol 81: 601–615
6. VAN DIJK C, 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
7. DILLON JT, D BAKER 1982 Variations in nitrogen fixing efficiency among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. New Phytol 92: 215–219
8. HALL RB, HS MCNABB JR, CA MAYNARD, TL GREEN 1978 Toward development of optimal *Alnus glutinosa* symbiosis. Bot Gaz 140: S120–S126
9. HOAGLAND DR, DI ARNON 1950 The water culture method for growing plants without soil. Calif Agric Exp Stn Circ 347 (rev ed)
10. MAYNARD CA 1980 Host-symbiont interactions among *Frankia* strains and *Alnus* open-pollinated families. PhD Thesis. Iowa State University, Ames
11. MIAN S, 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
12. MOORE AW 1964 Note on non-leguminous nitrogen fixing plants in Alberta. Can J Bot 43: 952–955
13. Deleted in proof.
14. NORMAND P, M LALONDE 1982 Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. Can J Microbiol 28: 1133–1142
15. SCHUBERT KR, HJ EVANS 1976 Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc Natl Acad Sci USA 73: 1207–1211
16. SCHWINTZER CR, JD TJEPKEMA 1983 Seasonal pattern of energy use, respiration, and nitrogenase activity in root nodules of *Myrica gale*. Can J Bot 61: 2943–2948
17. SUETIN SO, AN PARIJSKAJA, LV KALAKOUTSKII 1979 Structural aspects of endosymbiont development and interaction with host cytoplasm in *Alnus glutinosa* (L.) Gaertn. root nodules. Microbios Lett 12: 83–92
18. SUETIN SO, AN PARIJSKAJA, LV KALAKOUTSKII 1981 Electron-microscopic study of the growth cycle of an actinomycete endosymbiont in nitrogen-fixing nodules on *Alnus glutinosa* roots. Microbiology 49: 524–528
19. TJEPKEMA JD 1978 The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation by root nodules of *Myrica gale*. Can J Bot 56: 1365–1371
20. TJEPKEMA JD, W ORMEROD, JG TORREY 1980 Vesicle formation and acetylene reduction activity in *Frankia* sp. CpI1 cultured in defined media. Nature 287: 633–635
21. TJEPKEMA JD, W ORMEROD, JG TORREY 1981 Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. Can J Microbiol 27: 815–823
22. TJEPKEMA JD, LJ WINSHIP 1980 Energy requirement for nitrogen fixation in actinorhizal root nodules. Science 209: 279–281
23. TORREY JG, D BAKER, D CALLAHAM, P DEL TREDICI, W NEWCOMB, RL PETERSON, JD TJEPKEMA 1980 On the nature of the endophyte causing root nodulation in *Comptonia*. *In* WE Newton, WH Orme-Johnson, eds, Nitrogen Fixation, Vol II. University Park Press, Baltimore, pp 217–227
24. VANDENBOSCH KA, JG TORREY 1983 Host-endophytic interactions in effective and ineffective nodules induced by the endophyte of *Myrica gale*. Can J Bot 61: 2898–2909
25. VANDENBOSCH KA, JG TORREY 1984 The development of endophytic sporangia in field and laboratory-grown nodules of *Comptonia peregrina* and *Myrica gale*. Am J Bot. In press