JOURNAL OF BACTERIOLOGY
Vol. 87, No. 2, p. 416-421 February, 1964

ROOT NODULE SYMBIOSIS

I. ENDOPHYTE OF *MYRICA CERIFERA* L.[1]

WARREN S. SILVER

Department of Bacteriology, College of Agriculture, University of Florida, Gainesville, Florida

Received for publication 12 August 1963

ABSTRACT

SILVER, WARREN S. (University of Florida, Gainesville). Root nodule symbiosis. I. Endophyte of *Myrica cerifera*. J. Bacteriol. 87:416-421. 1964.—Electron microscopy of 0.1-μ thick sections of root nodules, fixed with permanganate and embedded with methacrylate, showed that infected plant cells were filled with a mycelial endophyte. The endophyte was filamentous, 1 μ in diameter, septate, and had an enlarged, club-shaped terminus. Although structurally the endophyte strongly resembles an actinomycete, it was not isolated in pure culture on a variety of appropriate media.

Since the discovery that nonleguminous plants bearing root nodules, such as those of the genera *Myrica*, *Casuarina*, and *Alnus*, can grow in the absence of fixed nitrogen, there has been considerable interest in the nature of the endophyte associated with nodular tissue. There have been reports of the isolation of the nodular endophyte of *Alnus* [reviewed by Schaede (1962) and Bond (1963)]; but, with one exception, such claims cannot be accepted, without reservation, because infection of bacteria-free plants with the isolated culture had not been achieved. Fletcher (1955), though unable to isolate the endophyte of *Myrica gale*, believed that paraffin sections revealed structures resembling filamentous bacteria, most likely actinomycetes. This was in agreement with an earlier description by Shibata (1902). Hawker and Fraymouth (1951) disputed similar claims and, after a review of the evidence, concluded on morphological grounds that the endophyte of *M. gale* was a member of the Plasmodiophorales.

We have been interested in the symbiotic association in the root nodules of *M. cerifera*, with the aim of both isolating the endophyte in pure culture and defining in biochemical terms some of the effects of the association on the host plant (Bendana, Powell, and Silver, Plant Physiol., *in press*). Although we have been unable to isolate the endophyte despite repeated attempts, the fine structure of root nodules, as observed with an electron microscope, supports our original premise in the isolation attempts—that the endophyte is a filamentous bacterium, most likely a member of the Actinomycetales. Since no studies of the fine structure of nonleguminous root nodules have been made previously with greater resolving power than that available with a light microscope, we have attempted to characterize better the endophyte of *M. cerifera* by using an electron microscope.

[1] A preliminary report of this work was presented at the 62nd Annual Meeting of the American Society for Microbiology, Kansas City, Mo., May 1962.

MATERIALS AND METHODS

All nodules were obtained from plants growing in the field in the vicinity of Gainesville, Fla. Field material was transported to the laboratory wrapped in moist paper towels. Nodules were washed thoroughly in cold tap water to remove adhering soil, and were dissected from roots with a sharp razor blade. Only healthy, nondecaying tissue was used. No attempt was made to separate nodules of different ages other than to discard obviously senescent tissue. The average time interval between removal of the nodules from the plant and immersion into fixative was 3 hr.

For observations with a light microscope, nodules were fixed in Craf's III solution (Sass, 1951), dehydrated, and embedded with Tissuemat (A. H. Thomas Co., Philadelphia, Pa.); sections, 6 μ thick, were cut with an A. O. Spencer model 820 microtome. Safranin and fast green were found to be suitable stains for distinguishing endophyte from host-cell tissue. Photographs were taken on Kodak high-contrast copy film with a Leica M-3 camera on a Zeiss phase microscope equipped with a Neofluar Ph 40/0.75 lens.

For observation of fine structure with an electron microscope, it was found that the most

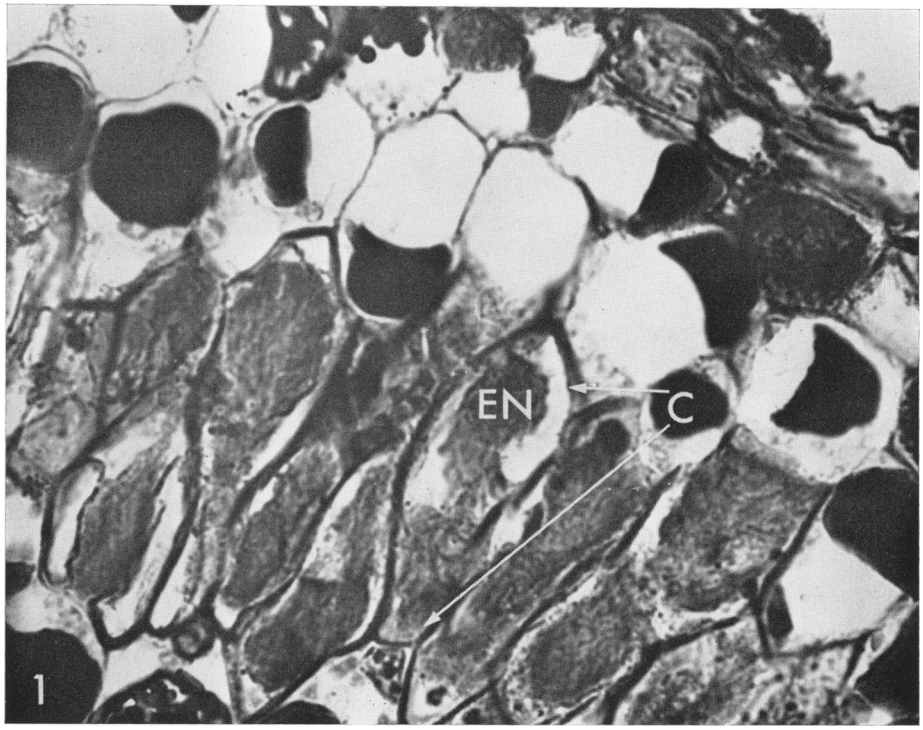

FIG. 1. *Photomicrograph of a 6 μ thick cross-section of a stained root nodule of Myrica cerifera. The endophyte (en) fills the enlarged cortical cells (c).* ×450.

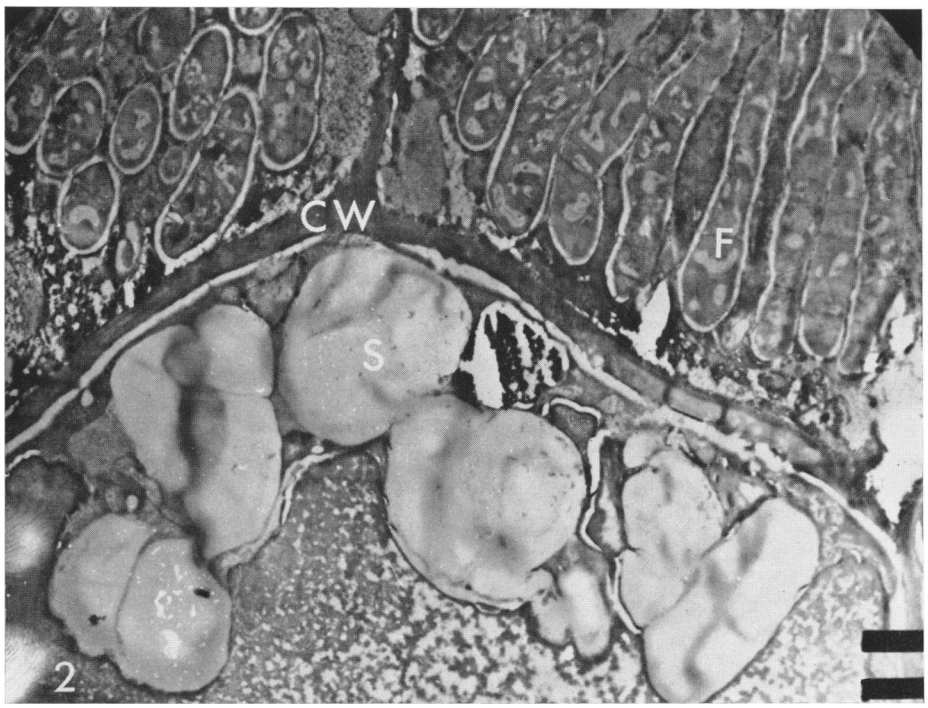

FIG. 2. *Electron micrograph of a thin section of a nodule of Myrica cerifera showing the enlarged terminus of endophyte filaments (f), host cell wall (cw), and starch (s) in an adjacent noninvaded cell. In all figures the marker represents 1 μ.* ×3,500.

critical factors in obtaining properly fixed tissue were the size of the tissue and the length of time in the fixative. Proper fixation of both the microorganisms and the host cell tissue was difficult, since plant tissue tended to be overfixed when the endophyte was adequately fixed. The following conditions, which represent a compromise, were found to suffice for the preparation of thin sections. Small cubes of nodules (about 1 mm^3) were cut under a dissecting microscope. This was done by first bisecting a nodule along its length and then cutting it transversely. The outer corky layer was thus left intact, and the fixative penetrated the tissue from the cut sides. Adequate fixation occurred within 85 to 110 min in 2% (w/v) aqueous potassium permanganate at room temperature (25 ± 2 C; Mollenhauer, 1959). The fixed tissue was embedded with methacrylate [butyl methacrylate, 3.5 parts; methyl methacrylate, 1 part (v/v)], catalyzed with Luperco CDB in gelatin capsules, and allowed to harden overnight at 60 C (Anonymous, 1959). Sections 0.1 μ thick were cut with a glass knife on a Porter-Blum microtome and observed in a Phillips EM 100 electron microscope.

In attempts to isolate the endophyte, nodules were thoroughly cleaned with tap water, soaked in 1:100 phenol for 20 min, rinsed thoroughly with sterile distilled water, soaked in cycloheximide (1 mg/ml) for 20 min, and then rinsed again with sterile distilled water. In some experiments, nodules treated in this manner were homogenized in 0.2 M phosphate buffer (pH 6.5) in a micro-Waring Blendor and ground briefly in a Ten Broeck glass homogenizer to release the endophyte from plant cells. Dilutions of the homogenate were then plated on a variety of media. It was necessary to employ such rigorous external treatment, for it was very difficult to reduce the external contaminant population to a point where the growth of colonies of the endophyte might be discerned. In several experiments, nodules were peeled aseptically under the dissecting microscope to remove the external corky layer (as well as contaminants) prior to cutting or squashing and implanting in isolation media.

The isolation media used were dextrose asparagine agar, Trypticase Soy Agar (BBL), Potato Dextrose Agar (Dajac Laboratories),

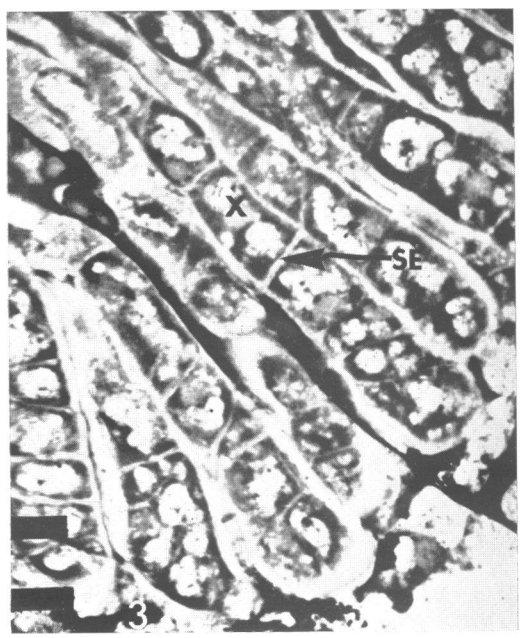

FIG. 3. *Electron micrograph of a thin section of a nodule of Myrica cerifera showing septa (se) and unidentified structures (x) (see text).* ×7,000.

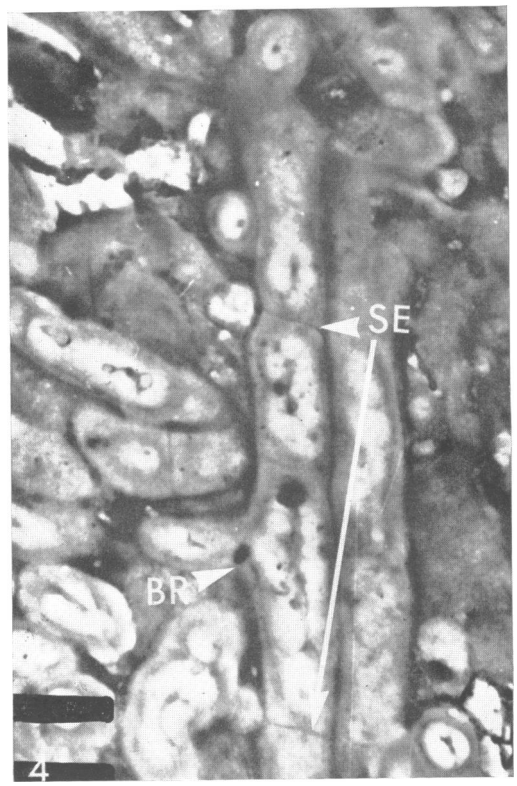

FIG. 4. *Electron micrograph of a thin section through an infected cortical cell showing branching of filament (br) and septa (se).* ×7,000.

Brain Heart Infusion Agar (Difco), mannitol nitrate mineral agar (Fred and Waksman, 1928), Czapek-Dox agar, mycological agar (BBL), and nutrient agar (Difco). Plates or tubes were incubated at 28 C aerobically, anaerobically, or under an elevated CO_2 tension (5%) for as long as 14 days. Since fungal contamination was a persistent problem, some of the above media were supplemented with 1 mg/ml of cycloheximide to retard fungal growth.

RESULTS AND DISCUSSION

In paraffin sections, the endophyte appeared to be filamentous (Fig. 1), but the thickness of the section rendered clearer observation difficult. In agreement with the results of Fletcher (1955) on *M. gale*, the organism was confined to the cortex, and cells filled with endophyte were greatly enlarged. The endophyte was never observed in the nodule roots which grow from the apices of nodules. The drawings of Shibata (1902), Arzberger (1910), and Bottomley (1912) reveal similar structures. It is apparent from Fig. 1 that the resolution attainable with relatively thick paraffin sections and a light microscope is insufficient for clear observations of the endophyte.

Electron photomicrographs of thin sections more clearly revealed the filamentous nature of the endophyte (Fig. 2). Filaments were regular, septate, approximately 1 μ in diameter, and had an enlarged club-shaped terminus. Septa are more clearly shown in Fig. 3. The cell wall at septa between units of a filament was similar in appearance to the outer wall of the filament. Each subunit of a hypha contained one or more nondense structures ("X" Fig. 3), which may represent nucleoids (Stuart, 1959). The branching, which was occasionally observed (Fig. 4), is quite similar to that noted in *Streptomyces griseus* by Hagedorn (1959) and in *S. noursei* by Stuart (1959), despite the use of different preparative methods. Also, discrete transverse membranes and cross walls (Fig. 2, 3, 4), reminiscent of electron micrographs of streptomycetes presented by Hagedorn (1959), Moore and Chapman (1959), and Stuart (1959) are evident. Host cells infected with endophyte showed little organized plant cell substructure (Fig. 5). The presence of even a small amount of endophytic mycelia

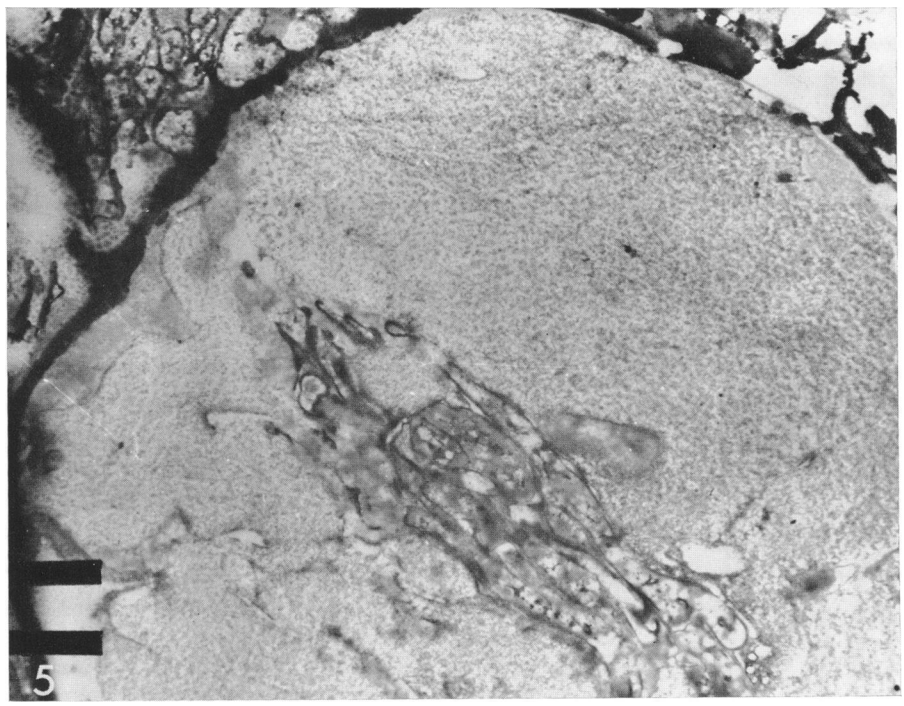

FIG. 5. *Electron micrograph of a thin section through a partially infected cortical cell. Note lack of normal host-cell substructure (compare with Fig. 6).* ×5,000.

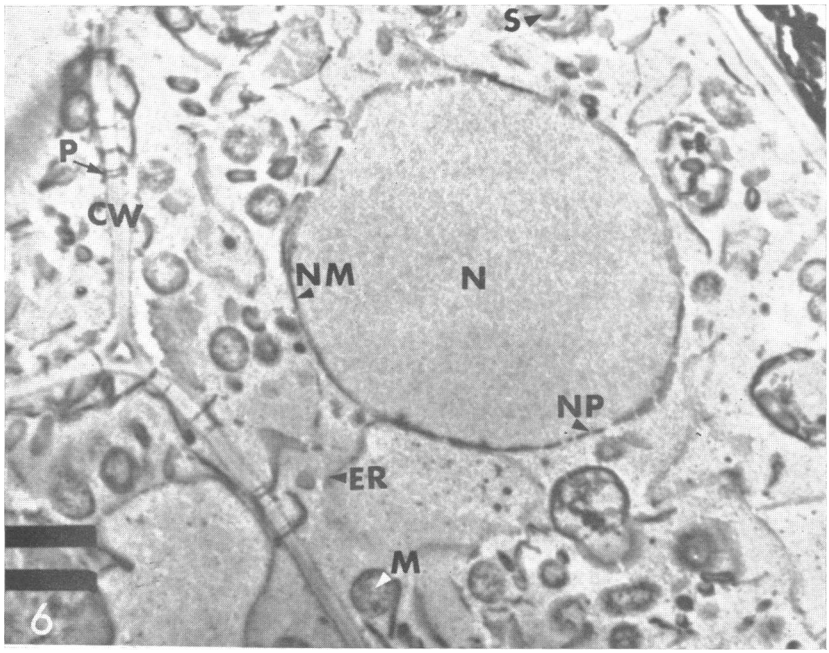

FIG. 6. *Electron micrograph of a thin section of an uninfected cortical cell showing typical plant-cell structure. Cell wall (cw), plasmadesma (p), nucleus (n), nuclear membrane (nm), nuclear pore (np), endoplasmic reticulum (er), mitochondrion (m), and starch (s).* ×*3,000.*

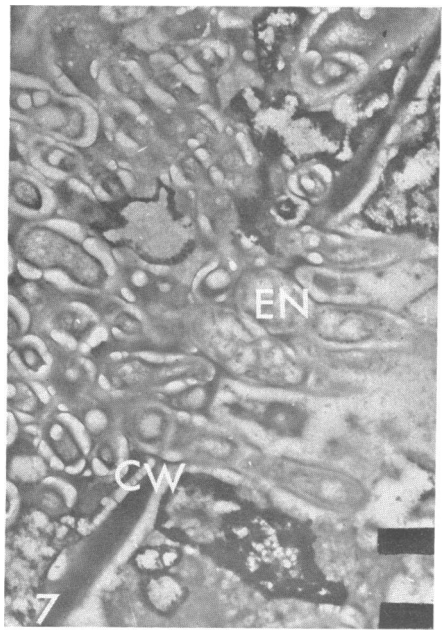

FIG. 7. *Electron micrograph of a thin section showing penetration of endophyte (en) through breach of host cell wall (cw) into adjacent cell.* ×*5,000.*

appears to preclude the presence of any host-cell structures other than the nucleus, which often appears distorted in infected cells (compare Fig. 5 and 6). A similar loss of host-cell substructure was noted by Arzberger (1910) in *M. cerifera* and Furman (1959) in *Ceanothus*. When adjacent cells were infected, a mass of filaments was observed in the area of the broken cell wall (Fig. 7), and the rupture of the cell wall between infected cells did not appear to be mechanical.

Our data, as well as that of the investigators mentioned above, do not support the contentions of Hawker and Fraymouth (1951) that the endophyte of *Myrica* is a slime mold. Indeed, their photographs of root nodule sections of *M. gale* reveal organisms more closely related to actinomycetes than to slime molds. Furman (1959) concluded from his studies of the endophyte of *Ceanothus* root nodules that all of the structures observed corresponded to a morphology characteristic of streptomycetes, and that the "sporangia" of Hawker and Fraymouth are really the vesicular ends of hyphae.

Recently, Becking (*personal communication*) demonstrated a very similar fine structure of nodules of *Alnus* with an electron microscope. It would appear then that the conclusions made

from observations reported here may be applied to other nodulated nonleguminous plants as well.

In spite of persistent attempts, no isolates were obtained which occurred with sufficient frequency or numerousness to warrant plant-root inoculation. If the evidence supports the idea that the endophyte is an actinomycete, why then did attempts at its isolation on a variety of appropriate media fail? It may be that special growth factors, not provided in the variety of media tried, are required for growth. However, addition of an aqueous extract of roots of *Myrica* to the isolation media had no beneficial effect upon the flora obtained.

Quispel (1960) demonstrated that a lipoidal fraction from *Alnus* roots increased the number of nodular foci in experimentally infected plants. The effect was noted only in vivo and attempts to obtain growth of the endophyte in vitro were unsuccessful. Since uninfected seedlings of *M. cerifera* were nodulated with difficulty no attempt was made to look for an in vivo effect as did Quispel.

The endophyte cannot be an obligate parasite since the symbiont is not seed-borne and the only known way in which natural nodulation occurs is via soil contact. Seedlings can be readily nodulated by planting in soil taken from the field where *Myrica* is indigenous. It may be that the nodular form of the organism requires soil passage to be recovered by conventional culture methods. Thus, direct isolation from nodules may not be possible.

Despite the claim of successful isolation of *Pseudomonas radicicola* as the endophyte of *M. gale* by Bottomley (1912), there are as yet no isolates which have the ability to induce the nodulation of bacteria-free roots, a necessary criterion for the fulfillment of Koch's postulates.

ACKNOWLEDGMENTS

The author is indebted to Pat K. Stewart for technical assistance, to T. C. Carlisle for the electron microscopy, and to Mildred M. Griffith for counsel in plant anatomy.

The investigation was supported in whole by U.S. Public Health Service research grant GM-08577 from the Division of General Medical Sciences.

LITERATURE CITED

ANONYMOUS. 1959. Thin sectioning and associated technics for electron microscopy. Ivan Sorval, Inc., Norwalk, Conn.

ARZBERGER, E. A. 1910. The fungous root-tubercles of *Ceanothus americanus*, *Elaeagnus argentea* and *Myrica cerifera*. Missouri Botan. Garden Rept. **21**:60–102.

BOND, G. 1963. The root nodules of non-leguminous angiosperms. Symp. Soc. Gen. Microbiol. **13**: 72–91.

BOTTOMLEY, W. B. 1912. Root nodules of *Myrica gale*. Ann. Botany (London) **26**:111–117.

FLETCHER, W. W. 1955. The development and structure of the root nodules of *Myrica gale* L. with special reference to the nature of the endophyte. Ann. Botany (London) **19**:501–513.

FRED, E. B., AND S. A. WAKSMAN. 1928. Laboratory manual of general microbiology. McGraw-Hill Book Co., Inc., New York.

FURMAN, T. 1959. The structure of the root nodules of *Ceanothus sanguineus* and *Ceanothus velutinus*, with special reference to the endophyte. Am. J. Botany **46**:698–703.

HAGEDORN, H. 1959. Elektronenmikroskopische Untersuchungen an *Streptomyces griseus* (Krainsky). Zentr. Bakteriol. Parasitenk. Abt. II **113**:234–253.

HAWKER, L. E., AND J. FRAYMOUTH. 1951. A reinvestigation of the root nodules of species of *Elaeagnus*, *Hippophae*, *Alnus* and *Myrica* with special reference to the morphology and life histories of the causative organisms. J. Gen. Microbiol. **11**:369–386.

MOLLENHAUER, H. H. 1959. Permanganate fixation of plant cells. J. Biophys. Biochem. Cytol. **6**: 431–436.

MOORE, R. T., AND G. B. CHAPMAN. 1959. Observations of the fine structure and modes of growth of a streptomycete. J. Bacteriol. **78**: 878–885.

PETRAS, E. 1959. Elektronenmikroskopische Untersuchungen an *Streptomyces purpurascens* Lindenbein. Arch. Mikrobiol. **34**:379–392.

QUISPEL, A. 1960. Symbiotic nitrogen fixation in non-leguminous plants. V. The growth requirements of the endophyte of *Alnus glutinosa*. Acta Botan. Neerl. **9**:380–396.

SASS, J. E. 1951. Elements of botanical microtechnic, 2nd ed. McGraw-Hill Book Co., Inc., New York.

SCHAEDE, R. 1962. Die Pflanzlichen Symbiosen, 2nd ed., revised by F. H. Meyer. G. Fischer, Stuttgart.

SHIBATA, K. 1902. Cytologishe Studien über die Endotrophen mykorrhiza. Jahrb. Wiss. Botan. **37**:643–684.

STUART, D. C., JR. 1959. Fine structure of the nucleoid and internal membrane systems of streptomyces. J. Bacteriol. **78**:272–281.