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ROOT HAIR INFECTION IN ACTINOMYCETE-INDUCED ROOT NODULE INITIATION IN CASUARINA, MYRICA, AND COMPTONIA

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The infection process leading to the development of root nodules of *Comptonia peregrina*, *Casuarina cunninghamiana*, *Myrica gale*, and *M. cerifera* was studied by light and electron microscopy. Deformed growth of root hairs was observed as early as 24 h after seedlings grown aeroponically or hydroponically were inoculated with suspensions of crushed nodules or cultures of the actinomycetous endophyte of *Comptonia*. The extent of root hair deformation showed a positive correlation with the number of nodules which subsequently developed. The essential features of infection in each of these species were very similar. The actinomycete entered a deformed root hair of the host in a region of folding of the cell wall. A convoluted elaboration of the root hair wall which occurred at this presumptive penetration site was continuous with the more evenly deposited capsule of the endophytic actinomycete. An associated feature of this wall deposition was thickening of the cell wall of the infected root hair and the adjacent prenodular cells. The actinomycete encapsulation was thickest at the presumed site of penetration and thinner in later stages of endophytic growth away from this site. These observations suggest a period of initial disequilibrium caused by the infection, followed by more harmonious symbiotic growth. The observation of a morphologically and cytologically similar root hair infection process in these three genera indicates that root hair infection involves a specific and orderly interaction which represents the common mode of invasion in the initiation of actinomycete-induced root nodules.

Introduction

The development of actinomycete-induced nodules on the roots of certain woody dicots represents a complex series of events. Three major stages in development can be distinguished: the infection of the root hair, the induction and invasion of a prenodular proliferation of the cortex, and the induction and invasion of primary and higher-order nodule lobe primordia. The anatomical details of root nodule morphogenesis have become better understood with studies of this process in *Alnus glutinosa* (POMMER 1956; TAUBERT 1956; ANGULO CARMONA 1974), *Casuarina cunninghamiana* (TORREY 1976), *Comptonia peregrina* (BOWES, CALLAHAM, and TORREY 1977; CALLAHAM and TORREY 1977; NEWCOMB et al. 1978), and *Myrica gale* (FLETCHER 1955; TORREY and CALLAHAM 1978, 1979).

The process of root infection by the actinomycete which initiates this developmental sequence is not clearly understood. Compelling evidence by TAUBERT (1956) and ANGULO CARMONA (1974) established that the actinomycete initially enters the roots of *A. glutinosa* by penetration of a deformed root hair. LALONDE (1977) observed root hair deformation within 24 h after inoculation of the plants. All elongating root hairs appeared to be affected, and each exhibited deformed growth with further elongation occurring in branches of the original root hair axis. The result of this response was a "slope" of deformed root hairs with newly initiated root hairs distal to this region remaining short and branched. The ultrastructural studies of LALONDE (1977) showed the endophyte within a root hair to be encapsulated by host-derived wall material as the actinomycete grows toward the root hair base. The infection of *C. peregrina* roots observed in the light microscope (CALLAHAM and TORREY 1977) occurred by penetration of a root hair at a site of invagination or folding of the cell wall.

Despite these observations of the actinomycete entering the host through root hairs, there has been little understanding of how this process occurs. The direct involvement of root hair deformation in the infection process was questioned by QUISPEL (1955, 1974), who claimed his experiments showed an inability of the actinomycete to proliferate outside of host cells; thus, he eliminated the possibility that the actinomycete was responsible for root hair deformation. Conclusions drawn from these results should be reconsidered in light of new evidence that at least one of these nodule endophytes can grow outside of host tissues (CALLAHAM, DEL TREDICI, and TORREY 1978).

The observations of LALONDE (1977) of a specific "exoencapsulation" process preliminary to the root hair infection in *A. glutinosa* were based on the assumption that the nodule endophyte exhibits rod-filament pleomorphism (LALONDE, KNOWLES, and FORTIN 1975; LALONDE 1977) when growing outside as opposed to within the host cells. However, the rhizosphere bacterium discussed by LALONDE (1977) was not identified by available immunological methods (LALONDE et al. 1975) or by direct observations of root hair penetration. In his exoencapsulation theory, LALONDE did not interpret the root hair deformation as directly involved in the mechanism of penetration of the root hair wall, although he did

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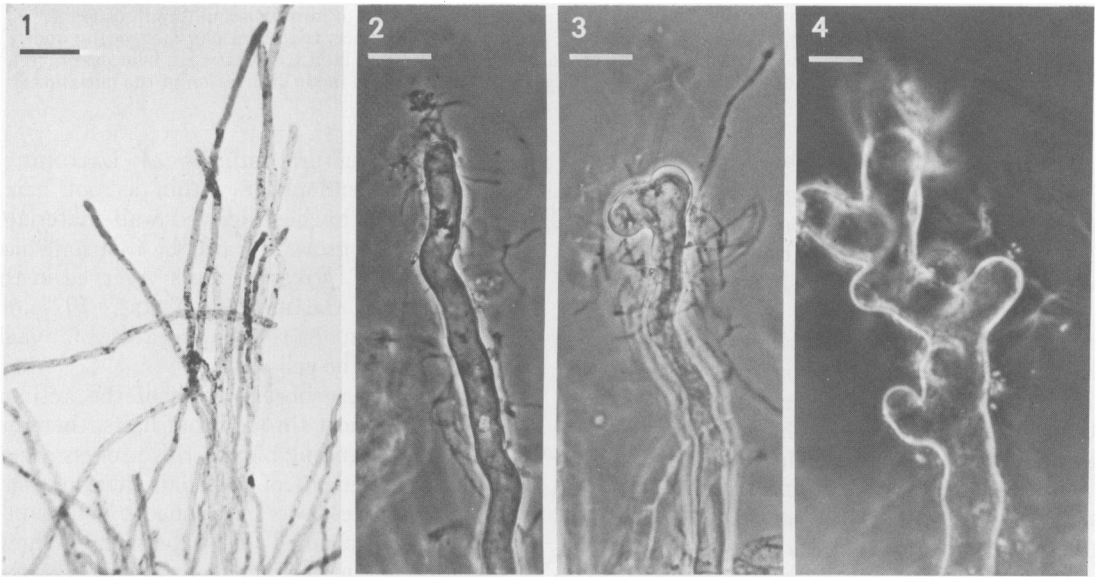
view the deformation as a plant response to the actinomycete.

There are consistent observations of root hair deformation associated with nodulation in *Alnus*, *Comptonia*, *Casuarina*, and *M. gale*. Convincing evidence for root hair infection associated with deformation was provided for *A. glutinosa* (POMMER 1956; TAUBERT 1956; ANGULO CARMONA 1974), *A. crispa* (LALONDE 1977), and *Comptonia* (CALLAHAM and TORREY 1977). The research reported here is an investigation of the infection process in the initiation of the root nodules of *M. gale*, *M. cerifera*, and *C. cunninghamiana* for which observations of the initial infection have not been recorded. Further evidence of root hair infection of *C. peregrina*, extending earlier observations (CALLAHAM and TORREY 1977), is also reported.

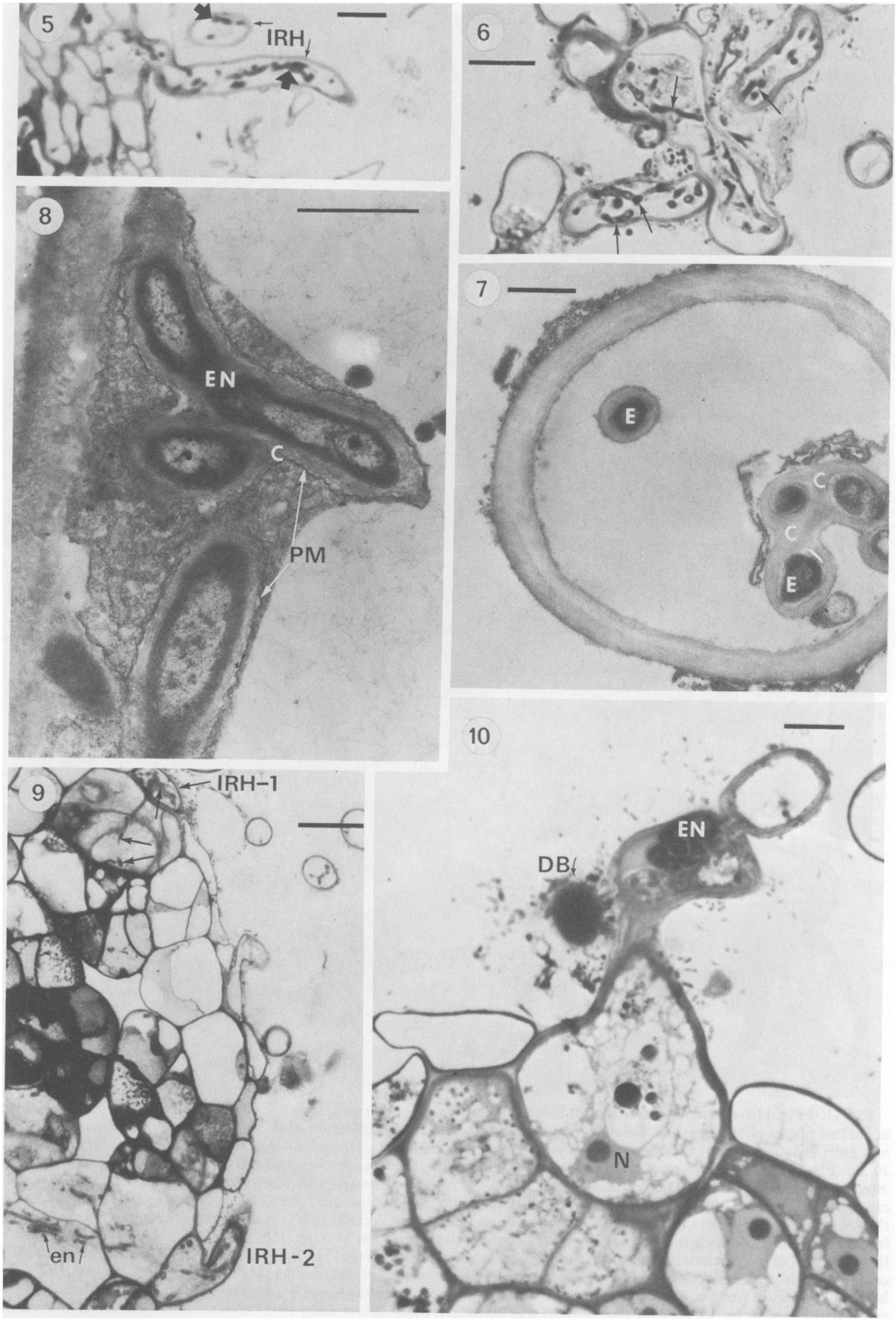
Material and methods

PLANT CULTURE.—Locally collected fruit of *Comptonia peregrina* were scarified, soaked for 24 h in 500 ppm gibberellic acid (GA_3), and germinated in flats of washed sand in the greenhouse at the Harvard Forest (DEL TREDICI and TORREY 1976). Locally collected fruit of *Myrica gale* and *M. cerifera* were treated similarly but without scarification of the fruit of *M. gale*. Seeds of *Casuarina cunninghamiana* were germinated in flats of washed sand in the greenhouse without scarification. All seedlings were transferred to aeroponic culture tanks (ZOBEL, DEL TREDICI, and TORREY 1976) when the shoots were about 3 cm high and were grown under conditions specified by CALLAHAM and TORREY (1977). Two weeks after germination, seedlings of *M. gale* were transferred to small test tube water cultures for



FIGS. 1–4.—Root hairs of *Myrica gale* from seedlings grown in water cultures. Fig. 1, Uninoculated root hairs which developed long and straight; scale = 200 μm. Fig. 2, Fully elongated root hair 24 h after application of CI inoculum; fragments of the inoculum are entwined about the root hair which still exhibited active streaming but failed to branch; phase contrast; scale = 50 μm. Fig. 3, Elongating root hair 24 h after application of CI inoculum, showing branching and continued growth from several points; phase contrast; scale = 50 μm. Fig. 4, Deformed root hair 24 h after inoculation with the CI; branching can be quite extensive; CI filaments are not associated with each branch point; anoptral phase contrast; scale = 20 μm.

FIGS. 5–10.—Fig. 5, Section of infected root hair (*IRH*) of *Casuarina cunninghamiana* cut longitudinally; the endophyte within the root hair (arrows) was traceable to the infected nodule cortex in other sections; scale = 10 μm. Fig. 6, Section through the highly branched distal part of the infected root hair of *Casuarina* in fig. 5; the filamentous endophyte (arrows) is present throughout this lobed root hair tip; scale = 10 μm. Fig. 7, Transmission electron micrograph (TEM) of a section of the *Casuarina* root hair cut adjacent to the section in fig. 6; the endophyte (*E*) within the root hair is encapsulated by a layer (*c*) which joins several filaments at the lower right into a large strand; scale = 1 μm. Fig. 8, TEM of the endophyte (*EN*) within an infected root hair of *Comptonia peregrina*. The hyphae of the endophyte are encapsulated within a fibrillar encapsulation material (*c*) which is continuous with the root hair wall as in later stages; the host plasmalemma (*PM*) separates the encapsulated hyphae from the host cytoplasm; scale = 1 μm. Fig. 9, Cross section of the root of *Comptonia peregrina* at the level of the prenodule which shows two root hair infections (*IRH*-1 and *IRH*-2) which have occurred after inoculation with cultures of the highly invasive *Comptonia* endophyte. The infecting hyphae (*en*) from *IRH*-2 penetrate deeply into the prenodule (infected cortex) while the infecting hyphae of *IRH*-1 (arrows) are localized to a few cells just below the root hair; scale = 25 μm. Fig. 10, Light micrograph of an infected root hair of *M. gale*, cut longitudinally; the endophyte (*EN*) has entered the root hair wall and appears closely associated with a densely staining body (*DB*) of segmented or particulate substructure just adjacent to the invasion site; the root hair nucleus (*N*) is at the root hair base and is not closely associated with hyphal growth. Note the thickness of the root hair wall relative to the adjacent uninfected epidermal cells; scale = 10 μm.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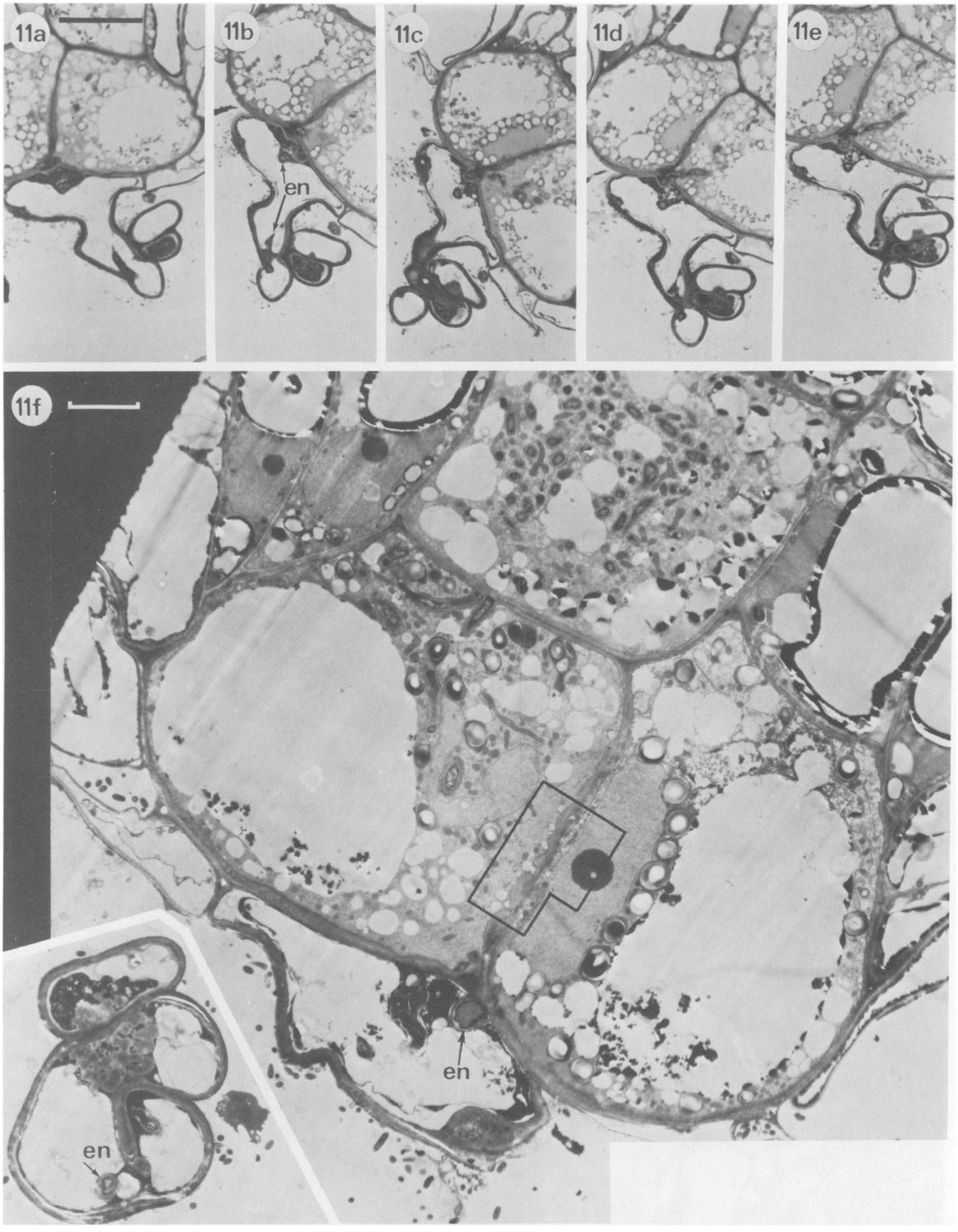

FIG. 11.—Fig. 11*a–e*, A series of sections cut through an infected root hair of *M. gale* demonstrating the different images obtained in various planes of sections; the distal portion containing convoluted wall material is continuous with the proximal portion of root hair; endophyte (*en*) hyphae are present in the proximal and distal portions and pass into the adjacent cortical cell; bar = 20 μm. Fig. 11*f*, TEM montage of the distal and proximal portions of the same infected root hair and adjacent infected and uninfected cortical cells in fig. 11*a–e;* the distal portion of the root hair contains convoluted elaborations of wall material and encapsulated endophyte (*en*); in the proximal portion the encapsulated hyphae are continuous with the host cell wall; compare the thickness of the encapsulating material in the root hair and in infected cortical cells; the cell wall between the infected and uninfected cells contiguous with the root hair shows sculptured thickenings; bar = 5 μm. Fig. 11*g*, High magnification of a portion of the distal part of the root hair in fig. 11*f;* the outer layers (arrows) of the cell wall are electron dense; the convoluted wall material is continuous with the host cell wall and contains many randomly arranged electron-dense fibrils; the folded portion (*FW*) of the host wall and a transection of an encapsulated hyphae (*en*) are also shown; bar = 2 μm. Fig. 11*h*, High magnification of an encapsulated hypha in fig. 11*f* showing that the host wall material is comprised of many electron-dense fibrils embedded in a less dense matrix; bar = 1 μm. Fig. 11*i*, High magnification of the common cell wall between the infected and uninfected cells continuous with the infected root hair in fig. 11*f;* many polyribosomes and profiles of rough endoplasmic reticulum are present near the sculptured cell walls of the infected cell; bar = 1 μm.

studies of root hair deformation and the time course of infection by the "*Comptonia* Isolate."

INOCULATION.—At about 1 wk after transfer to aeroponic culture, the plants were inoculated with crushed nodule suspension (BOND, FLETCHER, and FERGUSON 1954) from soil-grown or aeroponically grown nodules of the host plant, either by brushing the inoculum onto the root system in *M. gale* or by pouring it into the growth medium. Some observations were of plants of *Comptonia* and *M. gale* inoculated with a crushed, washed suspension of the cultured actinomycete isolated from *Comptonia* nodules, that is, the "*Comptonia* Isolate" or "CI" (CALLAHAM et al. 1978). This microorganism forms nodules on *Comptonia* with much higher frequency than crushed nodule suspensions and produces effective nitrogen-fixing nodules on *M. gale*, *M. cerifera*, and *Comptonia*.

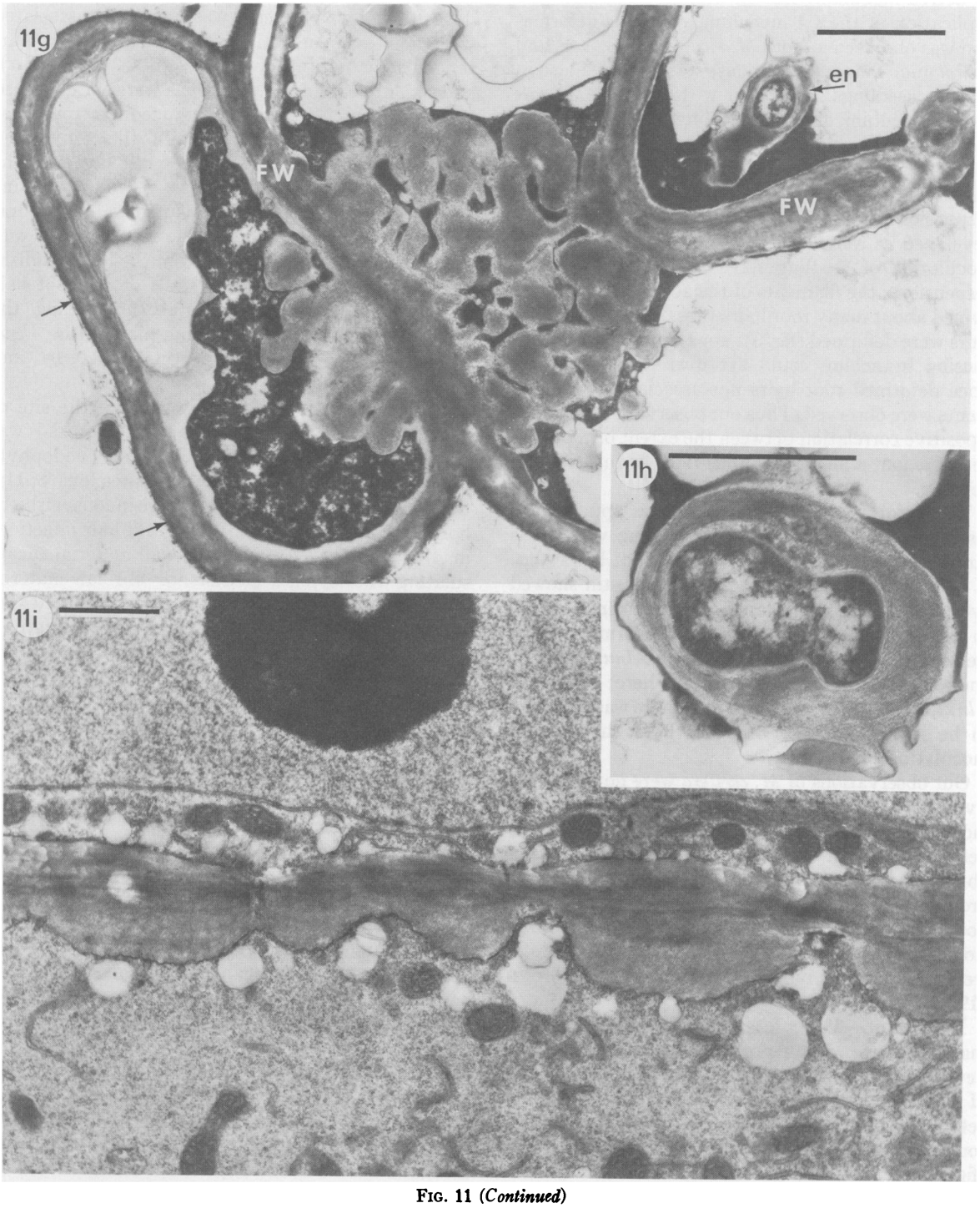

FIG. 11 (*Continued*)

MICROSCOPY.—Tissues were prepared for light microscopy and transmission electron microscopy as described by CALLAHAM and TORREY (1977) and NEWCOMB et al. (1978).

Observations

ROOT HAIR DEFORMATION.—All plants in either aeroponic or hydroponic culture developed long, straight root hairs if the roots were not exposed to an active inoculum (fig. 1). Within 24 h after the application of the CI inoculum, root hair deformation was observed in both *Myrica gale* (figs. 3, 4) and *Comptonia*. This rapid response was observed also in *M. gale* seedlings inoculated with crushed *M. gale* nodule inoculum, and such seedlings developed many root nodules within 8 days after inoculation. *Comptonia*, however, formed few deformed root hairs when inoculated with *Comptonia* crushed nodule suspensions, and very few nodules subsequently developed (CALLAHAM and TORREY 1977). After inoculation of seedling roots of *M. gale* with CI suspensions, the filaments of the actinomycete intertwined about many root hairs (figs. 2–4). Elongating hairs were deformed (fig. 3); apparently the stimulus causing branching could act over short distances since deformed root hairs not associated with filaments were observed. Thus our observations indicate a positive correlation between the extent of root hair deformation and the numbers of nodules which develop.

The deformed growth of root hairs appeared to result from branching and wall folding which was limited to young and still elongating root hairs (figs. 3, 4). Fully elongated root hairs which still showed cytoplasmic streaming failed to branch in response to the inoculum (fig. 2). Root hair deformation was well described by LALONDE (1977) for *Alnus* seedlings and was not significantly different here; just as in *Alnus*, a "slope" of deformed root hairs was observed to be related to root hair length at the time of inoculation.

Our observations of *M. cerifera* were not as extensive as for *Comptonia* and *M. gale*. *Myrica cerifera* inoculated with CI suspensions showed root hair deformation and effective nodules developed in abundance. When roots of *M. cerifera* seedlings grown in aeroponics were inoculated with a crushed nodule suspension from *M. gale*, root hair deformation occurred, but many small ineffective nodules were produced, as reported by GARDNER and BOND (1966).

INFECTION.—Using later stages of the same plant material, very young prenodular stages of nodule development were examined to determine the site of infection leading to nodule initiation. Actinomycete invasion of the root occurred exclusively by root hair infection in *Casuarina cunninghamiana* (figs. 5–7), *M. gale* (figs. 10–12), *M. cerifera* (inoculated with nodule suspensions of *M. gale*, not illustrated), and *Comptonia peregrina* (figs. 8, 9). In every plant infected root hairs were deformed, and the initial entry of the actinomycete was always traceable to a crook or sharply folded region of the root hair (figs. 11*f*, 12*a*). At such a site, the lobes of the deformed root hair come together at a point filled with dense polysaccharide deposits in which filaments may be embedded (figs. 11*a–g*, 12*a*). The presumed site of penetration is within such a folded root hair. In *M. gale* and *Comptonia* a pronounced deposition of wall material was frequently associated with the site of penetration. This wall material was arranged in elaborate convolutions (figs. 11*f*, *g*, 12*a*, *c*), and sometimes endophyte hyphae were encapsulated with it (fig. 12*c*). Randomly arranged electron-dense fibrillar material was present in the convoluted structures (fig. 12*c*) and in the outer layers of the root hair cell wall (figs. 11*g*, 12*a*, *c*). With toluidine blue O staining, both the convoluted wall configurations and the outer layers of the root hair cell wall appeared dark blue, differing from the light reddish-blue of the typical host cell walls of the root hair and the encapsulating material surrounding the actinomycete. The encapsulating material in *Alnus* is believed to consist of pectins (LALONDE and KNOWLES 1975).

Within the root hair and away from the site of penetration, the deposition of wall material was restricted to the capsule surrounding the endophyte hyphae (figs. 11*f*, *g*, 12*a–c*); in some sites (figs. 8, 11*f*) the encapsulating material was continuous with the host cell wall, as was observed in root hair infections in *Alnus* (LALONDE 1977). The wall material encapsulating the hyphae consists of fibrillar material arranged less randomly than in the convoluted wall material (cf. figs. 11*g*, *h*, 12*b*, *c*) and appears to be a more orderly continuation of the initial wall deposition. This stage may represent a period of equilibration in the establishment of an association between the host and endophyte; during this period the host and endophyte may not be in complete harmony. Further evidence favoring this interpretation is provided by the even thinner encapsulation of the endophyte in subsequently infected cortical cells (fig. 11*f*) and the pronounced thickening of the cell wall in the infected cell contiguous with the infected root hair cell but not in the adjacent uninfected cortical cell (fig. 11*f*, *i*). Because most of the infected root hairs were senescent, few observations were made on the organelles of these cells. One interesting finding, however, was the localization of the nucleus at the base of the infected root hair (fig. 10), apparently not associated with the growth of the penetrating endophyte. This is in contrast to the situation in infected root hairs of leguminous root nodules (DART 1977) and in *Alnus* (LALONDE 1977).

It is not clear how many root hair infections may be associated with a single root nodule. In earlier studies with *Comptonia*, which were conducted under

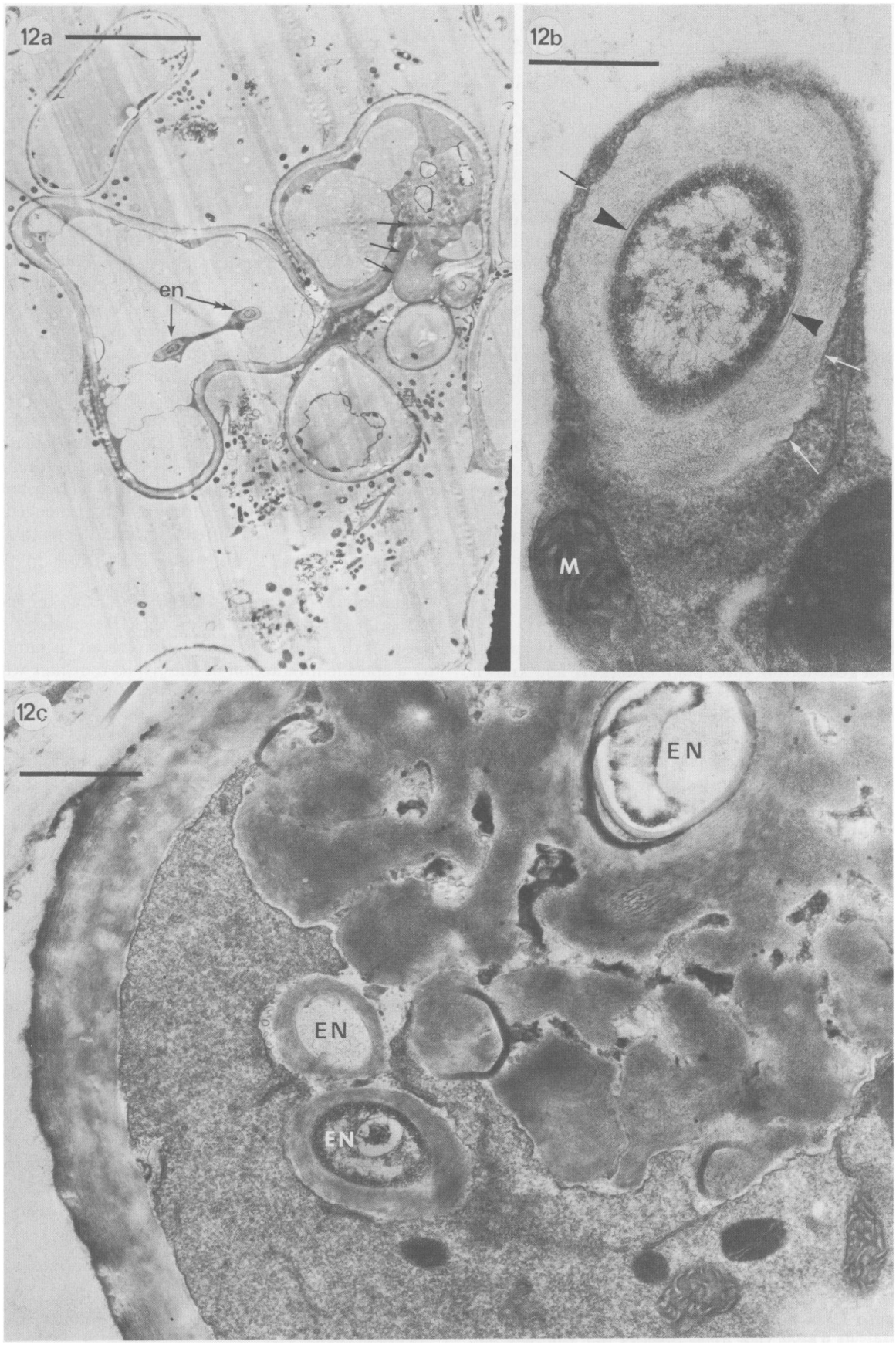

FIG. 12.—Fig. 12*a*, TEM of a deformed, infected root hair and contiguous cortical cells of *M. gale*, showing transection of encapsulated endophyte hyphae (*en*), the fold (arrows) which is the presumptive site of endophyte penetration, and the nearby mass of convoluted wall material; bar = 10 μm. Fig. 12*b*, High magnification of encapsulated hypha indicated by double arrows in fig. 12*a;* the wall material is fibrillar and is bounded by the host plasma membrane (small arrows) and the endophyte cell wall (large arrows); bar = 0.5 μm. Fig. 12*c*, High magnification of the convoluted material from an adjacent section to that illustrated in fig. 12*a;* many electron-dense fibrils are present in the convoluted material and in the outer layers of the cell wall; several sections of encapsulated endophyte hyphae (*EN*) are also shown; bar = 1 μm.

inoculum-limiting conditions and therefore only led to the establishment of few root nodules, only one root hair infection was associated with each nodule (CALLAHAM and TORREY 1977). In other plants, such as *M. gale* inoculated with a nodule suspension (TORREY and CALLAHAM 1979) or *Comptonia* seedlings inoculated with cultures of *Comptonia* endophyte, numerous root nodules were formed and root hair infections were sometimes observed close together but radially separated even within the same 1 μm thick section (fig. 9). One of these discrete infections could be traced to the infected cells of the prenodule and nodule lobes, while the second infection was limited to the root hair or outer cortical parenchyma. Since these observations are of fixed materials, it is not known if one infection occurred later than the other or was halted in development by an influence from the adjacent infection (LALONDE 1977). It seems clear that single infections are sufficient for nodule initiation and this is most likely the situation occurring in the field. The situation, however, may be more complex and endophytes from two sources might be present within one nodule.

Discussion

Including the present report, a total of five species in four genera from three plant orders are now known to be infected by this method, which appears to involve an orderly interaction of the actinomycete with the root hair cell. The repeated observations of root hair infection in the initiation of actinomycete-induced root nodules provide strong support for the view that this is the common mode of association establishment in this type of symbiosis. The sequence of events in the infection of each of these plants seems to be the same: a deformation of the hair elicited by substances produced by the microorganism, followed by penetration of the root hair cell wall accompanied by extensive formation of wall-like material by the host cell, including encapsulation of the endophyte filaments within the invaded cells.

The actual processes by which the root hairs become deformed and the actinomycete penetrates the cell wall are not known. Evidence here indicates that in *Comptonia* and *Myrica gale* the deformation is related to inoculation with active inocula which can include pure washed cultures of the *Comptonia* nodule actinomycete, in which case no extraneous materials are present. Such evidence argues in favor of a direct role of the actinomycete in causing the deformed root hair growth as well as nodulation under these conditions.

Root hair infections by actinomycetes appear analogous in certain ways to the infection process in legume-*Rhizobium* association, since they involve both a deformation of young growing root hairs attributable to the presence of activities of the microsymbiont in the rhizosphere (QUISPEL 1974) and also the penetration of the root hair in a folded region of the wall (NAPOLI and HUBBELL 1975; DART 1977). In root nodule initiation in legumes a role has been suggested for root hair–*Rhizobium* interactions in determination of the host-microsymbiont specificity through specific binding (BOHLOOL and SCHMIDT 1974; BHUVANESWARI, PUEPPKE, and BAUER 1977) or infection thread formation (LI and HUBBELL 1969). A common feature of both actinomycete and *Rhizobium* root hair infections is the penetration at a folded site of the root hair cell wall which in both cases may be involved in a localization of biochemical interactions so as to intensify their effect and aid wall penetration (HUNTER and ELKAN 1975; NAPOLI and HUBBELL 1975).

The model proposed by LALONDE (1977) for root hair interactions and penetration in *Alnus* nodulation suggests that an "exoencapsulation thread" is formed by a specific interaction of the *Alnus* root hair with a bacterial form of the actinomycete and that this structure may function in penetration of the root hair wall by concentrating hydrolytic enzymes produced by the invading bacteria. Our studies reported here have not produced evidence of such a mechanism in these plants. Preliminary studies now in progress indicate that the "*Comptonia* Isolate" exists in the hyphal form in the rhizosphere and that such hyphal forms invade deformed root hairs from within the fold, as suggested by the evidence presented here. Further work is needed to understand the process in detail.

Acknowledgments

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