Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity, and shoot development in *Myrica gale*

CHRISTA R. SCHWINTZER, ALISON M. BERRY, AND LYNN D. DISNEY
Harvard University, Harvard Forest, Petersham, MA, U.S.A. 01366
Received May 13, 1981

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.

Myrica gale L. populations growing in central Massachusetts were observed throughout the ice-free season. Nitrogenase activity appeared in mid-May shortly after budbreak, was at its maximum between late June and mid-August, and disappeared in late October after all leaves had fallen. Growth of overwintering nodules began in early May and was largely complete by mid-July. Most nodules (88%) lived for 3 years or less and 61% of the nodule biomass present in autumn was produced during the current season.

Colonizing hyphae of the *Frankia* sp. endophyte were seen throughout the year in partially expanded cortical cells near the nodule lobe apex. Vesicles first appeared in mature cortical cells coincident with the onset of nitrogenase activity in mid-May, occupied the bulk of the infected tissue during the summer, and disappeared as nitrogenase activity ceased in late October. Evidence is presented that the vesicles are the site of nitrogenase activity and are newly produced each season in freshly formed nodule lobe tissue. Sporangia frequently formed in mature infected cells in nodules at one site but were rare at another. The processes described here in *M. gale* are probably typical of winter-deciduous actinorhizal plants.

SCHWINTZER, C. R., A. M. BERRY et 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.

Des populations de *Myrica gale* L. du Massachusetts central ont été observées pendant toute la saison sans gel. L'activité nitrogénasique reprend à la mi-mai peu après le débourrement des bourgeons, elle est maximale entre la fin de juin et la mi-août et elle disparaît à la fin d'octobre, après la chute des feuilles. La croissance des nodules qui hivernent commence au début de mai et est à peu près complétée à la mi-juillet. La plupart des nodules (88%) vivent 3 ans ou moins et 61% de la biomasse nodulaire présente en automne est produite durant la saison courante.

Les hyphes du *Frankia* endophyte sont visibles toute l'année dans des cellules corticales partiellement dilatées près de l'apex des lobes nodulaires. Les vésicules apparaissent dans les cellules corticales matures au moment où l'activité nitrogénasique reprend à la mi-mai; durant l'été, elles occupent la plus grande partie du tissu infecté et elles disparaissent au moment où l'activité nitrogénasique cesse à la fin d'octobre. Des données montrent que les vésicules sont le site de l'activité nitrogénasique et qu'elles sont fraîchement produites chaque saison dans les tissus récemment formés des lobes nodulaires. Des sporanges se formaient fréquemment dans les cellules infectées matures des nodules dans un site, mais ils étaient rares dans un autre site. Les processus décrits ici chez *M. gale* sont probablement typiques des plantes actinorhiziennes à feuilles caduques en hiver.

[Traduit par le journal]

Introduction

Myrica gale L. is an actinorhizal dinitrogen-fixing shrub commonly found in open, minerotrophic peatlands and along the shores of lakes and streams in the northern United States, Canada, and Europe. It is an ecological dominant in several plant associations within its extensive range (Abbayes and Hamant 1946; Dansereau and Segadas-Vianna 1952; McVean and Ratcliffe 1962; Jeglum *et al.* 1974; Schwintzer 1978; and others) and is capable of fixing substantial amounts of nitrogen (24–34 kg $N \cdot ha^{-1} \cdot year^{-1}$) in dense stands (Schwintzer 1979).

The root nodules of *M. gale* develop from a local infection by the actinomycetous endophyte giving rise to one or more modified lateral roots, termed nodule lobes. The tissues of the nodule lobes consist of a central stele surrounded by a parenchymatous cortex and a corky periderm or exoderm. Repeated branchings of these primary nodule lobes form roughly spherical, coralloid perennial structures which can reach 3.5 cm in diameter. Single, short, negatively geotropic roots, termed nodule roots, arise from the tips of most nodule lobes. The endophyte is confined to the cortex of the nodule lobes where longitudinal files of infected cells intermingle with uninfected cells. The endophyte belongs to the genus *Frankia* of the Actinomycetales and has three morphological forms: hyphae, vesicles, and sporangia. All three forms may be found in a single nodule but often are not.

Earlier studies of the root nodules of *M. gale* and the endophyte have been reviewed by Fletcher and Gardner (1974). More recent reports describe the ultrastructure of the host cells and the endophyte (Gardner 1976; Henry 1977), the infection process leading to the development of nodules (Callaham *et al.* 1979), early nodule development (Torrey and Callaham 1979), and

0008-4026/82/060746-12$01.00/0

development of the nodule roots (Torrey and Callaham 1978). Recent reports also describe the function of the nodule roots (Tjepkema 1978; Sprent and Scott 1979) and the seasonal pattern of nitrogenase activity (Sprent *et al.* 1978; Schwintzer 1979).

Little is known about the seasonal pattern of nodule growth and endophyte morphology in *M. gale* or any other actinorhizal plant. In the present study we tested the hypothesis that there are seasonal patterns of nodule growth and that the expression of the morphological forms of the endophyte is correlated with the seasonal patterns of nitrogenase activity and phenology of the host shoots. To do this, we observed nodule growth and endophyte morphology at regular intervals throughout the ice-free season in 1979 while also monitoring nitrogenase activity, shoot phenology, and soil temperature. Nodule age and size distribution were also examined.

Study area

The study site is located near a small lake known locally as Harvard Pond (42°30′ N; 72°12′ W, elevation approximately 252 m) in the Harvard University Forest at Petersham, MA. Most observations were made in an area designated "Open Mat" in an earlier study (Schwintzer 1979) and located in an extensive open peatland (about 400 × 400 m) on a partially floating mat at the northern end of the lake. Time-lapse photographic observations of nodule growth were made on the west-facing shore of the lake approximately 100 m east of the Open Mat site. In addition some observations of endophyte morphology were made on nodules collected in an area previously designated "Pond Side." This site is located on a south-facing shore of the lake and is approximately 2.1 km from the Open Mate site.

The vegetation of the Open Mat site is dominated by *M. gale* (mean cover 53%) and *Chamaedaphne calyculata* (12%). The ground layer covering the hummocks and hollows on the mat surface consists primarily of *Sphagnum* spp. (mean cover 35%) and leaf and woody litter. The peat is weakly minerotrophic and has acidic (pH 3.9) shallow ground waters low in metal ions (Schwintzer 1979). Further details of the Open Mat and Pond Side sites including information on other studies in the area are given by Schwintzer (1979).

Materials and methods

Nodule growth and morphology

Nodule growth in the field was examined with time-lapse photography of 6 nodules on the lakeshore and periodic measurement and description of an additional 20 nodules at the Open Mat site. Shortly after the soil thawed in mid-April, six nodules on the lakeshore were exposed *in situ* by removing the organic soil from above and beside them without disturbing the roots to which they were attached. The resulting shallow hole containing the nodule was then covered with a piece of fiberglass screening covered with a layer of living peat moss which remained moist throughout the growing season. The covers remained in place except when the nodules were being photographed. Photographs were taken at approximately 2-week intervals from late April through early August and then at longer intervals. The 20 nodules at the Open Mat site were prepared for observation during the preceding November in the same way as those described above and were examined at 3- to 4-week intervals beginning in mid-June. General vigor of each nodule, color of the nodule roots, and diameter of the nodule cluster were recorded.

Several population characteristics were examined in nodules collected at the Open Mat site in late November in seven randomly distributed 1/8 m^2 circular plots. The whole substrate layer containing nodules was removed from each plot, returned to the laboratory, and carefully hand sorted to obtain all nodules. The nodules were counted and assigned to a size class. A random subsample of 10 nodules was then taken from each plot. Their diameter was measured and their age estimated based on the number of annual rings in the xylem of the subtending root (Akkermans 1971).

Endophyte morphology and nitrogenase activity

Endophyte morphology and nodule lobe anatomy were examined in the light microscope using plastic sections of lobes from nodules which had been assayed for nitrogenase activity at the time of their collection in the field and then fixed and embedded. Nodules were collected at the Open Mat site at 1-week intervals throughout May, 2-week intervals in June and July, and 3-week intervals from August through November. Additional nodules were collected at the Pond Side site in late March and in October. Nodules attached to 2–5 cm long root segments were carefully dug out of shallow holes (15 cm deep) in the peat in late morning and placed individually in 245-mL glass canning jars with metal lids fitted with a rubber septum. Enough distance was allowed between collection points to ensure that the nodules came from different *Myrica* clones. The nodules were returned to the laboratory (15 min from the field) and assayed for nitrogenase activity by the acetylene reduction method (Hardy *et al.* 1973). The nodules were preincubated at 15°C for 30 min and then incubated with 10% acetylene at 15°C for 60 min. Further details of the assay are given by Schwintzer (1979).

After completion of the acetylene reduction assay, individual lobes were removed from representative nodules and fixed in 3% glutaraldehyde in 0.025 *M* sodium phosphate buffer, pH 6.8, overnight at 4°C. The material was dehydrated and embedded in Spurr's (1969) resin and sectioned at 1–2 μm with glass knives on a Porter Blum ultramicrotome. The sections were stained with 0.5% toluidine blue 0 in 0.1 *M* sodium phosphate buffer, pH 6.8, with 1% w/v sodium borate.

Seasonal pattern of soil temperature, shoot phenology, and nitrogenase activity

Soil temperature was recorded at the Open Mat site weekly at 9 a.m. throughout the growing season. Measurements were made with a mercury-in-glass thermometer at 15 cm below the surface in the center of a small depression.

Shoot phenology was observed at the Open Mat site in 24 representative shoots marked in late April and examined weekly during periods of rapid change and biweekly at other times. The following events were monitored: flowering and budbreak, length of the new shoot developing from the uppermost vegetative bud, and the number of green leaves present on this new shoot at various times after its full expansion.

The seasonal pattern of nitrogenase activity was examined in large samples of nodules collected at approximately 3-week intervals at the Open Mat site from late May through late October. On each sample date 20 jars of nodules, each containing the equivalent of four medium-sized nodules (dry weight of nodule tissue 0.4–0.7 g), were collected and assayed by the acetylene reduction method at the temperature prevailing in the soil at the time of nodule collection as described by Schwintzer (1979) except that all nodules were obtained in a single, 128-m^2 area immediately south of one of the two areas used previously.

Effect of temperature on initiation of shoot and nodule growth

The effect of increased temperature on shoot phenology, nodule growth, and nitrogenase activity under natural light regimes was examined in twelve 2- to 4-year-old potted plants growing in a greenhouse heated to maintain a minimum temperature of 6°C. The maximum temperature was not controlled and reached a high of 24°C but was usually much less. Only natural light reached the greenhouse. The plants were placed in the greenhouse during the previous autumn and shoot phenology was monitored at weekly intervals from late February to late March. At the end of this period the nodules were removed from six representative plants and assayed for nitrogenase activity at 20°C.

Results

Nodule growth and morphology

Externally visible growth of nodule lobes as observed with time-lapse photography at the lakeshore usually consisted of the following two steps: (1) a nodule root grew out from the tip of a preexisting nodule lobe slightly preceded and accompanied by expansion of the tip of the old lobe (Figs. 1A–1D) and (2) one or two new lobes developed at the tip of the old lobe near the new nodule root. Finally, a papilla, a caplike structure (Bowes *et al.* 1977), formed at the tips of some new lobes at the point where a nodule root would emerge later (Figs. 1E–1G). The nodule roots were white as they grew out and turned tan relatively rapidly after reaching their final length. In early summer the nodule roots made their complete growth and turned tan in less than 3 weeks. Completion of steps 1 and 2 was termed one cycle of nodule growth. Thus each of the two nodule lobes shown in Figs. 1B–1G, completed one cycle of growth. A total of 15 nodule lobes on four nodules could be followed throughout the season. Of these, seven completed one cycle of growth, seven completed two cycles, and one enlarged and formed a papilla but showed no further growth.

Externally visible growth began about mid-May and all six nodules were actively growing by early June. Within a given nodule, the time of beginning of growth varied from lobe to lobe. For example, lobe 1 shown in Figs. 1B–1G produced a new nodule root in early May and lobe 2 in mid-June. Most externally visible nodule growth was complete by late July and only limited growth was observed after this. Eleven of 15 nodule lobes showed no growth after late July, 3 showed only lobe initiation and (or) lobe expansion, and only 1 showed extension of a new nodule root which occurred in September.

Anatomical observations in resin-embedded sections and freehand sections of fresh nodules were consistent with the external observations just described. A meristematic region was found at the nodule lobe apex underneath the papilla and just above the distal end of the stele. As nodule growth resumed in May, this gave rise to additional cells at the apex of the old nodule lobe and then to a nodule root. Later one or two new nodule lobe meristems arose at the periphery of the stele near the base of the nodule root and gave rise to additional nodule lobes.

Field observations on 20 nodules in screen and moss-covered cavities at the Open Mat site generally support observations made by time-lapse photography at the lakeshore. Step 1 growth, as indicated by the presence of white nodule roots, was complete by early July in 67% of the nodules surviving until the end of the season, while limited step 1 growth occurred after this in the remaining 33%, some as late as mid-September. It was not possible to detect step 2 growth with these field observations but presumably this followed relatively rapidly after step 1 growth as in the nodules observed by time-lapse photography. The nodules suffered substantial mortality with 40% dying during the growing season.

Limited growth and a relatively short life span of field nodules are also indicated by the size and age distributions of nodules at the Open Mat site in November 1979 (Fig. 2). Most nodules (87%) were less than 10 mm in diameter and none exceeded 20 mm. However, in extensive field work over three seasons, occasional nodules were found that exceeded this size, the maximum being 35 mm. In addition most nodules (88%) were 1–3 years old and none more than 5 years.

The nodule diameter increases as new lobes are added at the periphery of the nodule. If each nodule lobe contributes 1 mm to radial growth (Fig. 1) and two cycles of growth occur per year then each year approximately 4 mm would be added to the nodule diameter (2 mm added to the radius). Actual yearly increments in nodule diameter were smaller than this except during the 1st year when they were slightly larger (Table 1). Factors contributing to slower diameter growth are some

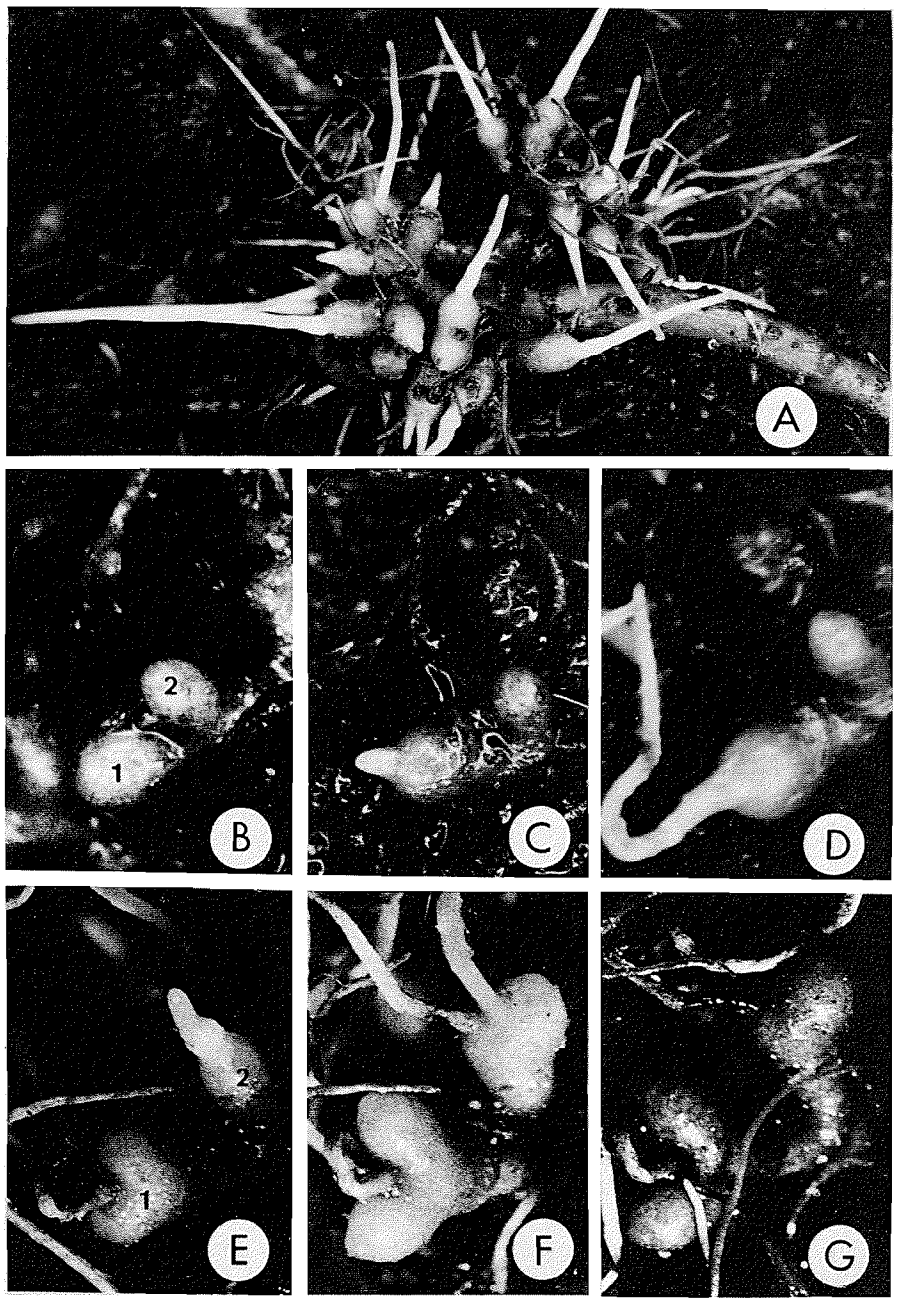


FIG. 1. Seasonal pattern of nodule lobe growth in the field 1979. (A) Nodule No. 6 on June 6 showing fresh nodule roots growing from the tips of nodule lobes produced last year (step 1 growth), the subtending root, and withered nodule roots produced the previous year. ×4.2. (B–G) Two lobes from nodule No. 2 at various times during the year. All ×12.2. (B) April 25, both lobes are dormant. (C) May 9, a nodule root is beginning to grow from lobe 1. (D) June 6, lobe 1 is expanding at its apex and the nodule root continues to grow. (E) June 27, new lobes have formed beside the nodule root growing from lobe 1, a new nodule root is growing from lobe 2. (F) July 25, the new lobes formed on lobe 1 have grown further, a new lobe and second nodule root have grown from lobe 2. (G) October 19, there has been no further growth in either lobes 1 or 2.

TABLE 1. Age, diameter, and volume of *Myrica gale* nodules at the Open Mat site in November 1979

Age, years	N^a	Diameter, mm $\bar{x} \pm SE$	Volume,[b] mm^3	New tissue,[c] mm^3	New tissue,[d] %
1	21	4.4±0.3	45	45	100
2	20	5.8±0.4	102	57	56
3	20	9.5±0.7	449	347	77
4	6	11.3±1.3	756	307	41
5	2	12.3±1.2	974	218	22

[a]Number of nodules examined.
[b]Volume per nodule calculated from mean diameter assuming spherical nodules with a constant ratio of lobes to airspaces.
[c]Volume of new tissue per nodule calculated as the difference between the volume of the current year and the previous year.
[d]New tissue as percent of total nodule volume.

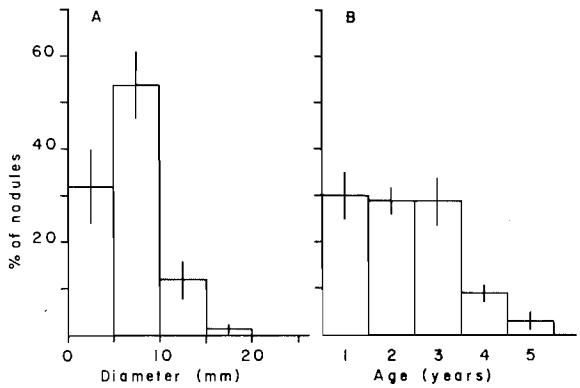


FIG. 2. Size and age distribution of *Myrica gale* nodules at the Open Mat site in November 1979. The nodules were sampled in 1/8-m^2 plots (see text); $\bar{x} \pm$ SE; $N = 7$.

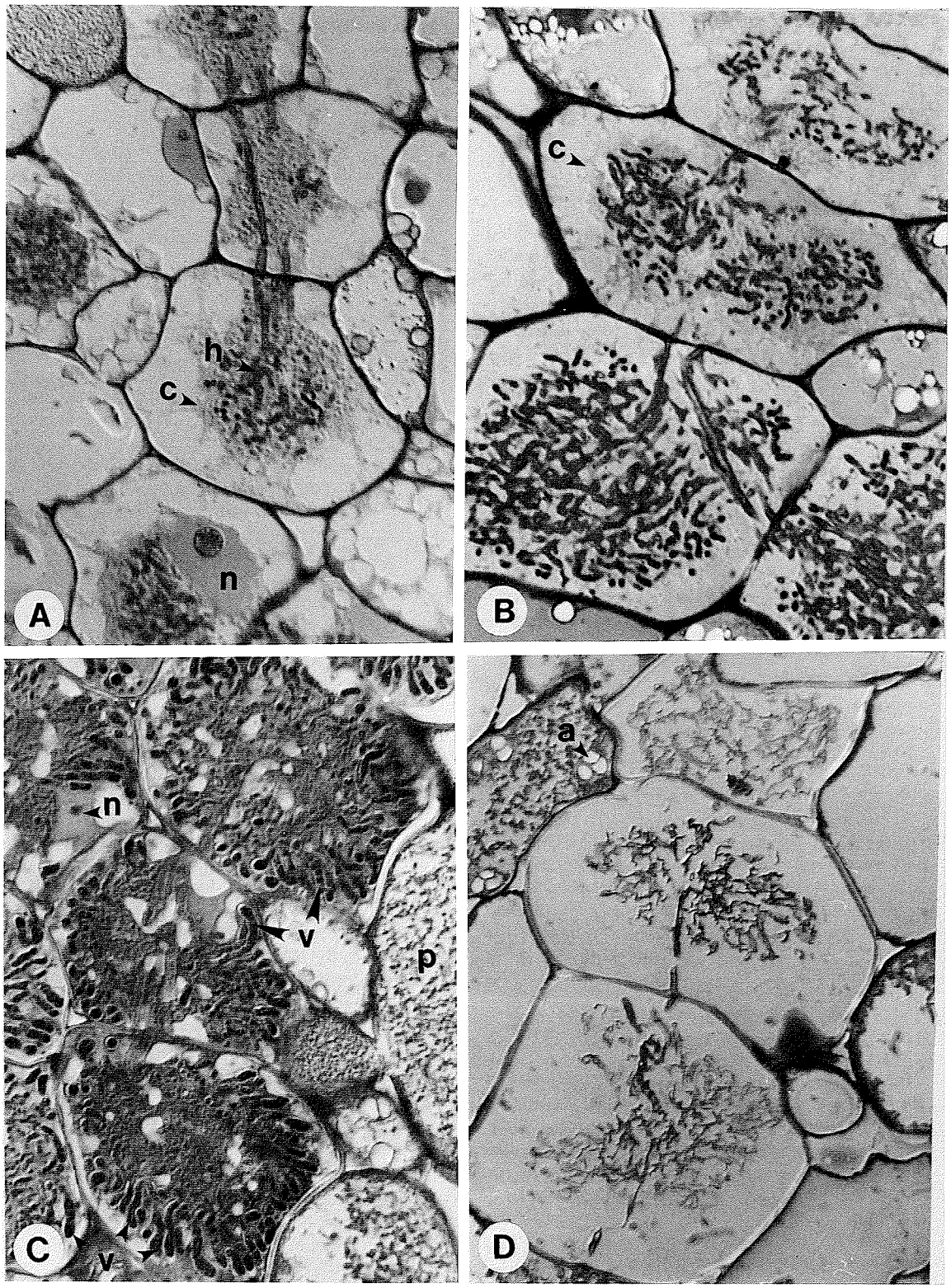
nodule lobes undergo only one cycle of growth and portions of nodules often die, resulting in subsequent growth in a limited number of directions.

The age structure of the population of nodules represented in Fig. 2 and Table 1 appears to be stable with the number of nodules newly initiated in 1979 (1-year nodules) being balanced by death of nodules in the older age groups. The exact balance between nodule initiation and death is probably fortuitous since there is likely to be year-to-year variation in both nodule initiation and death. The fraction of the total nodule volume present at the end of the growing season that was produced during the current year can be calculated from the data in Table 1 as the total volume of new tissue produced divided by the total volume present (Akkermans 1971). In 1979 these were 11 301 and 18 441 mm^3, respectively, and consequently 61% of the nodule volume present at the end of 1979 had been formed during that year.

Endophyte morphology and nitrogenase activity

All three morphological forms of the endophyte, namely hyphae, vesicles, and sporangia, previously described in *M. gale* nodules (Schaede 1938; Fletcher and Gardner 1974; Gardner 1976) were present in our material. We recognized two stages of the hyphal form: colonizing hyphae and proliferating hyphae. Colonizing hyphae occurred in the infected cells nearest the nodule lobe apex and were found centrally within the host cell (Fig. 3A). There were only a few predominantly unbranched hyphae in each cell and they frequently penetrated through the cell wall into successive cells in the same cell file. The host cells thus infected were partially expanded cortical cells and contained normal nuclei, nucleoli, cytoplasm, and some starch grains but lacked phenolic vacuoles and abundant large starch grains regularly seen in nearby uninfected cells. Proliferating hyphae occurred in expanding cells below

FIG. 3. Stages in the developmental morphology of the *Frankia* sp. endophyte in *Myrica gale*. (A) Colonizing hyphae. The earliest identifiable phase of hyphal invasion is found invading acropetally in subapical expanding cells in the cortex. The hyphae (*h*) are found centrally within the matrix of the host cytoplasm (*c*). Note nucleus (*n*) with prominent nucleolus in an infected cell (nodule collected May 23). (B) Proliferating hyphae. While the infected host cells are expanding, the endophyte hyphae proliferate and extend outward from the cell center (May 23 material). (C) Vesicles. As the infected host cells mature, the endophyte vesicles (*v*) form as darkly staining, club-shaped termini of peripheral branch hyphae. Accumulations of polyphenols (*p*) are commonly found in uninfected cells (July 27 material). (D) Endophyte disintegration. Infected cells corresponding positionally to former vesicle-containing cells contain only faintly staining endophyte remains. Amyloplasts (*a*) are prominent in uninfected cells (October 31 material). All nodules are from the Open Mat site. The cells are from near median longitudinal nodule lobe sections, Nomarski interference contrast optics. ×1100.



(proximal to) those containing colonizing hyphae. These hyphae were concentrated near the center of the host cell but branched frequently and gradually filled the cell with hyphae from the center outward (Fig. 3B).

Vesicles formed as club-shaped termini of peripheral branch hyphae in cells below those containing proliferating hyphae. They were septate and filled the periphery of the host cell while the center was occupied by the already proliferated branched hyphae (Fig. 3C). The vesicle-containing host cells were fully expanded with representative dimensions of 33–42 μm parallel and 47–67 μm perpendicular to the long axis of the nodule lobe. These cells no longer contained starch grains but did contain nuclei which were often lobed and sometimes found pressed against the cell wall. Cells containing colonizing hyphae, proliferating hyphae, and vesicles formed a developmental continuum from the apex of the nodule lobe downward.

Sporangia formed in some cells in the vesicle-containing region of the lobes, but not in every nodule. Immature sporangia consisted of cuboidal cells closely packed into globose to obovate bodies. Mature sporangia (Fig. 4) were unstained to lightly stained structures filled with well-stained, somewhat angular spores.

The morphology of the endophyte showed a clear seasonal pattern which correlated closely with nitrogenase activity (Table 2). Nodules having nitrogenase activity were termed "active" and those without "inactive." In late March, when the soil was still frozen, all nodules were inactive and had a few infected cells with colonizing hyphae near the lobe apex. The infected cells in the remainder of the cortex were dead and contained empty hyphae or encapsulation of host origin which had collapsed (Fig. 3D, typical of nodules from the Open Mat site) or were tubular in shape (common in nodules from the Pond Side site). In some nodules, mature sporangia were present in this region.

In early May nodule growth resumed. Colonizing hyphae were present in cells immediately behind the growing apex and proliferating hyphae were present in many nodules (Table 2). Nitrogenase activity appeared in mid-May and both active and inactive nodules were present until mid-June with the frequency of inactive nodules declining rapidly.

Active nodules collected from May through October contained in order from the apex downward: colonizing hyphae, proliferating hyphae, and vesicles (Table 2). The two hyphal stages each occupied a relatively narrow band of tissue while vesicles occupied the bulk of the cortex of the newly formed lobes after the early stages of lobe development.

In late July the endophyte began to senesce in the first formed cells at the base of the current-year's tissue in some nodules. In nodules from the Open Mat site, densely staining vesicles and hyphae decreased in frequency and the hyphae appeared increasingly fragmented until no recognizable endophyte structures remained. Thereafter endophyte senescence continued moving from the lobe base towards the tip with the bulk of the endophyte senescing in unison during late September and October. Nitrogenase activity disappeared in the second half of October when vesicle disintegration was nearly complete. A few recognizable vesicles were still present in inactive nodules in late October but none remained in mid-November (Table 2), when both the endophyte and host cells had apparently died.

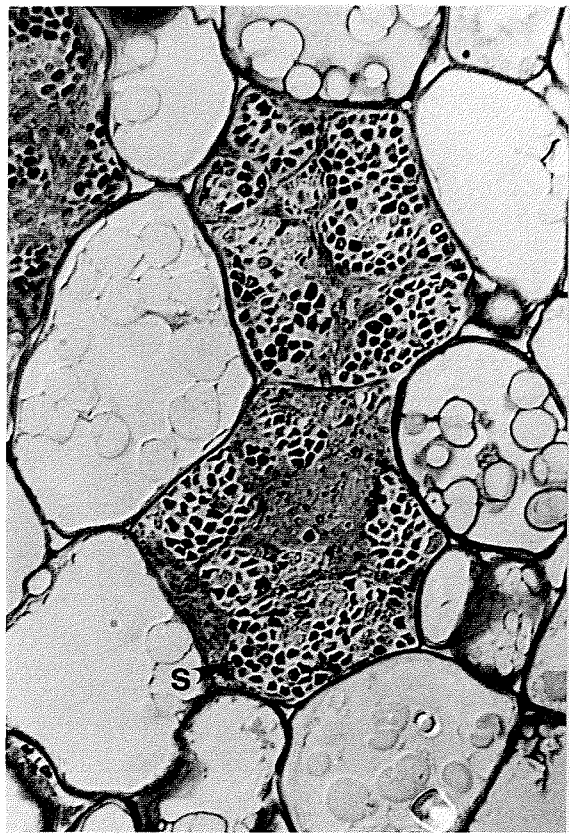


FIG. 4. Sporangia containing spores. The spores are darkly staining polyhedral granules. The cells are from a near median longitudinal lobe section and correspond positionally to former vesicle-containing cells (nodule collected October 31 at the Pond Side site). Nomarski interference contrast optics. ×1100.

The seasonal behavior of the endophyte forms discussed so far and the uninfected host cells can be summarized as follows. Colonizing hyphae were present in living host cells near the nodule lobe apex throughout the year, proliferating hyphae were present from early May before the onset of nitrogenase activity through early September, and the appearance, disappearance,

TABLE 2. Endophyte morphology[a] and nitrogenase activity of *Myrica gale* nodules from the field during 1979[b]

Date[c]	N[d]	Nitrogenase activity	Colonizing hyphae, % present[e]	Proliferating hyphae, % present[e]	Vesicles % present[e]
3/25[f]	3	−	100	0	0
5/3	5	−	100	60	0
5/10	3	−	100	67	0
5/23	2	−	100	50	0
5/23	3	+	100	100	100
6/1	5	+	100	100	100
6/27	3	+	100	100	100
7/11	2	+	100	100	100
7/27	3	+	100	100	100
8/8	3	+	100	100	100
9/4	3	+	100	67	100
9/19	3	+	100	0	100
10/10[f]	4	+	100	0	100
10/31	3	−	100	0	33
11/15	4	−	100	0	0

[a]See text for description of morphological forms.
[b]Nodules were from the Open Mat site unless otherwise indicated.
[c]Month/day.
[d]Number of nodules examined.
[e]Percent of nodules containing form.
[f]Nodules were from the Pond Side site.

and period of extensive development of vesicles closely coincided with the appearance, disappearance, and period of high rates of nitrogenase activity. Vesicle-containing cells died in autumn and were newly produced each year. Mature uninfected host cells showed no discernable seasonal changes. Most contained abundant starch grains and prominent vacuoles filled with phenolic compounds as well as occasional druses throughout the year.

The seasonal behavior of sporangia could not be completely determined because of their infrequent occurrence at the Open Mat site where most materials were collected for this study. In 1979 sporangia were found in only 4 of 41 nodules from the Open Mat site but were much more common at the Pond Side site where they occurred in 6 of 8 nodules examined. Examination of freehand sections of additional nodules collected in November 1980 confirmed this pattern. Sporangia were present in 1 of 30 nodules at the Open Mat and in 19 of 20 at the Pond Side. Sporangia always occurred in cells in the middle and lower regions of the cortex among cells that presently or had previously contained vesicles. At the Open Mat site mature sporangia were present in the previous-year's tissue in single nodules collected in May and June and in the current-season's tissue in two nodules collected in July. At the Pond Side site mature sporangia were present in the previous-year's tissue in March and sporangia in all stages of development occurred in the current-season's tissue in October. No Pond Side materials were collected between March and October. These observations indicate that sporangia begin to form in the present-year's tissues sometime after the first vesicle containing cells have fully developed and persist in the previous-year's growth throughout the year.

Seasonal pattern of soil temperature, shoot phenology, and nitrogenase activity

Soil temperatures at the Open Mat site (Fig. 5) increased rapidly from freezing in mid-April to about 15°C in mid-May, maintained values between 14 and 21.5°C through June, July, and August and then declined through September and October. Several aspects of shoot phenology are shown in Fig. 6A. The plants flowered in late April and budbreak followed about 1 week later. The new shoots expanded rapidly in May and June and reached their final length in early July. Leaf senescence and loss began with the small, first produced leaves at the shoot bases as the shoots reached their final length. It continued slowly through the rest of the summer and early fall and became rapid in September. There was little leaf loss due to herbivory.

Nitrogenase activity appeared in May, had a high rate from late June through early August, and disappeared in mid-October (Fig. 6B). It was first measurable in individual nodules examined for endophyte morphology

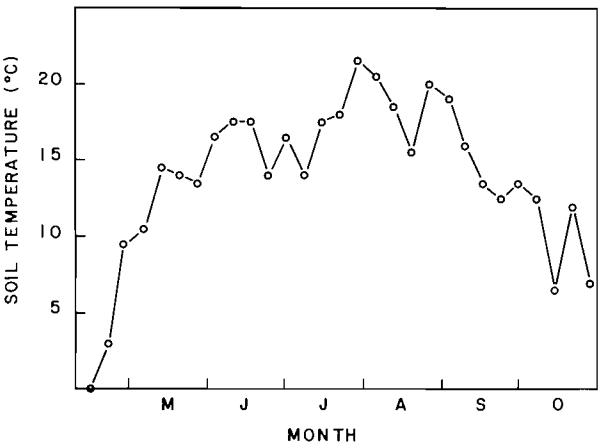


FIG. 5. Soil temperature at 15 cm below the surface at the Open Mat site in 1979. Measurements were made at 9 a.m.

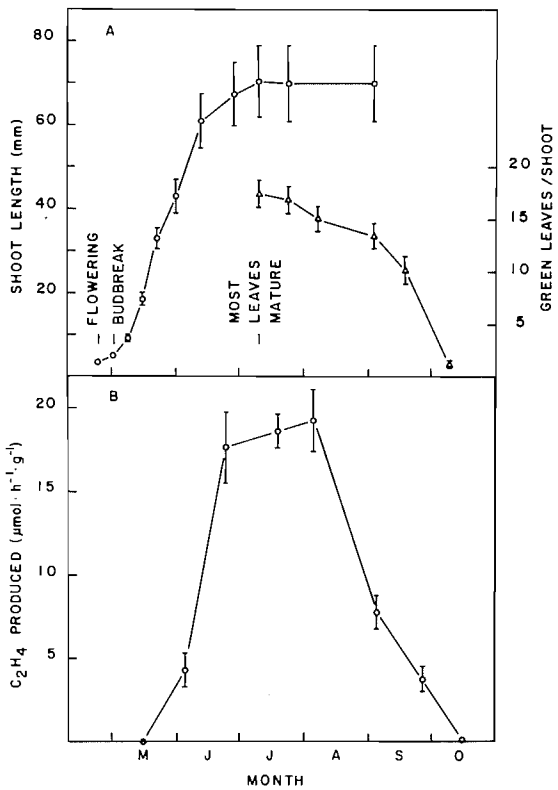


FIG. 6. Phenology of *Myrica gale* shoots and seasonal pattern of nitrogenase activity at the Open Mat site in 1979. (A) ○, shoot length; △, number of green leaves; mean ± SE; N = 24. (B) Nitrogenase activity was measured in excised nodules at temperatures prevailing in the soil at the time of nodule collection; activity is expressed on the basis of ovendry weights; mean ± SE; N = 20.

on May 16 when 1 of 10 nodules was active and increased substantially by May 23 when 6 of 10 nodules were active. The annual rate of nitrogen fixation calculated for 1979 from the curve in Fig. 5B was 37.2 kg N·ha^{-1}·year^{-1} assuming a nodule biomass of 104 kg dry weight ·ha^{-1} as measured in 1978 and a ratio of 3.0 C_2H_4 produced per N_2 fixed (Schwintzer 1979).

Effect of temperature on initiation of shoot and nodule growth

Shoot and nodule growth began approximately 3 months earlier in the greenhouse maintained at a minimum temperature of 6°C under natural light regimes than in the field. On February 28 all plants were in full flower and budbreak had occurred in 50%. By March 29 the growing shoots had reached a length of 38.2 ± 5.7 mm ($\bar{x}$ ± SE; N = 12) and the nodules had new nodule roots and a nitrogenase activity of 9.4 ± 4.0 μmol C_2H_4·h^{-1}·g dry weight^{-1} ($\bar{x}$ ± SE; N = 6).

Discussion

The two-step growth cycle observed in *M. gale* field nodules in their second or a subsequent year of growth is essentially the same as the growth pattern of 1st-year nodules on *M. gale* seedlings grown in water culture or aeroponics. In 1st-year nodules three morphological stages are recognized following nodule initiation: namely, nodule lobe formation, followed by a transition state, and nodule root elongation (Torrey and Callaham 1978). The same stages are present in field nodules ≥ 1 year old but the order of the steps shows a seasonal pattern. Most field nodules overwintered in the transition state and then resumed growth in the spring with nodule root elongation accompanied by expansion of the tip of the old lobe and followed by nodule lobe formation.

The vesicles of the endophyte are almost certainly the site of nitrogenase activity in *M. gale* nodules since their appearance, disappearance, and time of strong expression was closely correlated with that of nitrogenase activity. Additional evidence is provided by the cultured endophyte, *Frankia* sp. CpI1, originally isolated from *Comptonia peregrina* (Callaham *et al.* 1978). Formation of vesicles in *Frankia* sp. CpI1 can be controlled by cultural conditions and nitrogenase activity correlates with presence of vesicles (Tjepkema *et al.* 1980, 1981). Moreover, in young, nodulating *Alnus glutinosa* seedlings, the onset of nitrogenase activity coincides with production of vesicles by the endophyte (Mian and Bond 1978) and in *Elaeagnus umbellata* inoculation with a crushed nodule suspension produces nodules possessing both vesicles and nitrogenase activity while the cultured strain, *Frankia* sp. EuI1 produces nodules which lack both (Baker *et al.* 1979).

Colonizing hyphae and spores are the only living

forms of the endophyte that overwinter within nodules. Colonizing hyphae must be the source of the endophyte which invades the newly formed cortical cells when growth resumes in the spring because they are present in all nodules while spores are absent from many.

Spores have been referred to by various terms in the past, i.e., bacteroids, bacterialike cells, polyhedral-shaped cells, and granulae (van Dijk 1978) reflecting uncertainty as to their development and function within the life cycle of the endophyte. However, recent light- and electron-microscopical descriptions of the development of sporangia and spores in *Alnus glutinosa* nodules (van Dijk and Merkus 1976) and the cultured endophyte, *Frankia* sp. CpI1 (Newcomb *et al.* 1979), have shown that these cells are spores.

Schaede (1933) first noticed that two types of nodules, now designated Sp(+) and Sp(−), could be distinguished in *A. glutinosa*. Spores and sporangia are abundant in Sp(+) nodules and hardly present in Sp(−) nodules (van Dijk and Merkus 1976). In *A. glutinosa* this difference is due to strain differences in the *Frankia* endophyte (van Dijk 1978). It is likely that Sp(+) and Sp(−) nodules also exist in *M. gale*. The populations at the Open Mat and Pond Side sites are probably Sp(−) and Sp(+), respectively. Sporangia were present in only 7% of the nodules at the Open Mat but occurred in 89% at the Pond Side. It is possible, however, that the nodules at both the Open Mat and Pond Side sites represent mixtures of Sp(+) and Sp(−) *Frankia* strains.

Death of vesicle-containing cells and the enclosed endophyte in autumn and formation of new vesicle-containing tissues in spring may be typical of the nodules of winter-deciduous actinorhizal plants. In addition to being observed here in both Sp(+) and Sp(−) *M. gale* nodules, it has been described in *Alnus glutinosa* (Krebber 1932; Käppel and Wartenberg 1958) and *A. incana* (Krebber 1932). Krebber (1932) noted that in *A. glutinosa* and *A. incana* the presence of vesicles coincides with presence of leaves on the shoots. Käppel and Wartenberg (1958) made similar observations in naturally growing Sp(−) nodules of *A. glutinosa* but found that in Sp(+) nodules disintegration of vesicles is incomplete in autumn and production of new vesicles occurs in March about 2 months before budbreak. Further evidence that death of vesicle-containing cells and the enclosed endophyte is common is provided by Schaede (1933) who observed dissolution of the endophyte contents leaving only empty "membranes" followed by host cell death in the older portions of nodule lobes in *A. glutinosa, A. japonica,* and *Hippophäe rhamnoides*.

A relatively short life span may also be typical of the nodules of actinorhizal plants. Most of the *M. gale* nodules (88%) in our study did not live more than 3 years and none more than 5 years. Moreover, most nodules on a 20-year-old *Alnus glutinosa* tree growing in a peatland in the Netherlands did not live more than 3 years and none more than 8 years (Akkermans 1971; Akkermans and van Dijk 1976). Since the proportion of new, i.e., nitrogenase active, nodule tissue decreases rapidly with increasing nodule age (Table 1), early nodule death results in a favorable balance of active to inactive tissues within the nodule population. The relatively high death rate of nodules suggests that actinorhizal plants must produce large amounts of new nodule tissue each season to maintain a constant nodule biomass. This is indeed the case. Newly produced nodule tissue comprised 61% of the *M. gale* nodule biomass at the Open Mat site and 52–62% of the nodule biomass on individual *A. glutinosa* trees in the Netherlands (Akkermans 1971; Akkermans and van Dijk 1976).

The seasonal pattern of nitrogenase activity in *M. gale* observed here and its relationship to shoot phenology is probably also typical of winter-deciduous actinorhizal plants. Similar patterns of appearance of nitrogenase activity after budbreak, maximum activity in early or midsummer after full expansion of the leaves, and disappearance of activity at about the time of leaf fall in autumn have also been observed in a variety of other winter-deciduous actinorhizal plants including: several *Alnus* species (Akkermans 1971; Pizelle 1975; Fleschner *et al.* 1976; Tripp *et al.* 1979; Moiroud and Capellano 1979), *Hippophäe rhamnoides* (Akkermans 1971), and *Purshia tridentata* (Dalton and Zobel 1977).

The shape of the seasonal nitrogenase activity curve in *M. gale* is probably due to interaction of several factors including nodule temperature, production of carbohydrates by photosynthesis, nodule phenology, and competition between nodules and other plant parts for photosynthate. Although nitrogenase activity of *M. gale* nodules is strongly temperature dependent, the seasonal pattern of soil temperature is not a major factor in determining the seasonal pattern of nitrogenase activity (Schwintzer 1979). Photosynthesis is related to shoot phenology and the observed pattern of shoot phenology agrees well with the period of initiation and increasing nitrogenase activity in spring and early summer. However, it does not coincide with the rapid decline of nitrogenase activity in August when day length, shoot phenology, and temperature are still favorable for photosynthesis. The pattern of endophyte development and nodule lobe growth observed here also agrees well with the period of initiation and increasing nitrogenase activity and no doubt interacts with developing photosynthetic capacity to determine the first half of the nitrogenase activity curve. However, it too fails to coincide with the rapid decline of nitrogenase activity in August although a gradual decline in nitrogenase activ-

ity can be expected at this time. In late July the first produced vesicles began to senesce and relatively few new vesicles were formed since there was little nodule lobe growth. Sprent *et al.* (1978) have suggested that the rapid decline may be due to competition for photosynthate by rhizomes.

Increasing photoperiod and soil and air temperatures probably interact to stimulate the initiation of shoot and nodule growth and the return of nitrogenase activity in the spring in winter-deciduous actinorhizal plants. Wheeler and McLaughlin (1979) have shown that the photoperiodic response is the major factor controlling the breaking of dormancy in *Alnus glutinosa*. Our observations in a naturally lighted greenhouse with a minimum temperature of 6°C and in the field indicate that photoperiods permitting breaking of dormancy in *M. gale* are reached in late February but dormancy is not broken in the field until the second half of April. Thus under field conditions, temperature should be the major factor that determines the actual time that dormancy is broken. Soil temperatures began to rise above 0°C about 2 weeks earlier in the spring in 1979 than in 1978 (Schwintzer 1979). Consequently flowering, budbreak, and the return of nitrogenase activity would be expected to be earlier in 1979 than 1978. This was indeed the case. In 1979 flowering and budbreak occurred about 2 weeks earlier than in 1978. Nitrogenase activity also returned earlier in 1979, when it was first detected May 16, than in 1978 when all nodules were still inactive on May 20 but had activity when next measured on June 1 (Schwintzer 1979).

Acknowledgements

We thank all of the people at the Harvard Forest who have aided this study. Special thanks are due to John G. Torrey and John D. Tjepkema for stimulating discussions, use of their laboratory facilities, and valuable comments on the manuscript; William Ormerod for photographing the field nodules, darkroom assistance, and assistance with photomicrography; Susan A. Lancelle for examining nodules collected in 1980 for presence of sporangia; Frances O'Brien for typing the manuscript. This work was supported in part by National Science Foundation grant No. DEB 77-02249 and a Charles Bullard Fellowship to C.R.S.

ABBAYES, H. DES, and C. HAMANT. 1946. Repartition et comportement de *Myrica gale* L. dans le Massif américain. C. R. Seances Soc. Biogeogr. **23**(195): 12–17.

AKKERMANS, A. D. L. 1971. Nitrogen fixation and nodulation of *Alnus* and *Hippophäe* under natural conditions. Ph.D. thesis, State University of Leiden, The Netherlands.

AKKERMANS, A. D. L., and C. VAN DIJK. 1976. The formation and nitrogen-fixing activity of the root nodules of *Alnus glutinosa* under field conditions. *In* Symbiotic nitrogen fixation in plants. *Edited by* P. S. Nutman. Cambridge University Press, London. pp. 511–520.

BAKER, D., J. G. TORREY, and G. H. KIDD. 1979. Isolation by sucrose-density fractionation and cultivation *in vitro* of actinomycetes from nitrogen-fixing root nodules. Nature (London), **281**: 76–78.

BOWES, B., D. CALLAHAM, and J. G. TORREY. 1977. Time-lapse photoghapic observations of morphogenesis in root nodules of *Comptonia peregrina* (Myricaceae). Am. J. Bot. **64**: 516–525.

CALLAHAM, D., W. NEWCOMB, J. G. TORREY, and R. L. PETERSON. 1979. Root hair infection in actinomycete-induced root nodule initiation in *Casuarina*, *Myrica* and *Comptonia*. Bot. Gaz. (Chicago), **140**(Suppl.): S1–S9.

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

DALTON, D. A., and D. B. ZOBEL. 1977. Ecological aspects of nitrogen fixation by *Purshia tridentata*. Plant Soil, **48**: 57–80.

DANSEREAU, P., and F. SEGADAS-VIANNA. 1952. Ecological study of the peat bogs of eastern North America. I. Structure and evolution of vegetation. Can. J. Bot. **30**: 490–520.

FLESCHNER, M. D., C. C. DELWICHE, and C. R. GOLDMAN. 1976. Measuring rates of symbiotic nitrogen fixation by *Alnus tenuifolia*. Am. J. Bot. **63**: 945–950.

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

GARDNER, I. C. 1976. Ultrastructural studies of non-leguminous root nodules. *In* Symbiotic nitrogen fixation in plants. *Edited by* P. S. Nutman. Cambridge University Press, London. pp. 485–495.

HARDY, R. W. F., R. C. BURNS, and R. D. HOLSTEN. 1973. Applications of the acetylene–ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. **5**: 47–81.

HENRY, M.-F. 1977. Cytologie ultrastructurale de l'endophyte présent dans les nodosités radiculaires de *Myrica gale* L. Bull. Soc. Bot. Fr. **124**: 291–300.

JEGLUM, J. K., A. N. BOISSONNEAU, and V. F. HAAVISTO. 1974. Towards a wetland classification for Ontario. Can. For. Serv. Dep. Environ. Inf. Rep. O-X-215.

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

KREBBER, O. 1932. Untersuchungen über die Wurzelknöllchen der Erle. Arch. Mikrobiol. **3**: 588–608.

MCVEAN, D. N., and D. A. RATCLIFFE. 1962. Plant communities of the Scottish Highlands. Her Majesty's Stationery Office, London.

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.

MOIROUD, A., and A. CAPELLANO. 1979. Etude de la dynamique de l'azote à haute altitude. I. Fixation d'azote (réduction de l'acétylène) par *Alnus viridis*. Can. J. Bot. **57**: 1979–1985.

NEWCOMB, W., D. CALLAHAM, J. G. TORREY, and R. L.

PETERSON. 1979. Morphogenesis and fine structure of the actinomycetous endophyte of nitrogen-fixing root nodules of *Comptonia peregrina*. Bot. Gaz. (Chicago), 140(Suppl.): S22–S34.

PIZELLE, G. 1975. Variations saisonnières de l'activité nitrogénasique des nodules d'*Alnus glutinosa* (L.) Gaertn., d'*Alnus incana* (L.) Moench, et d'*Alnus cordata* (Lois.) Desf. C.R. Hebd. Seances Acad. Sci. Ser. D, **23**: 1829–1832.

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. 1978. Vegetation and nutrient status of northern Michigan fens. Can. J. Bot. **56**: 3044–3051.

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

SPRENT, J. I., and R. SCOTT. 1979. The nitrogen economy of *Myrica gale* and its possible significance for the afforestation of peat soils. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Forest Research Laboratory, Oregon State University, Corvallis, OR. pp. 234–242.

SPRENT, J. I., R. SCOTT, and K. M. PERRY. 1978. The nitrogen economy of *Myrica gale* in the field. J. Ecol. **66**: 657–668.

SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31–34.

TJEPKEMA, J. D. 1978. The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation of *Myrica gale*. Can. J. Bot. **56**: 1365–1371.

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., and D. CALLAHAM. 1978. Determinate development of nodule roots in actinomycete-induced root nodules of *Myrica gale*. Can. J. Bot. **56**: 1357–1364.

——— 1979. Early nodule development in *Myrica gale*. Bot. Gaz. (Chicago), **140**(Suppl.): S10–S14.

TRIPP, L. N., D. F. BEZDICEK, and P. E. HEILMAN. 1979. Seasonal and diurnal patterns and rates of nitrogen fixation by young red alder. For. Sci. **25**: 371–380.

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 microscopical 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**: 73–91.

WHEELER, C. T., and M. E. MCLAUGHLIN. 1979. Environmental modulation of nitrogen fixation 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. Forest Research Laboratory, Oregon State University, Corvallis, OR. pp. 124–142.