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MORPHOGENESIS AND FINE STRUCTURE OF THE ACTINOMYCETOUS ENDOPHYTE OF NITROGEN-FIXING ROOT NODULES OF COMPTONIA PEREGRINA

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The morphogenesis and fine structure of the cultured endophyte isolated from root nodules of *Comptonia peregrina* have been studied, utilizing scanning electron microscopy, transmission electron microscopy, and Nomarski interference microscopy; comparisons were made with the endophyte in vivo. The actinomycetous nature of the endophyte was confirmed. Prokaryotic, branched septate hyphae, less than 1 μm in diameter, are the predominant growth form. Some hyphae increase in diameter (ca. 1–1.5μm) and undergo morphogenesis, forming club-shaped sporangia in vitro. The sporangia produced numerous sporangiospores which are presumed to be the propagative state of the microorganism. Sporangia were formed by the growth of longitudinally and transversely oriented septa which compartmentalize the wider hyphae. Sporangia were not observed in vivo in *Comptonia*. Club-shaped septate vesicles formed in vivo but not in vitro. The mature spores contained electron-dense inclusions resembling eukaryotic nucleoli, numerous vesicular structures, nucleoid regions, and a thick multilayered wall. The microorganism in vitro lacked a capsule which the organism possessed in vivo, providing further evidence for the role of the host in the deposition of this encapsulating wall material.

Introduction

The isolation and culture on a relatively simple medium of the actinomycete from root nodules of *Comptonia peregrina* have been reported (CALLAHAM, DEL TREDICI, and TORREY 1978). Inoculation of seedling roots of *Comptonia* resulted in the formation within 8 days of numerous nodules which possessed normal morphology and were capable of reducing acetylene, signifying symbiotic nitrogen-fixing capacity. The same microorganism was then reisolated from these seedling nodules; thus, the requirements of Koch's postulates were fulfilled, establishing this microorganism as the causal agent of root nodules in *Comptonia*. The cultured actinomycete grows mainly as a hyphal form which undergoes morphogenesis to form large (10 μm × 30–50 μm) club-shaped sporangia containing many sporangiospores. The sporangia have not been observed in vivo within *Comptonia* nodules (NEWCOMB et al. 1978), possibly because young nodules were preferentially studied, since they showed better preservation with our present microtechnical procedures. Structures resembling sporangia have been observed in the older regions of the nodules of *Alnus glutinosa* (VAN DIJK and MERKUS 1976) and have been observed by us in sectioned nodules of *Myrica gale*.

In this study transmission electron microscopy (TEM), scanning electron microscopy (SEM), and light microscopy utilizing Nomarski interference optics were correlated to provide a more detailed account of the structure and morphogenesis of the filamentous soil bacterium which initiated the actinomycete-*Comptonia* symbiosis.

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Material and methods

Cultures of the microorganism isolated from *Comptonia* root nodules (CALLAHAM et al. 1978) were fixed for about 2 h in 2% glutaraldehyde in 0.025 M potassium phosphate buffer, pH 6.8, at room temperature, washed with buffer, postfixed in 1% osmium tetroxide for 2 h at 0 C, and subsequently dehydrated in a graded acetone series and embedded in Spurr's resin (SPURR 1969). Ultrathin sections for TEM were cut with glass knives and mounted on either uncoated 200 mesh or formvar-coated 100 mesh copper grids, stained for 30 min in saturated uranyl acetate in 50% ethanol and 5 min in 0.02% lead citrate (VENABLE and COGGESHALL 1965), and examined with a Philips EM 200 microscope.

For observation with SEM, cultures were fixed in 2% glutaraldehyde and 0.5% osmium tetroxide in distilled water at 0 C for 1–2 h, washed five times in distilled water at room temperature, placed in 1% thiocarbohydrazide for 15 min, rewashed, postfixed in 0.5% osmium tetroxide in distilled water at 0 C for 1 h, rewashed, dehydrated over 3 h in acetone, and critically point dried (KELLEY, DEKKER, and RHUNEN 1973). The preparations were mounted on stubs using a solution prepared by soaking cellotape in chloroform, coated with gold and palladium with a Techon Hummer II sputterer, and examined with an ETEC SEM.

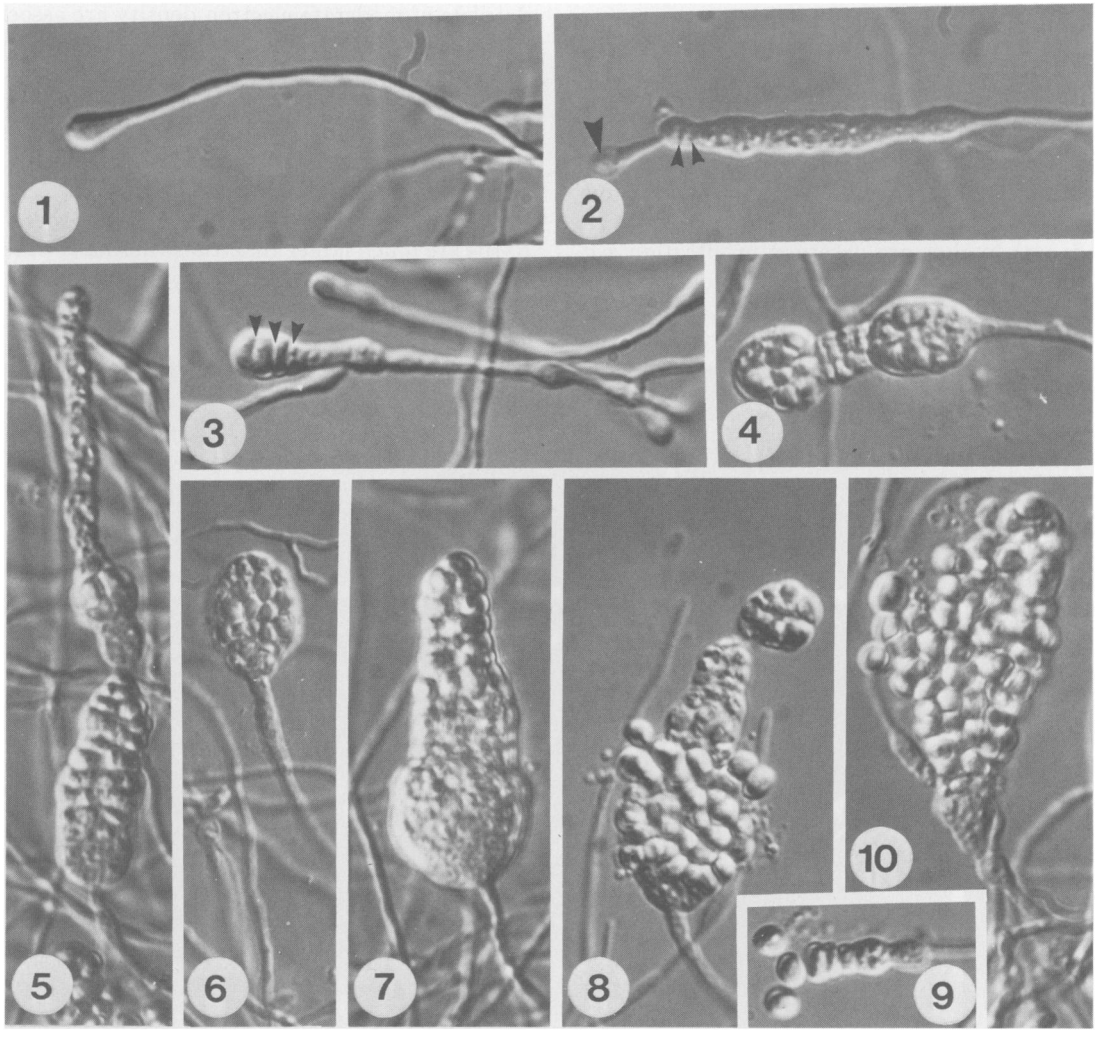
A Leitz orthoplan light microscope equipped with Nomarski interference optics was used to examine living cultures.

Observations

MORPHOGENESIS.—The cultured endophyte isolated from *Comptonia* root nodules is characterized by growing predominantly as hyphae, of which

portions may undergo morphogenesis to form sporangia containing many spores. The sporangia may develop terminally at the ends of hyphae (figs. 1–10, 19) or in intercalary positions within hyphal strands (figs. 11–18, 20, 21). When sporangia develop at the end of hyphae, the initial developmental changes involve the formation of pear-shaped (fig. 1) or spherical (fig. 32) swellings; normally the ends of a hypha are not swollen but are similar in diameter to the remainder of the hypha (fig. 8). Occasionally an elongated swollen area, an early sporangium, forms a short distance from the swollen hyphal tip (fig. 2). Intercalary sporangia develop initially by the formation of small oval swellings, occurring singularly (figs. 11, 20) or in groups (figs. 12, 21). Subsequent development of both types of sporangia involves an increase in size and the formation of transverse septa (figs. 2, 3, 14, 16, 22), followed by the growth of longitudinal septa (figs. 7–9, 17, 18, 23, 24).

The sporangia are of many different sizes and shapes. Intercalary sporangia may be oriented as branches (figs. 13, 15, 17, 18) of the parental hypha or as a radially symmetrical enlarged region (fig. 16) at the hypha. Terminally positioned sporangia may

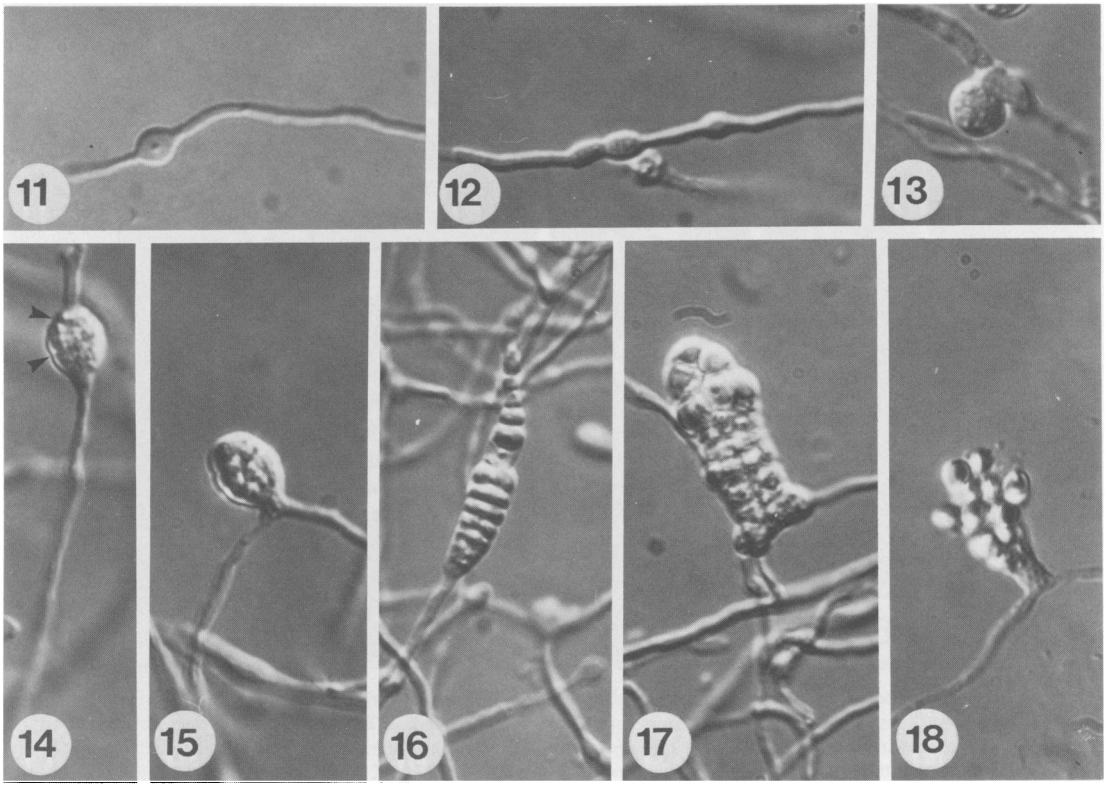


FIGS. 1–10.—Terminally positioned sporangia (living cells photographed with Nomarski interference optics). Fig. 1, Pear-shaped swelling at end of hypha is an early stage of sporangium formation; × 1,420. Fig. 2, Terminal swelling (large arrow) similar to that in fig. 1 and an elongated widened region in which two septa (small arrows) are present; × 1,490. Fig. 3, Developing sporangium contains several transverse septa (arrows); note increase in size over stage in fig. 1; × 1,420. Fig. 4, Developing sporangium, shaped like a dumbbell, has both transverse and longitudinal septa forming immature spore cells; developing spores are larger in bulb-shaped regions than in connecting portion; × 1,420. Fig. 5, Elongate developing sporangium has larger growing spores in center and near proximal end than in distal portion; × 1,420. Fig. 6, Spherically shaped sporangium with transverse and longitudinal septa; × 1,140. Fig. 7, Pear-shaped sporangium has largest sporangiospores at distal region; outer surface of sporangium is distended and has taken the shape of the underlying spores; × 1,440. Fig. 8, Pear-shaped sporangium has mature spores in central and proximal regions; spores are being released from the central region of the sporangium; × 1,450. Fig. 9, Sporangium has mature spores being released from distal end; other parts of structure have prominent transverse septa but no obvious longitudinal septa; × 1,490. Fig. 10. Sporangiospores are ready to be released from the distal end of a mature sporangium; × 1,490.

be dumbbell shaped (fig. 4), elongated (fig. 5), spherical (fig. 6), pear shaped (figs. 7, 8, 10), or straight (fig. 9). During the early stages of development the outer surface of the sporangium appears smooth, as does the surface of the connecting hypha (figs. 1, 2, 11–13, 19–22, 24). During the formation of septa and subsequent spore enlargement, the outer covering of the sporangium becomes distended and outlines the spherical shape of the underlying spores (figs. 3–7, 15–17, 22, 23). Just prior to spore dispersal the surface layer of the sporangium appears rough (fig. 23). Eventually this outer covering ruptures, resulting in the release of the spores (figs. 8–10, 18, 24).

FINE STRUCTURE.—In ultrathin sections hyphae and sporangia are cut in many planes because the microorganism grows in an anastomosing manner, forming a loosely woven mycelium; this, of course complicates the correlation of the ultrastructural observations with those obtained by Nomarski optics and SEM. Both narrow (ca. 0.5 μm) and wide (ca. 1.5 μm) hyphae are observed in ultrathin sections (fig. 26); it appears that most of the wide hyphae are early stages of nonseptate sporangium formation (figs. 1, 2, 11–13). The narrow hyphae predominate in cultured material and are characterized by being septate, branched, and having cytoplasm that is usually more electron dense than that of the wide hyphae (figs. 26, 28, 29). In addition, the narrow hyphae contain large nucleoid areas, abundant ribosomes, a plasma membrane, and two-layered cell wall (figs. 25–27).

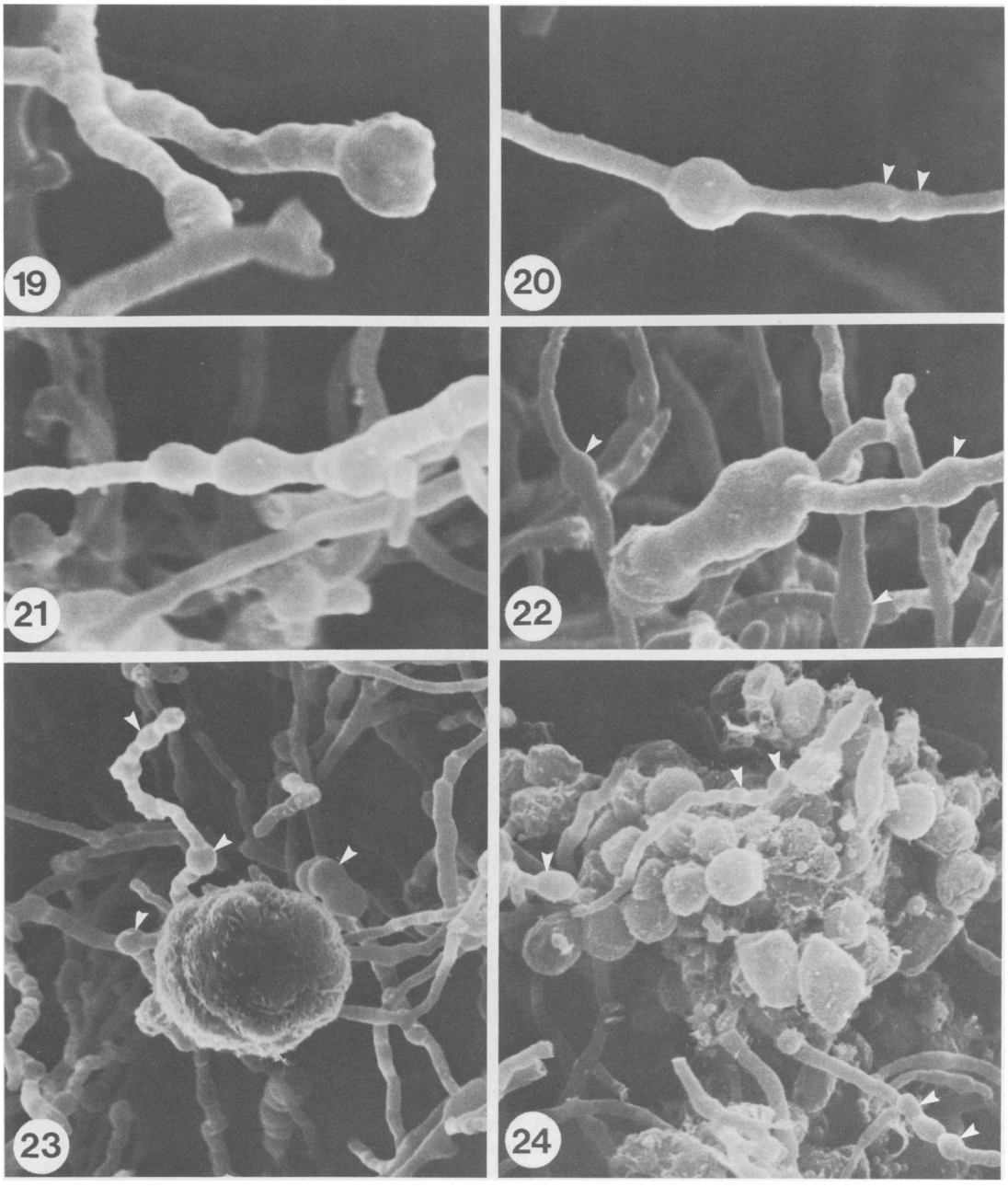
The cell wall consists of an inner, uniformly thick (ca. 270 nm) layer of low electron density; the outer layer is not continuous and consists of thin electron-dense membrane-like layers which may be several in number and arranged in stacks (fig. 25). Often droplets of moderate electron density are associated with these outer wall layers which appear eventually to be sloughed off the surface of the microorganism (fig. 25). A few hyphae do not have these outer layers and droplets. Both fine and wide fibrils are present in the nucleoid regions of the narrow hyphae; usually the fine fibrils radiate out from the centrally located wider fibrils (figs. 25–27). Since these wide fibrils are much larger than those normally observed in the nucleoid areas of prokaryotes, the possibility that they are a fixation artifact must be considered.



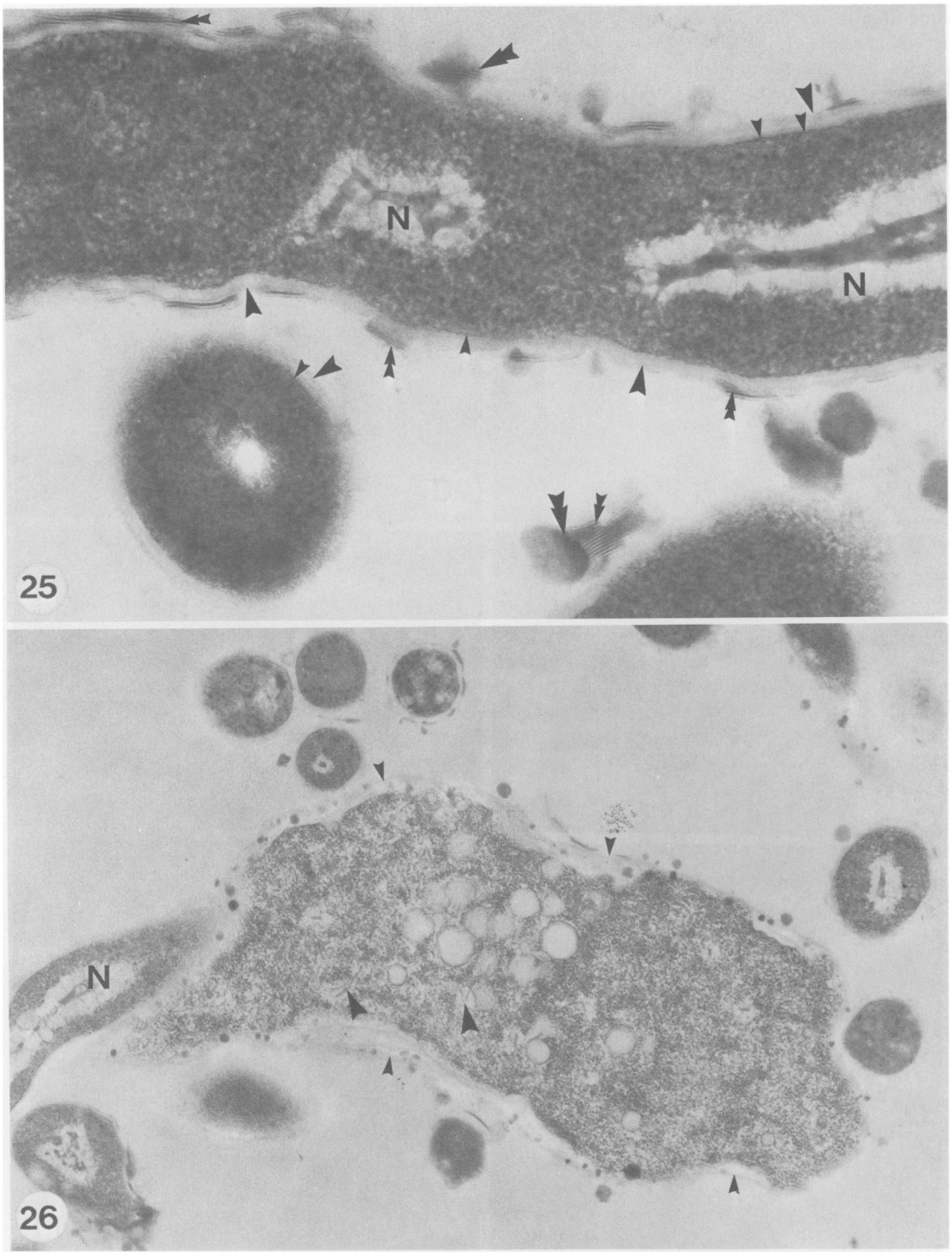
FIGS. 11–18.—Intercalary sporangia (living cells photographed with Nomarski interference optics). Fig. 11, Early stage of intercalary sporangium is a bilaterally symmetrical swelling; × 1,490. Fig. 12, Three sporangia are in an early stage of development; two sporangia are contiguous and separated from the third; × 1,480. Fig. 13, Early stage of sporangium is oriented as a branch of the hypha; × 1,420. Fig. 14, Bilaterally symmetrical sporangium contains small developing sporangiospores as evidenced by the small distension (arrow) of the surface; × 1,450. Fig. 15, Developing sporangium, oriented as a branch of the hypha, contains both longitudinal and transverse septa; × 1,470. Fig. 16, Elongate sporangium containing prominent transverse septa; × 1,470. Fig. 17, Sporangium, arranged as a branch, containing transverse and longitudinal septa and large spores at the distal end; × 1,400. Fig. 18, Sporangium, arranged as a branch, has spores ready to be released; × 1,260.

Only fine fibrils are present in the nucleoid areas of the same organism when it is located within *Comptonia* root nodules (NEWCOMB et al. 1978). No mesosomes are present in this microorganism in vitro or in vivo (NEWCOMB et al. 1978).

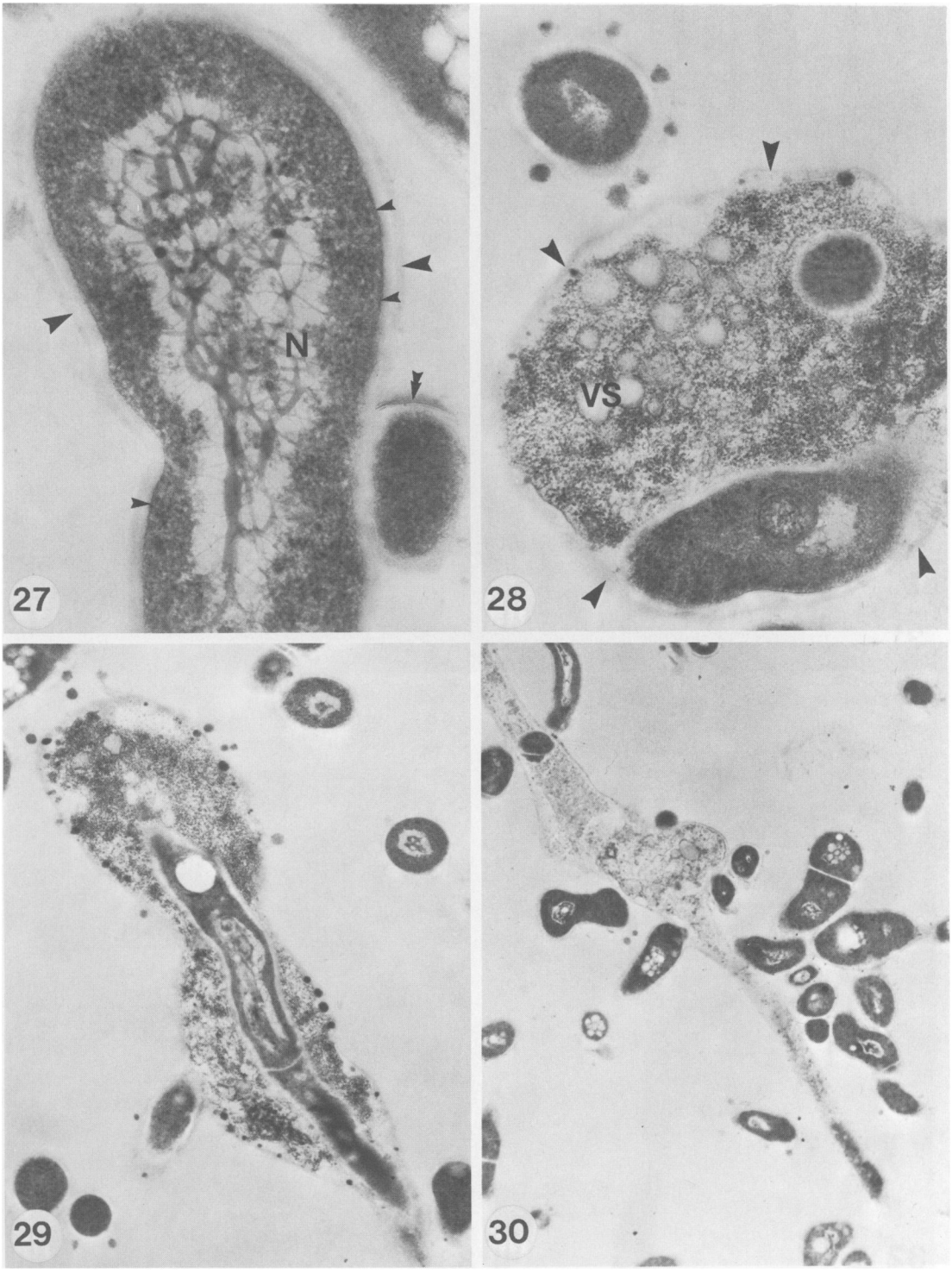
The wide hyphae are considered to be the earliest stage of sporangia formation because of their similar size to those observed by other techniques (figs. 1, 2, 11, 12); in addition, their wall structure and the numerous vesicular structures (figs. 26, 31, 32) closely resemble those observed in early septate sporangia (figs. 33–35). The electron density of the cytoplasm in early sporangia varies considerably, but even the structures having low electron densities contain an abundance of ribosomes (figs. 26, 28). The vesicular structures are only rarely observed within the narrow hyphae but are almost always present in developing sporangia or wide hyphae



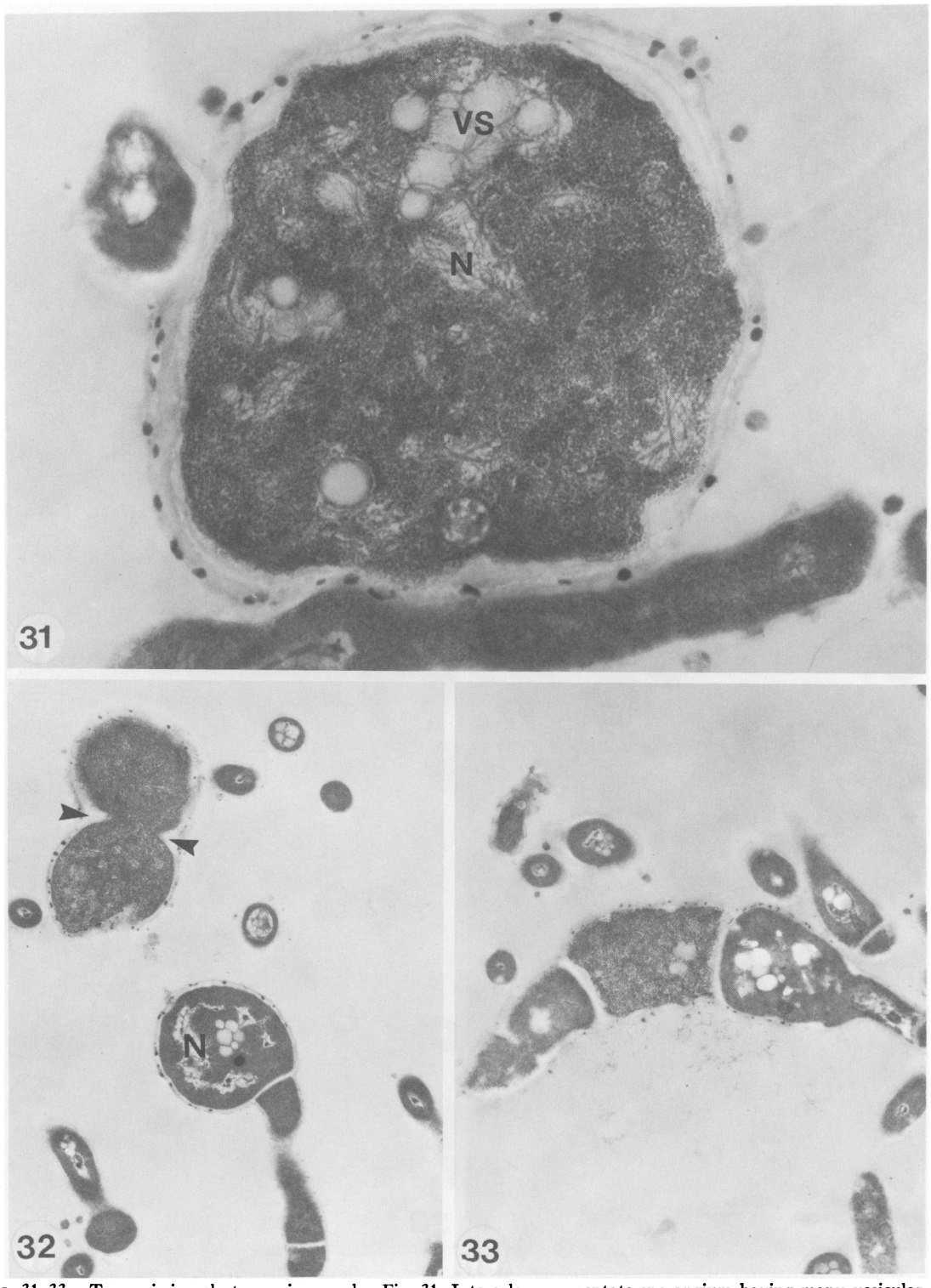
FIGS. 19–24.—Scanning electron micrographs. Fig. 19, Early stages of terminally positioned sporangia; × 8,730. Fig. 20, Early stage of intercalary sporangium; smaller swellings (arrows) may be earlier stages of sporangia; × 8,400. Fig. 21, Intercalary sporangia located close to one another; × 8,400. Fig. 22, Surface of intercalary sporangium is distended by underlying spores; small (arrows) sporangia are in an earlier stage of development; × 5,300. Fig. 23, Sporangium containing growing spores is distended and has a rough surface; sporangia (arrows) in earlier stages of development are also shown; × 4,240. Fig. 24, Sporangium whose outer covering is absent contains many spores apparently ready for release; also shown are smaller sporangia (arrows); × 4,320.



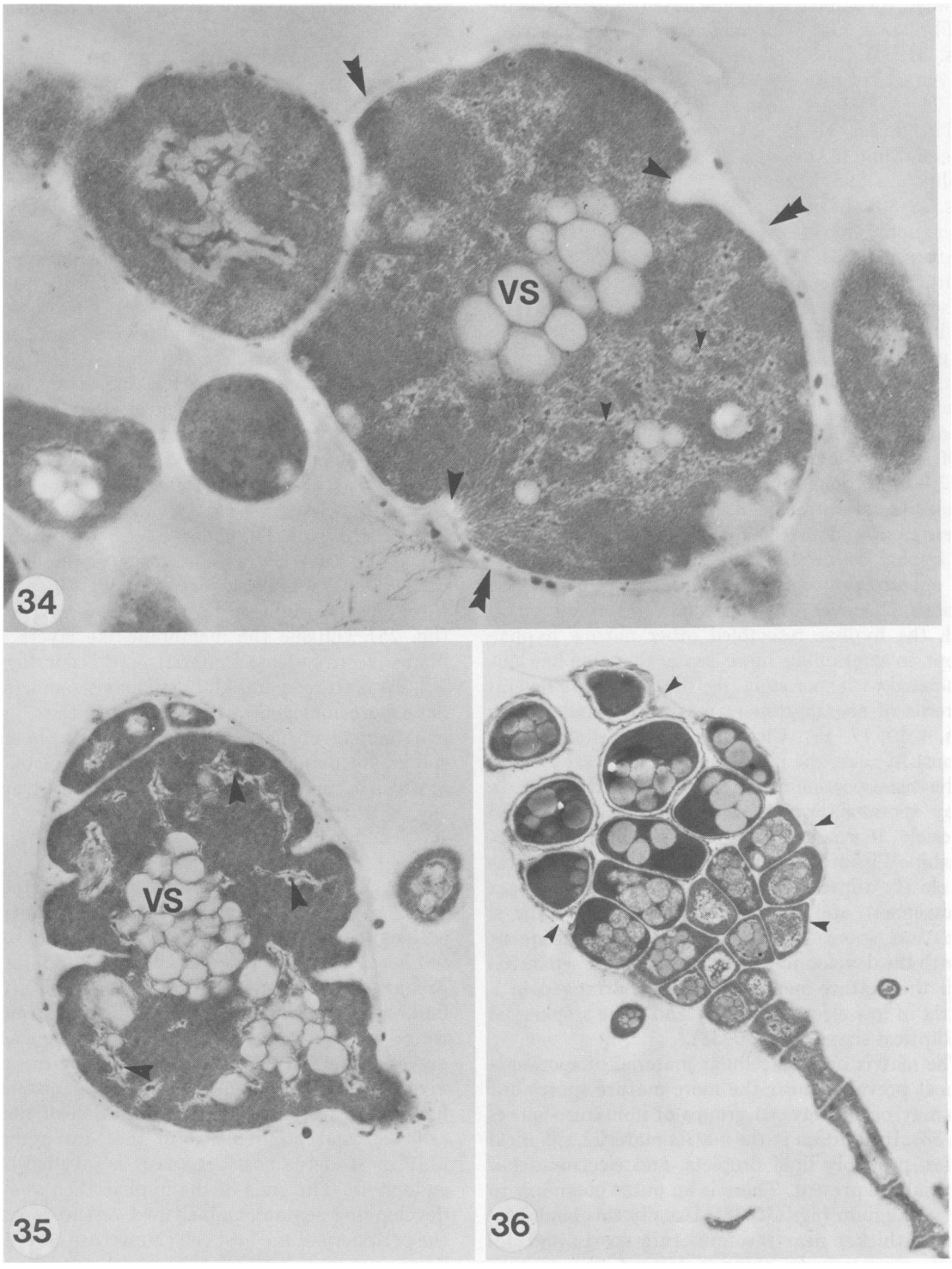
FIGS. 25–26.—Transmission electron micrographs. Fig. 25, Sections of hyphae illustrating fine and wide fibrils in nucleoid (*N*) regions, many ribosomes in cytoplasm, plasma membrane (single small arrows), cell wall (single large arrow), electron-dense layers (double small arrows) on outer surface of cell wall, and electron-dense layers of hyphal surface; some of these droplets are attached (double large arrows) to the outer electron-dense wall layers; × 73,000. Fig. 26, A wide hypha, containing many ribosomes and vesicular structures, is connected to a narrow hypha; the fibrillar regions (large arrows) of the wide region correspond to the nucleoid regions (*N*) of the narrow hypha; note the uneven thickness of the cell wall (small arrows) surrounding the wide hypha; × 26,000.



FIGS. 27–30.—Transmission electron micrographs. Fig. 27, Oblique section of hypha illustrating the fine and wide fibrils of the nucleoid (*N*) region, abundant ribosomes in the cytoplasm, plasma membrane (single small arrows), cell wall (large arrows), and outer electron-dense wall layers (double small arrows); × 56,760. Fig. 28, Wide hypha contains abundant ribosomes, many vesicular structures (*VS*), an unevenly thick cell wall (arrows), and two narrow hyphae growing within it; × 31,200. Fig. 29, Longitudinal section of narrow hypha growing within a wide hypha; × 19,690. Fig. 30, Narrow hyphae are growing in and near a senescent wide hypha; × 11,960.



FIGS. 31–33.—Transmission electron micrographs. Fig. 31, Intercalary nonseptate sporangium having many vesicular structures (*VS*), fibrils in the nucleoid region (*N*), abundant ribosomes, and thick multilayered cell wall with many osmiophilic droplets; × 34,050. Fig. 32, Terminally positioned nonseptate sporangium, many narrow hyphae, and a structure which is probably an elongate sporangium with growing septa (arrows). Fibrillar material in nucleoid regions (*N*) of terminal sporangium and the numerous electron-dense droplets in the walls of the sporangia; × 13,610. Fig. 33, Developing sporangium contains two complete septa and one incomplete septum; several holes are present in the section because this stage is difficult to infiltrate; × 11,850.



FIGS. 34–36.—Transmission electron micrographs. Fig. 34. The early sporangium has septa (single large arrows) starting to grow inward, several vesicular structures (*VS*), and fibrillar nucleoid regions (small arrows). The cell wall (double large arrows) is uniform in thickness; × 35,100. Fig. 35, Early sporangium containing complete and incomplete septa, numerous vesicular structures (*VS*), and several fibrillar nucleoid regions (arrows); × 20,480. Fig. 36, Sporangium is connected to hypha; the most mature sporangiospores are located distally and have a thicker two-layered cell wall than the less mature spores at the proximal end of the structure; the outer layer (arrows) of the sporangium follows the contours of the underlying spores; × 9,520.

(figs. 26, 28, 31, 32). The vesicular structures are bounded by a membrane and often have reticulate or fibrillar contents of moderate electron density (figs. 31–34). The wall of the wide hyphae or the early stages of sporangia have a low electron density and electron-dense droplets embedded within the wall itself (figs. 31–35). The nucleoid areas have both wide and fine fibrils (figs. 21, 32, 35). Often narrow hyphae are found growing within the wide hyphae (figs. 28–30); a similar occurrence has been noted in other actinomycetes (WILLIAMS, SHARPLES, and BRADSHAW 1973). Many of these wide hyphae appear healthy but some are clearly senescent (fig. 30). The nonseptate sporangia become compartmentalized by the inward growth of septa (figs. 34, 35). Initially the growing septa are thick, but as they transect the middle region of the developing sporangium, they become thinner (fig. 35). Cytologically these sporangia appear similar to the earlier nonseptate stages.

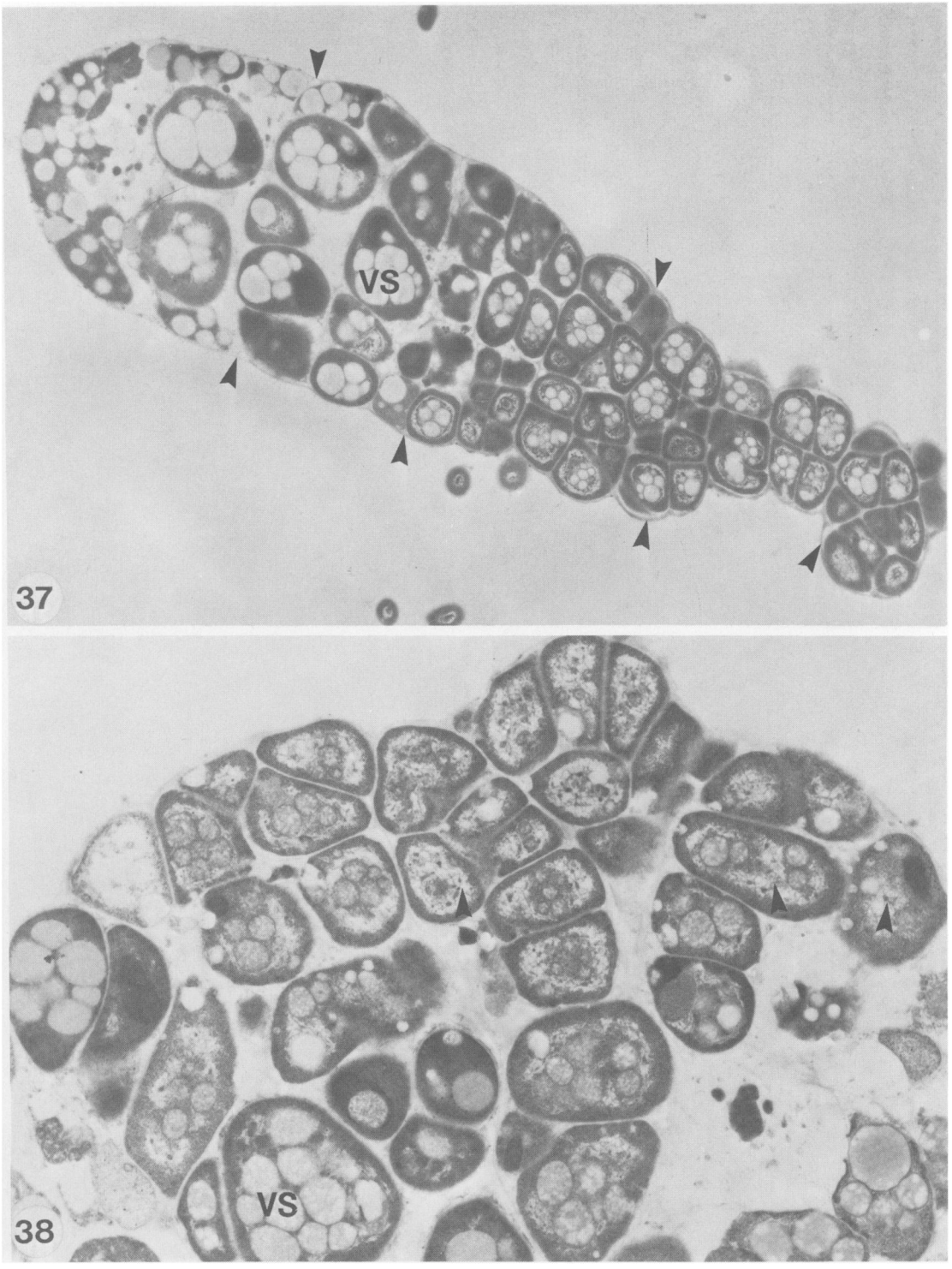
Often the sporangia containing immature spores are 30–60 μm long, making it difficult or impossible to obtain an ultrathin section through both the sporangium and the attached hyphae. As a result, the hyphal connection and the complete length of the sporangium were usually missing from these sections. However, in the hyphal connections examined, the hyphae resembled other narrow hyphae except in containing more septa than hyphae not associated with sporangia (fig. 36). There are several patterns of sporangiospore maturation and release (figs. 4–10, 17, 18). Ultrathin sections showed that in most hyphae, the most mature spores are located in the distal portion of the sporangium. However, in many sporangia which were apparently sectioned obliquely, it was not possible to establish this relationship. Within the sporangium the immature spores are closely adpressed to one another in a compact arrangement, and as a result, many of the walls of immature spores are straight. With subsequent growth the developing spores become more separated until the mature spores are loosely arranged in a matrix of low electron density and have a spherical or elliptical shape (figs. 36–38).

The matrix or extracellular material of sporangia is most prevalent near the more mature spores but also may occur between groups of immature spores (fig. 38). In addition to the matrix material, spherical bodies, probably lipid droplets, and electron-dense material are present. There is an outer boundary to the sporangium (figs. 35–38). Usually this boundary layer is thicker near the immature spores and becomes progressively thinner toward the mature spores. Presumably this layer is wall material derived from the original narrow hyphal wall which is uniformly thick (fig. 25); no plasma membrane is associated with the outer boundary of the sporangium (fig. 38). As the sporangia increase in size, this wall becomes distended, possibly because it is being stretched with limited or no additional deposition of its components. Eventually this layer ruptures and the spores are released (fig. 24).

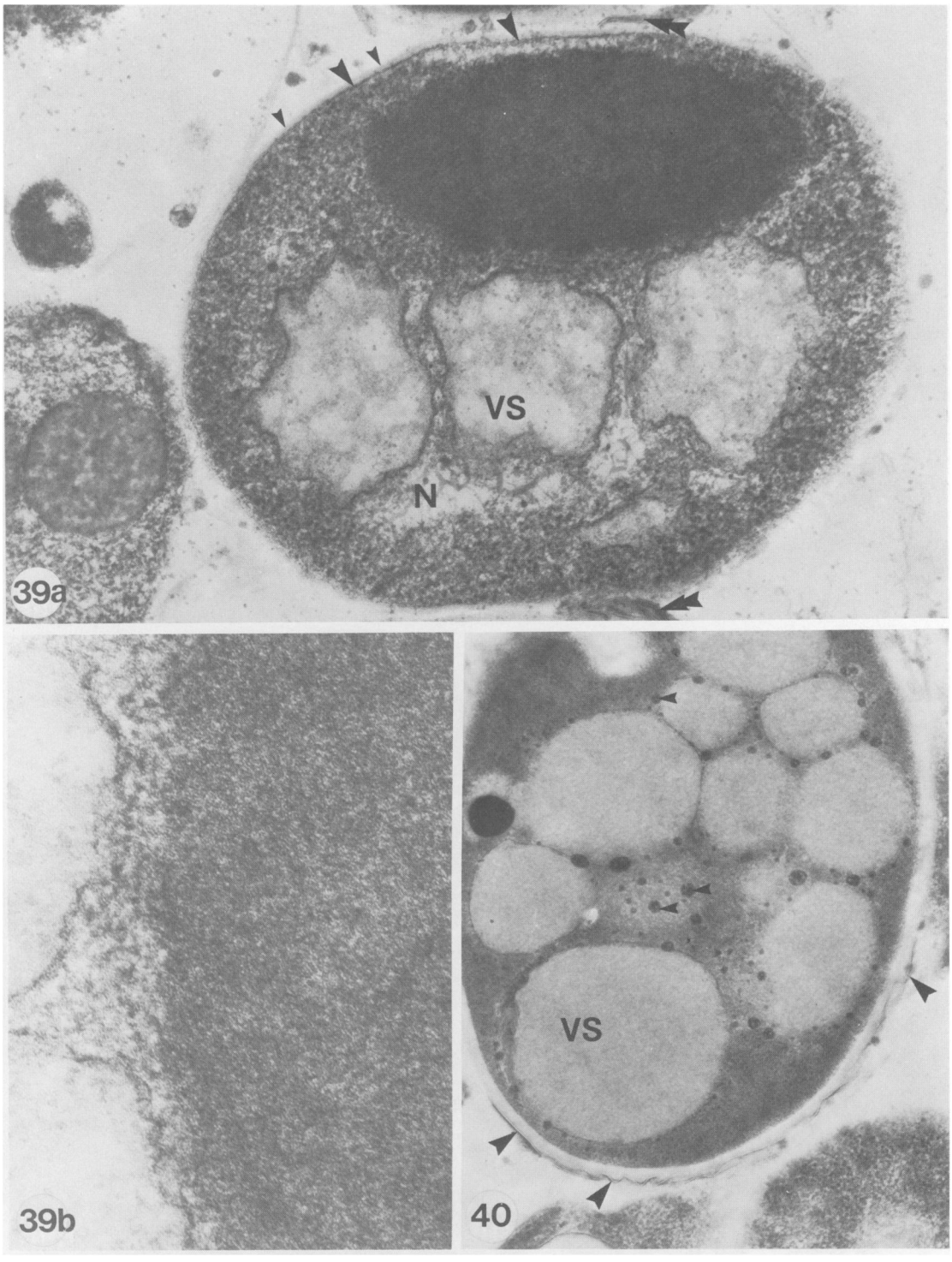
The development and maturation of the sporangiospores involve cytological changes in addition to growth. The immature spores generally have an outer layer of ribosome-rich cytoplasm surrounding a less electron-dense nucleoid area containing numerous fine and wide fibrils and vesicular structures with fibrillar and reticulate contents (figs. 37, 38). The cell walls of the immature spores are usually thin and of low electron density. More mature spores usually have smaller nucleoid areas and fewer vesicular structures (figs. 35, 37, 38). These vesicular structures are often larger and fewer in number than those found in less mature spores, suggesting that they may coalesce. The most striking change involves the formation of large electron-dense structures which have a particulate and fibrillar substructure and are not membrane bound (figs. 35, 39*a*, *b*). The cell wall is still relatively thin at this stage. Mature spores are characterized by having a thick two-layered cell wall. The inner layer is of low electron density and overlaid with one or more thin electron-dense membrane-like layers (figs. 36, 40) similar to those on the outer surface of the narrow hyphae (fig. 25). Outside the membrane-like layers amorphous electron-dense material may occur (figs. 36, 40). In mature spores the vesicular structures may have more homogeneous contents than those present in earlier stages (figs. 38, 40). Also present in mature spores are numerous electron-dense droplets, some of which occur at the edges of the vesicular structures (fig. 40).

Discussion

The cultured microorganism, which is the causal agent of *Comptonia* root nodules, has a typical actinomycetous structure (LECHEVALIER and LECHEVALIER 1967; WILLIAMS et al. 1973), that is, it is prokaryotic and grows in the form of septate, branched, gram-positive hyphal filaments, most of which are less than 1 μm in diameter. This actinomycete differs in its morphogenesis in vitro and in vivo. Within the nodule the microorganism has a hyphal morphology during penetration of the host cell wall and proliferation in the host cytoplasm until most of the host cytoplasm is invaded by the endophyte. The ends of the hyphae then swell and develop into septate, club-shaped vesicles located at the periphery of the host cell (NEWCOMB et al. 1978). Some authors believe that the vesicles are the site of nitrogenase and thus are the actual site of dinitrogen reduction (AKKERMANS 1971; ANGULO, VAN DIJK, and QUISPEL 1976; BECKING 1977; VAN STRATEN, AKKERMANS, and ROELOFSEN 1977). LALONDE (1979) reported that vesicle morphology appears to be specified by the host cell in cross inoculation. The



FIGS. 37–38.—Transmission electron micrographs. Fig. 37, Club-shaped sporangium has loosely packed mature sporangiospores at distal end and more compactly arranged immature spores at proximal end; outer layer (arrows) of sporangium appears thicker and denser at proximal end; much extracellular material, including numerous vesicular structures (*VS*) which are possibly droplets, are present in the distal region; × 9,200. Fig. 38, Immature spores are surrounded by extraspore material in a sporangium. The developing spores are tightly adpressed to one another and contain fibrillar regions (arrows), electron-dense cytoplasm, and vesicular structures (*VS*) with a reticulate matrix; × 15,700.



FIGS. 39–40.—Transmission electron micrographs. Fig. 39*a*, Maturing spore containing a large electron-dense region, vesicular structures (*VS*) with reticulate contents, a nucleoid region (*N*), numerous ribosomes in the cytoplasm, a plasma membrane (single large arrows), and a thin cell wall (single small arrows); note electron-dense layers (double large arrows) of adjacent spore (cf. fig. 25); × 56,300. Fig. 39*b*, Higher magnification of a portion of the spore in fig. 39*a*. The electron-dense region is comprised of particulates and fibrillar materials and is not membrane bound as are the vesicular structures; × 119,630. Fig. 40, Mature spore contains many electron-dense droplets (small arrows), vesicular structures (*VS*) with reticulate contents, and a thick two-layered cell wall; outer electron-dense wall layers (large arrows) are similar to those in fig. 25; × 46,900.

cultured microorganism does not form vesicles in vitro even though plants inoculated with these cultures form nodules which are capable of reducing acetylene and contain vesicles.

Conversely, the sporangia which develop in vitro have not been observed in vivo within *Comptonia* nodules (NEWCOMB et al. 1978), although sporangia have been reported in the nodules of *Alnus glutinosa* (VAN DIJK and MERKUS 1976) and various species of *Myrica*, *Hippophaë*, *Casuarina*, and other genera (TORREY 1978). It is possible that the failure to observe sporangia of *Comptonia* endophyte in vivo is related to our preferential examination of young nodules because older nodules were difficult to preserve and section.

In vivo the actinomycete is surrounded by the capsule, a layer of wall material probably pectinaceous (LALONDE and KNOWLES 1975), deposited most likely by the host cell because a large proliferation of rough endoplasmic reticulum containing a fibrillar matrix is associated with the early growth of the endophyte in newly infected cells (NEWCOMB et al. 1978). In vitro the actinomycete lacks a capsule; this provides further evidence that the synthetic activities of the host are the source of this material.

The formation of the sporangia in the cultured endophyte of *Comptonia*, as described here, and in vivo with *A. glutinosa* nodules (VAN DIJK and MERKUS 1976) is very similar. In both situations wide hyphae are compartmentalized by the formation of septa. The fine structure of the sporangium differs in some details. The electron-dense structures observed in the *Comptonia* endophyte in vitro have no counterpart in vivo in the *A. glutinosa* actinomycete. These dense structures have a fibrillar and particulate substructure remarkably similar to the fine structure of eukaryotic nucleoli; it is not suggested, however, that these structures in the actinomycete serve any similar function or even that they contain ribonucleoproteins. The biochemical nature of the dense bodies in the endophyte in vitro is unknown. VAN DIJK and MERKUS (1976) observed multilayered outer membranes on the spores in vivo of the *A. glutinosa* endophyte. The spores of the *Comptonia* endophyte have membrane-like electron-dense layers located on the outer surface of the cell walls, but these layers do not resolve as a trilamellate unit membrane. Similar noncontinuous layers are found on the surface of some hyphae, but again they are not resolvable as a unit membrane. In vivo the *Comptonia* actinomycetous hyphae contain numerous lipid droplets and glycogen particles, both of which are not common in the organism in vitro. In vitro the nucleoid areas contain fine and wide fibrils while in vivo only fine fibrils are observed. It is very likely that the greatly different cultural conditions in vivo and in vitro are reflected in the physiological activities of the organisms. In addition, the conditions for fixation also differ greatly in these two very different cultural regimes.

With a precise knowledge of the morphogenesis and structure of the *Comptonia* endophyte both in vivo and in vitro, it should now be possible to examine nodules of other genera and to develop a better understanding of infection and host-actinomycete interactions leading to nodule development. Such information may be useful in attempts to extend this symbiosis to other genera and species which at present do not bear nitrogen-fixing root nodules.

Acknowledgments

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NOTE.—We wish to call attention to the following paper on a closely related subject: LALONDE, M. 1978. Confirmation of the infectivity of a free-living actinomycete isolated from *Comptonia peregrina* (L.) Coult. root nodules by immunological and ultrastructural studies. Can. J. Bot. **56**:2621–2635.