INITIATION AND DEVELOPMENT OF ROOT NODULES OF CASUARINA (CASUARINACEAE)[1]

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ABSTRACT

Roots of seedlings of the "beefwood" tree, Casuarina cunninghamiana Miq. grown in nitrogen-free nutrient solution were inoculated with a suspension prepared from crushed root nodules taken from mature plants. Marked deformation of root hairs was evident but no infection threads were observed in root hairs. The mode of infection remains undetermined. Root nodules were initiated within three weeks and thereafter numerous upward-growing nodule roots developed from each nodule. Nodules in this symbiotic nitrogen-fixing plant resulted from an infection caused by an unidentified actinomycete-like soil microorganism. Anatomical analysis of nodule formation showed that nodules are the result of repeated endogenous lateral root initiations, one placed upon another in a complexly branched and truncated root system. The endophyte-infected cortical tissues derived from successive root primordia form the swollen nodular mass. Nodule roots develop from nodule lobes after escaping from the initial inhibitory effects of the endophyte. Included is a discussion of the anatomical similarities between nodules of Casuarina which produce nodule roots and those of Alnus which form coralloid nodules usually lacking nodule roots.

CASUARINA (Casuarinaceae) is one of the genera of non-leguminous angiosperms whose species are capable of forming root nodules in response to invasion from the soil by an actinomycete-like organism. The genus comprises some 45 species, most of them trees inhabiting the Old World tropics and Australia, but present as introductions in many other areas, including Florida and California. Evidence that the nodules are nitrogen-fixing has been provided by Aldrich-Blake (1932), Mowry (1933) and Bond (1957a, b).

The root nodules of Casuarina are of complex morphology, comparable to those of Alnus, Myrica and several other genera. From a single infection point in a root there can be formed eventually a spherical mass several cm in diameter composed of close-packed, radiating nodule lobes. In Casuarina, as well as in Myrica and Comptonia, fairly normal, uninfected roots ("nodule-roots") emerge from the tips of the swollen nodule lobes.

The initiation, development and structure of nodules on roots of nitrogen-fixing non-leguminous angiosperms have been reviewed by Bond

(1963, 1967) and Becking (1968, 1970a). Nodule formation among these plants has been studied most carefully in species of Alnus. Relatively little detailed information is available concerning the process of nodulation in the genus Casuarina. Miehe (1918) described the general structure of the nodules of Casuarina equisetifolia, and noted its resemblance to that of the nodules of Alnus. Microscopic study of the branching process in the nodule lobes led him to conclude that branching was initiated in the apex by a segmentation of the meristem, though he included no illustrations to support this view. Miehe failed to observe nodule roots in his material. McLuckie (1923), unaware of Miehe's work, made a similar general study of the nodules of C. cunninghamiana. Although he observed thread-like endophytic structures in newly infected cells, he believed that they were bacteria present in an infection thread. McLuckie paid particular attention to the method of branching of the nodule lobe, and to the manner in which the nodule-roots arose. He reported that after the apical meristem of the nodule lobe had produced a certain amount of elongation, it divided into several distinct groups of cells, commonly four in his material. Of these, a central group produced the nodule-root, whereas the others gave rise to new nodule lobes. He described the branching as due to meristem bifurcation. As will be mentioned later, the branching of the nodule lobes in other genera, e.g., Alnus, has been interpreted by various authors as the result of an apical process of di- or polychotomy, though not on the basis of any rigorous study of the phenomenon.
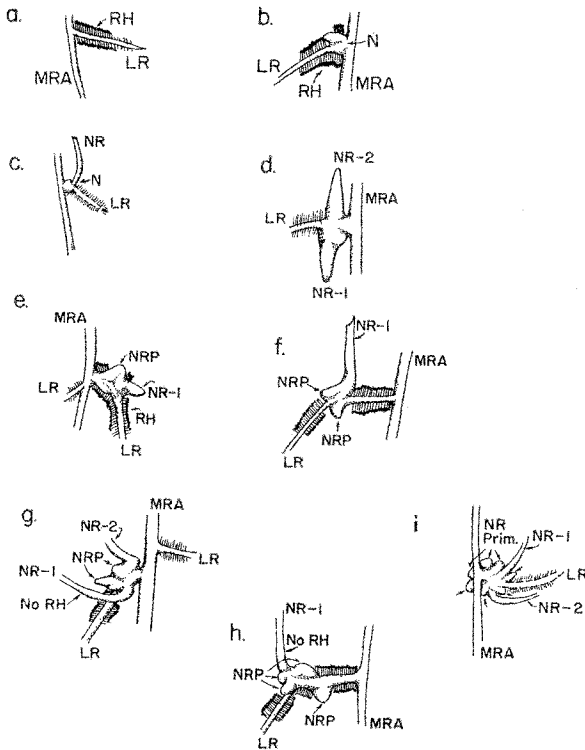
Fig. 1a–i. Drawings from living specimens showing stages in the initiation and development of nodules of seedling roots of *Casuarina cunninghamiana* grown in water culture. RH, root hairs; LR, lateral root; MRA, main root axis; N, nodule; NR, nodule root; NRP, nodule root primordium. Stages included range from the early infection (a) up to 6 wk after infection (i). Stages (b) and (c) represent nodules about 24 days after inoculation.

Fig. 2a–b. Diagrams of nodule initiation and development. a. Diagrammatic representation of the sequential development of a nodule and nodule roots. Abbreviations are given under the legend for Fig. 1. The attachment sites for lateral roots are spread apart for clarity. Stippling represents internal vascular strands of the main root and lateral root branches. b. Diagrammatic representation of a newly initiated nodule, showing a transection of a lateral root (LR), the basal infected tissue with the first nodule root (NR1) growing out through it and the primordium of the second nodule root (NRP) both seen in longitudinal section. Compare with Fig. 18.

When plants of *Casuarina* and *Myrica* were grown under conditions of water culture, where the nodule-roots are particularly prominent, it was observed that they grew in an upwards direction (Bond, 1952, 1956, 1971), and the study of this phenomenon in *Myrica gale* indicated that of the various external factors which could have induced this unusual direction of growth, gravity was in fact responsible. Silver, Bendana and Powell (1966) suggested an explanation for this
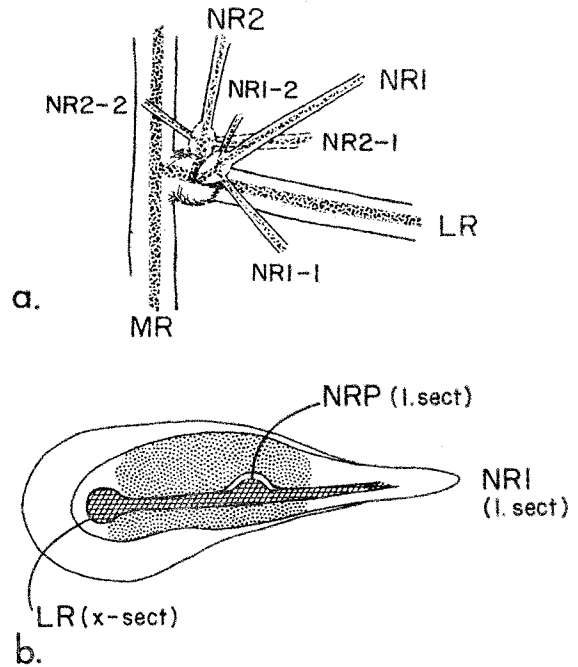
negative geotropism on the basis of their auxin studies in the nodule-roots of *Casuarina* and *Myrica*.

The objectives of the present study were as follows: a) to find the method by which the *Casuarina* endophyte, provisionally named *Frankia casuarinae* by Becking (1970b), infects the host root, and b) to study nodule initiation and determine the morphological processes by which the repeated branching of the nodule lobes and the

Fig. 3–11. Nodules and root hairs on seedling roots of *Casuarina cunninghamiana*. **3.** A young nodule attached to the base of a lateral root off from the main root axis. Note the nine vertically upright nodule roots which originate from the root nodule. Normal lateral roots grow downward. × 2. **4.** An older nodule of a plant grown in water culture, showing the swollen nodule and numerous nodule roots exhibiting negative geotropism. × 2.—Fig. 5–7. Root hairs on uninoculated seedling roots. Note that all of these root hairs are straight. They show different cytoplasmic contents varying from large spherical organelles to diffuse, finely glandular cytoplasm. All stages occur on the same lateral root. × 1600. **8.** Swollen and branched root hairs on a lateral root inoculated with nodule suspension when root hairs had just begun to develop. × 400.—Fig. 9–11. Branched root hairs on lateral roots of seedlings inoculated with nodule suspension when root hairs were partially developed. Note cytoplasm is similar to that in root hairs of untreated roots. No infection threads were observed in any branched root hair. × 400.
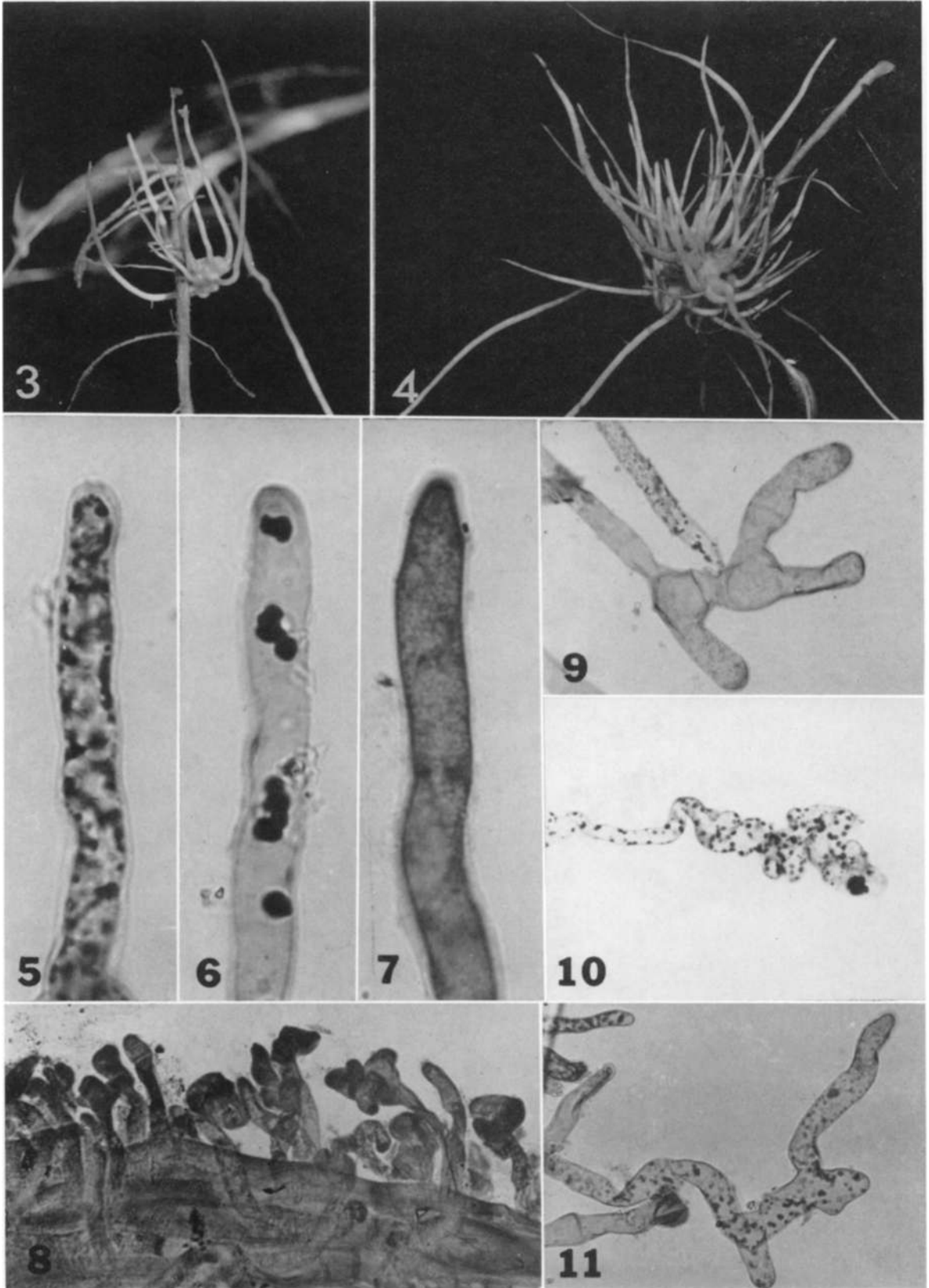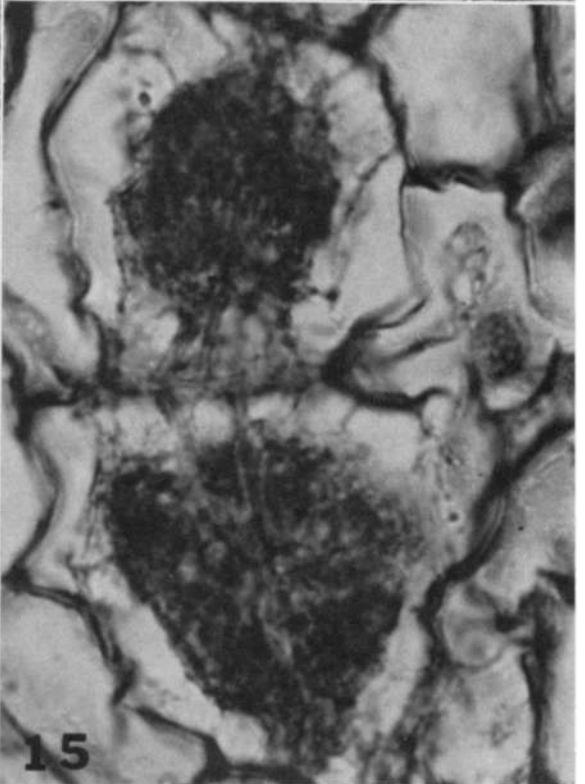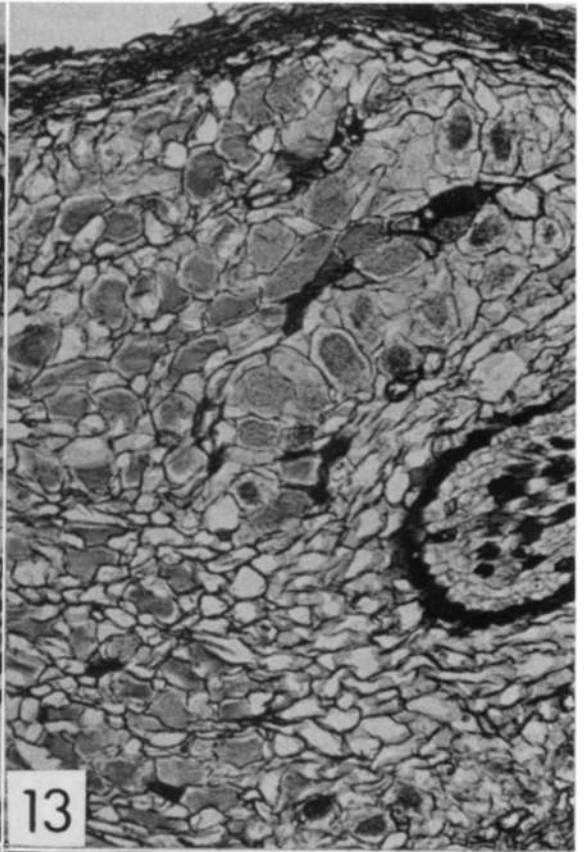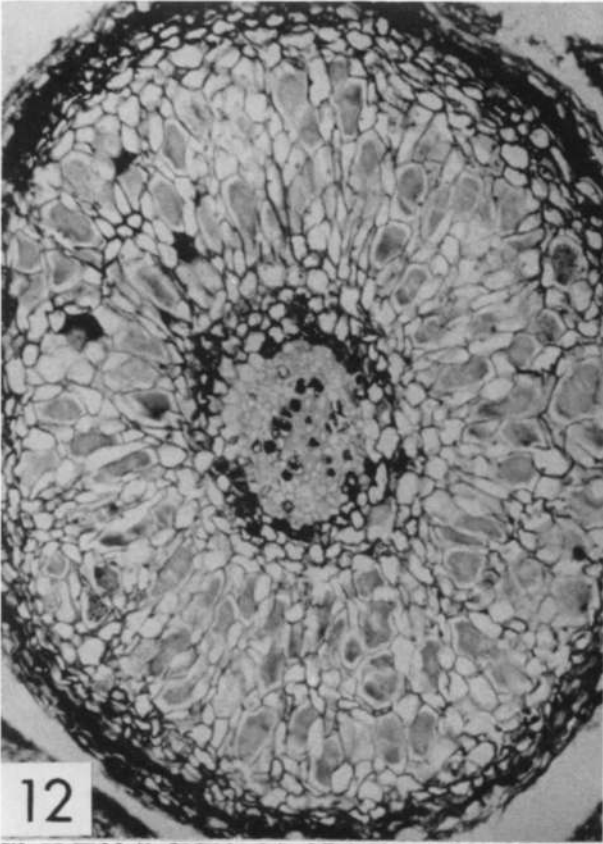
production of nodule-roots occur. In the analysis of nodule formation in *Casuarina*, earlier studies on *Myrica gale* by Fletcher (1955) provided a useful basis for comparison, as did anatomical studies of nodule formation in *Alnus* made by Pommer (1956), Taubert (1956) and Becking (1970a).

MATERIALS AND METHODS—Seeds of *Casuarina cunninghamiana* Miq. were germinated in perlite watered with ⅓-strength Crone solution. Two-month-old seedlings were transferred to water culture containers with ⅓-strength Crone solution lacking nitrogen at *p*H 5.4. Plants were grown either in the greenhouse with supplementary incandescent light or in a growth chamber at 20 C with 16 hr of fluorescent light per day. The modified Crone solution (Bond, 1951) was prepared as follows (in grams per liter of distilled water): 0.75 KCl; 0.5 $CaSO_4 \cdot 2H_2O$; 0.5 $MgSO_4 \cdot 7H_2O$; 0.25 $Ca_3(PO_4)_2$; 0.25 $Fe_3(PO_4)_2 \cdot 8H_2O$. Trace elements based on Hoagland's A to Z solution were also supplied.

Inoculation of the seedling root system was performed as follows: Nodules from mature plants of *Casuarina* maintained in water culture were excised, placed in a small volume of distilled water (1 g of nodule material per 20 g water), and ground with a mortar and pestle to a fine suspension. The suspension was brushed freely onto the whole seedling root system, covering the main axis and the lateral branches. Uninoculated plants produced no nodules. Inoculated seedlings first showed nodules in about three weeks.

At intervals after inoculation, whole root systems were fixed in different fixatives with vacuum aspiration and stored at 4 C. Fixatives included: 45% acetic acid; glacial acetic acid : absolute ethyl alcohol (1:3) for 18 hr, then transfer to 70% ethyl alcohol; or 3% glutaraldehyde in sodium cacodylate buffer at *p*H 7.2. For root hair preparations, root samples were hydrolyzed in 1N HCl at 60 C for 15 min after acetic-alcohol fixation, then squashed in 45% acetic acid, rinsed and stained with 0.01% azure B in *p*H 4.5 citrate buffer solution. Some material for anatomical study was fixed in Bouin's fluid, embedded in paraffin, sectioned at 6 μm, and stained with tannic acid, iron alum, safranin and orange G. Other roots were fixed in glutaraldehyde, embedded in Araldite, sectioned at 2 μm, and stained with toluidine blue 0 in borax buffer. I am indebted to William Newcomb for sections of nodules embedded in resin.

OBSERVATIONS—*Root hair deformation and the initial infection*—*Casuarina* seedling roots grown in water culture show an abundance of root hairs on the newly formed lateral roots and along the primary root axis near the root apices. The root hairs are smooth, straight and single-celled (Fig. 1a, 5–7), increasing progressively in length from near the apex proximally until they attain maturity. In contrast, seedling roots exposed to the suspension of crushed *Casuarina* nodules show regions of strikingly deformed root hairs. Newly developing root hairs near the root apices become curled or club-shaped at an early stage in their development and fail to elongate normally (Fig. 8). Root hairs already having begun to elongate show candelabra-like branching and terminal deformations (Fig. 9–11). Thus the normal conical tip-growth of the root hairs becomes distorted and reminiscent of root hairs treated with extracellular polysaccharide from *Rhizobium* extracts (Hubbell, 1970). Root hairs which have completed their elongation are unaffected by the nodule extract. Under the specific conditions of water culture used in the present experiments, root hair deformation was especially localized at the base of young lateral roots and on the main axis near the apex, that is, in regions of new root growth.

The locus of nodule initiation—On seedling roots treated in water culture, every nodule was initiated in association with a lateral root. The initial infection site was most typically located at the base of the lateral root near its attachment to the main root axis (Fig. 1b, c). Some nodules were observed a few millimeters along the lateral root from the base of the lateral root attachment. These initiation points were always sites of markedly deformed root hairs. Despite careful microscopic study of hundreds of fixed and stained root hairs from these sites, no deformed root hairs which contained an infection thread were observed. Since only one infection point would be

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Fig. 12–15. Photomicrographs of transections of root nodules of *Casuarina cunninghamiana* embedded in paraffin. **12.** Section cut through the swollen base of a nodule root, showing extensive infection of cortical cells by the filamentous endophyte. Heavily suberized layer of the endodermis limiting the inner cortex is apparent as well as the several suberized cell layers of the outer cortex and epidermis. × 160. **13.** An oblique section at the base of a nodule root, showing successive stages in the cortical infection from the center of the photograph toward the upper right. × 160. **14.** A transection of the lateral root showing two attachments of nodule root branches with cortical infection surrounding all the central vascular strands. The endophyte was excluded from the central cylinder, presumably by the endodermis with its black tannin deposits, and from the innermost and outermost cell layers of the cortex. × 160. **15.** Enlarged view of cortical cells showing massive occupation of cells by the endophyte mycelia and cell-to-cell connections of the filaments, presumably via plasmodesmatal connection. × 1600.
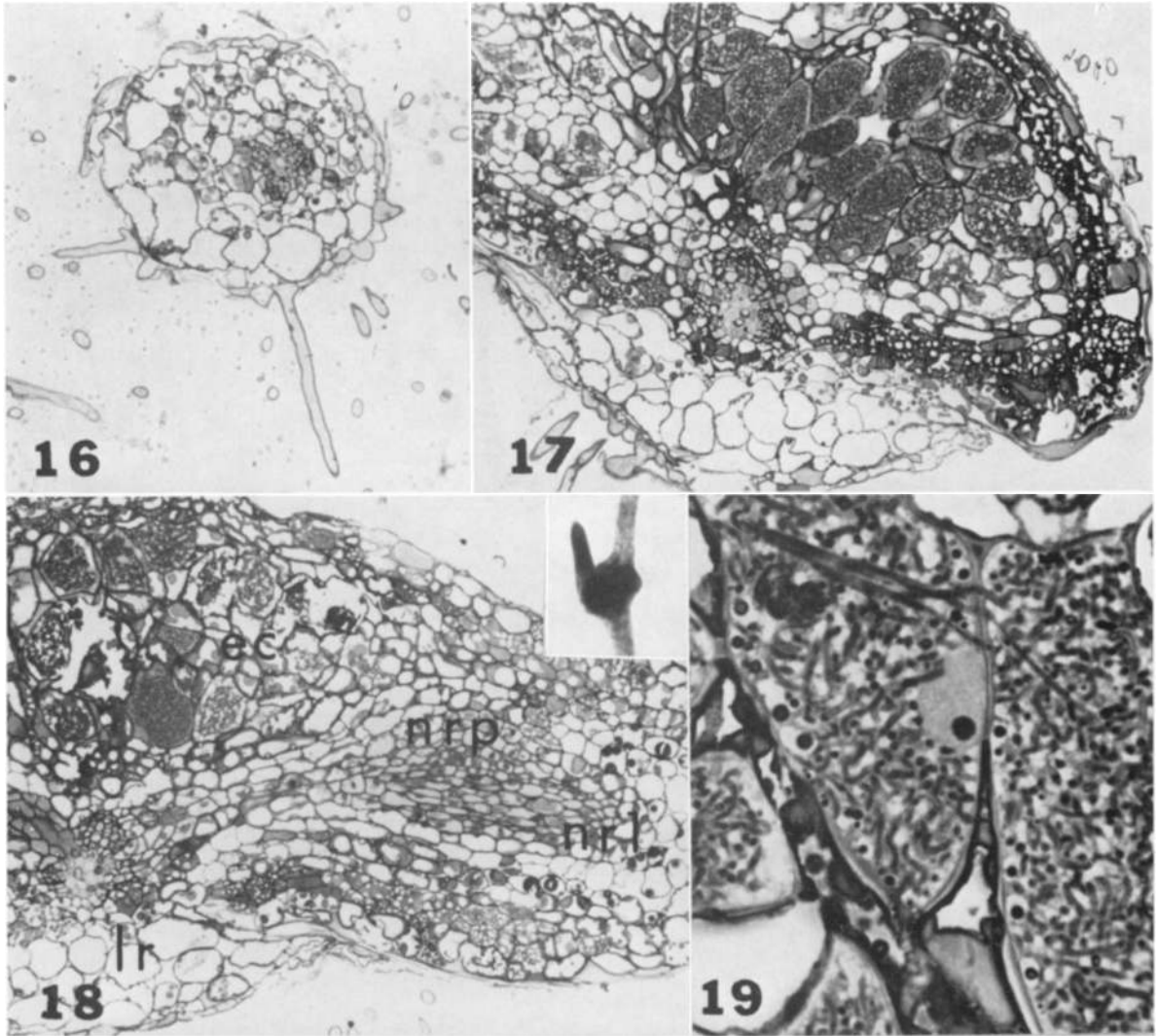
Fig. 16–19. Transections of plastic-embedded roots showing stages of early nodule initiation in *Casuarina*. **16.** Transection of a small lateral root showing the central vascular tissues, enlarged cortical cells and root hairs. No infection is evident. × 192. **17.** Transection through a newly infected lateral root showing the vascular cylinder of the lateral root, the swollen endophyte-containing cortical cells, and the tannin-filled cell layer surrounding the infected nodular tissue. × 192. **18.** Transection of a lateral root (LR) cut at the level of the first-formed nodule tissue showing endophyte-containing cortical cells (EC). The first nodule root (NR1) seen in off-median longitudinal section projects to the right having vascular connection to the lateral root. Within about 200 μm of its attachment to the lateral root, the first nodule root shows the primordium of the second nodule root (NRP). See diagram of similar stage in Fig. 2b. × 192. **18.** (Inset). Whole mount of young nodule before preparation of sections shown in Fig. 17, 18 and 19. Compare with Fig. 1c. × 20. **19.** Enlarged view of transection of infected, densely packed cortical cells of early formed nodule tissue. Note nucleus, thickened endophyte strands passing between two adjacent cells and vesicle-like structures. × 1200.

necessary for each nodule, it is statistically difficult to observe such an infection thread among hundreds of hairs. If infection is via a root hair in *Casuarina* it is a rare event and probably does not involve multiple infection threads for each nodule formed.

Initiation of the nodule—In the earliest stages of nodule initiation the endophyte was observed in cortical cells of the lateral root in the region of club-shaped root hairs. Some cortical cell proliferation appears to occur in association with this early infection and also the infected cortical cells enlarge considerably so that a swelling or protuberance develops (Fig. 17). When examined in the seedling stage, this region of the lateral root typically appears bright red in color externally, presumably due to anthocyanin pigmentation.

The initiation of nodule roots—Very soon after the development of the swollen, pigmented zone forming the young nodule, one or two conical, nonpigmented primordia become apparent in the nodule (Fig. 1c, d and inset Fig. 18). These are the first external signs of the nodule roots. The initiation of nodule roots occurs endogenously, in proximity to the infection tissue (Fig. 18). Initiation is in the pericycle opposite the protoxylem points in tetrarch roots or, in diarch roots, at an angle of forty-five degrees from the line made by the primary xylem tissue. Thus, nodule roots arise in the fashion of lateral roots. They develop, however, into the midst of infected cortical tissue. Although their rate of elongation has not been determined, it is apparent that their elongation is arrested early and the young primordia increase in size by cell division and enlargement within the infected cortical tissues of the parent root. Each primordium is partially encased initially in a tannin-filled endodermal cell layer so that the tissues of the primordium remain free of the infective endophyte.

After nodule formation has begun, one can observe at any given time thereafter several primordial nodule roots in a temporarily arrested state (Fig. 1e–i). In section, each primordium is a squat, rounded structure with compact tissues internally free of endophyte, surrounded by enlarged, endophyte-filled cortical cells and outer tannin-filled and suberized cell layers of a cork-like tissue. Some time after initiation, the arrested lateral root primordium begins to elongate rapidly, typically growing upward (Fig. 3, 4, 18 and inset). The first two nodule roots from a nodule site frequently develop as a pair (Fig. 1d). In sections of the earliest stages of nodule formation one can see the swollen infection site in cross sections of a lateral (compare Fig. 16 and 17), the first nodule root formed as a modified lateral branch of the lateral with vascular connection to the lateral and the primordium of the next nodule root just developing (Fig. 18).

At the base of each nodule root, new nodule-root primordia form within a few hundred micrometers of the earlier branch point and the same developmental sequence is repeated. New nodule tissue is thus derived from the basal cortical and outer tissues of each new nodule root and such tissues allow the continued invasion of the endophyte via the cortex. New nodule root primordia in turn form in pairs or multiples at the base of each nodule root by initiation within the pericycle. The new primordia always arise endogenously, free of endophyte, develop through infected cortical tissue and finally escape and elongate as nodule roots. Infected cortical tissue is restricted to the base of each nodule root. The early sequence is shown diagrammatically in Fig. 2b. In older nodules, a transection may cut across a mature vascular cylinder of the branching system (Fig. 14) in a region of nodule root attachment, showing the oblique attachment of nodule roots at their bases.

At the macroscopic level, swollen, red-pigmented root nodules were first observed approximately 19 days after the application of crushed nodule tissue to the roots growing in water culture. By 24 days after treatment, these swollen regions had produced several elongating nodule roots and thereafter new nodules were most easily discovered by the appearance of the white upward-growing nodule roots. By actual count in one experiment, nodules were observed with five nodule roots at 24 days, as many as 10 nodule roots from a single nodule by 6 wk (Fig. 3) and as many as 30–40 nodule roots from a single nodule site by 3 months (Fig. 4). Nodule roots elongate to 5–6 cm and then stop growing. Nodule roots are white when grown in water culture, usually showing no root hairs. The apical structure of the nodule root is modified as compared to a typical lateral root. From long-term cultures, it seems apparent that such nodule roots eventually deteriorate and a progression of new nodule roots occurs, presumably on a seasonal basis. Thus the nodule is perennial, built up largely of the infected cortical root tissues and internal vascular system of many successions of nodule roots.

Technically, the nodule has no meristem of its own but is perpetuated by the continuous succession of newly initiated lateral root primordia, one placed upon another in a complex, truncated branched root system. Order is retained by the succession of primordial root initiations, each group of new initials occurring as secondary branches upon earlier root structures, but retaining structural communication via a common central vascular tissue system (see Fig. 2a). In some respects the *Casuarina* root nodule can be likened to a "witch's broom" on a shoot system, having perhaps more order imposed by the succession of lateral root initiation. One is tempted to interpret new nodule root initiation in terms of normal lateral root initiation. At the outset of nodule formation it appears as if the infection site served as a physiological decapitation of the lateral root on which the nodule is to develop, since the lateral root distal to the infection site ceases elongation and thereafter atrophies or functions weakly. As each nodule root escapes from the endophyte-containing tissue, new nodule-root primordia are initiated at the base of the existing nodule roots (Fig. 18). Each new origin is close upon existing nodule root structures so that a very compact and condensed branch system develops. The internal vascularization gives witness to the past succession of repeated endogenous branchings. Diagrammatic representations of the early stages of this process are shown in Fig. 2a, b.

The endophyte is restricted to the cortical tissues by unknown internal barriers. Presumably,

a densely-filled, tannin-containing endodermal layer perpetuated in continuity with each new primordium formation serves as the barrier to endophyte-entry into the central cylinder or into newly-formed root primordia (Fig. 13). Restrictions of the endophyte to specific cell layers within the root cortex must depend on other perhaps more subtle physiological barriers.

Nodule proliferation—Nodule proliferation involves two separate stages. An initial phase probably involves proliferation of cortex and pericyclic derivatives forming the asymmetry of the lateral root at the early stage of nodule initiation (Fig. 17). Thereafter, with the initiation of nodule-root meristems in the pericycle, new tissue is derived from meristematic activity which is basically nodule-root proliferation (Fig. 18). Most tissues of the nodule are derived from endophyte-free tissue of pericyclic origin and can be thought of as the product of lateral root meristem activity.

Once the endophyte has begun to occupy cortical cells of the newly initiated nodule root, one can see in section a progression of cortical cells increasingly occupied and filled with a filamentous endophyte mass. The nucleus in the cell is displaced to the side and pressed against the cell wall (Fig. 19). The endophyte mycelium later occupies most of the cell (Fig. 17, 19) and the normal cytological features of the cell disappear, although the nucleus persists quite late in this sequence.

The endophyte apparently hydrolyzes the primary cell wall of the host and invades one cortical cell from an adjacent cell, first in thin tubular threads, perhaps through plasmodesmata, and then via more massive filamentous bridges (Fig. 15, 19). Finally, the cell wall disappears between adjacent occupied cortical cells so that in section two or three or more radially aligned cortical cells appear as one large cavity occupied by the endophyte (see radially elongate elements in the cortex in Fig. 12). The endophyte usually is restricted to the middle cortical layers between one or two uninfected cortical layers bordering the endodermis internally and several endophyte-free outer, corky layers. From the anatomical studies, it would appear that invasion involves penetration of the apical transverse walls as well as tangential walls of cortical cells.

After infected cortical cells are occupied completely by the endophyte, one can distinguish small, dense, rounded bodies on some filaments which take up stain more intensely and are about twice the diameter of the filament. These vesicle-like structures represent the only evident differentiation within the filamentous structure of the endophyte (Fig. 19). Because the filaments and the vesicle-like structures are very small, ultrastructural studies will be necessary to define them more precisely.

DISCUSSION—The infection of roots by actinomycetes and the initiation of root nodules in non-leguminous symbiotic nitrogen-fixing plants need further study. Analysis would be markedly simplified if one knew specifically the nature of the infective agent and could handle the organism in pure culture. It is only in *Alnus glutinosa* that the method of infection has been closely studied. Pommer (1956), Taubert (1956) and Angulo Carmona (1974) have illustrated hyphae of actinomycete dimensions within deformed root hairs in that species and have evidence that the hyphae proceed thence into the root cortex. Thus, it can be accepted that in *Alnus glutinosa* this is one method of endophyte entry from the soil. Although root hair deformation occurs generally on *Casuarina* roots exposed to the presence of nodule suspensions and is lacking in uninoculated plants, no unequivocal evidence of infection threads was obtained despite extensive study of root hairs fixed at various stages after inoculation up until the period when macroscopic nodules were visible. At the time the first swollen cortical structure of the young nodule was apparent, infected cortical cells were already present.

It is possible that infection occurs through a break in the root epidermis and cortex caused by lateral root emergence. As noted, however, in a few cases the nodule site was several millimeters from the base of the lateral root. Alternatively, infection might occur directly through the root epidermis. Since no direct observations were made of infection, it is not possible to decide among these possibilities.

Studies of nodule initiation are also rare. In the past, attention has been concentrated on *Alnus glutinosa*. According to the recent account given for that species by Angulo Carmona (1974), which differs in some respects from earlier ones, the entry of the endophyte into the cortical cells of a root stimulates them to enlarge and divide, leading to the formation of a small but visible swelling which he termed the primary nodule. Further development depends on the initiation of a lateral root within the pericycle of the infected root, a process which, according to his evidence, is stimulated by factors produced by the endophyte. As the lateral root grows through the primary nodule, its own cortex becomes infected and as an apparent result the further development of the lateral root is modified. It emerges from the mother root to form the first lobe of the nodule proper. In *Alnus* the nodule lobe ceases development and new nodule lobes are formed successively thereafter.

The stages in nodule initiation described in this account of *Casuarina* are essentially similar to those reported by Angulo Carmona (1974) for alder. There is the same initial formation of what can be termed a primary nodule and then the successive formation of modified lateral root branches

which in the case of *Casuarina* first form nodule lobes (in a state of temporary arrest) and then develop into elongate nodule roots. In *Casuarina*, infected tissue is restricted to cortical tissues of the lateral root axis and to the basal-most tissues of the nodule roots; the endophyte fails to invade the central cylinder or the apex of the elongating nodule root even as the nodule root primordium penetrates the cortex of the lateral root axis. Once having passed through the cortex and outer suberized layers of the root, the nodule root escapes further growth inhibition and elongates normally, free of endophyte. There still persists, however, the physiological effect of the endophyte since the root grows vertically upward. It would seem that the presence of the endophyte stimulates nodule root formation directly or acts to block the usual inhibitory effect of a root tip on root branching. One, two or three new primordia may occur at one time at the same level and these in turn pass through the same sequence of inhibited elongation while penetrating the cortex of infected tissues and then escaping to form elongate nodule roots. Each new nodule root in turn may form multiple branches at its infected base. Each nodule root can be traced to an endogenous origin in the central cylinder and the vascular tissues of the nodule root have continuity with the central cylinder of the root from which it was derived. Thus, initiated lateral root primordia serve as the source of new tissues for infection sites within the expanding nodule.

As pointed out by Becking (1968), there are two morphological types of non-leguminous root nodules with actinomycete-like endophytes. The *Alnus*-type nodule is composed of modified, frequently branched roots arrested in growth, which when young are primarily two-dimensional, but as root branching continues in different planes, form a large, roughly spherical coralloid mass. The *Myrica*-type nodule is a basically similar branched lateral root system, but differs in that each lateral root meristem escapes the inhibition produced by the presence of the endophyte and elongates, growing vertically upward, forming a clump of nodule roots. This type of nodule occurs in *Myrica gale* and other *Myrica* species, in the closely related *Comptonia peregrina*, and in species of *Casuarina*. Fully elongated nodule roots typically have a reduced vascular system and persist only for a single season. Large field-collected coralloid nodules may show the presence of dried-up nodule roots still attached to the nodule mass or they may show no nodule roots, so that accounts of the presence or absence of nodule roots from field collections are less to be trusted than are accounts of plants grown under controlled conditions.

However, there may exist intergradations between the *Alnus*-type and the *Myrica*-type nodule and also between the simple, dichotomously branched nodules, and complexly branched coralloid structures. Variations depend upon the host, upon the particular infective endophyte, and perhaps even upon the environmental conditions in which the nodules develop. Thus, Rodriguez-Barrueco (1966) reported that in water cultured plants of *Alnus jorullensis* nodule-roots were consistently produced, though they grew downwards. Becking (1968) observed the formation of nodule-roots in *Alnus rubra* associated with an unusual endophyte, as did Mackintosh (1969) in several *Alnus* species which had been grown with endophytes from other *Alnus* species.

McLuckie (1923) reported observing both simple, dichotomously branched nodules and large, coralloid nodules in *C. cunninghamiana*. It seems unlikely, however, that branching in an angiospermous root, even though highly modified, should occur by a division of the apical meristem in the way suggested by McLuckie. Rather, most of the evidence suggests that these are false dichotomies from modifications of lateral root formation which involve endogenous origins of each nodule lobe or nodule root. No evidence for bifurcated root apical meristems was found in the present study of *Casuarina* nodules. The account given by Fletcher (1955) of branching in *Myrica gale* nodules points strongly to an endogenous origin for new nodule lobes. And a close study of McLuckie's drawings allows them to be interpreted on the basis of an endogenous initiation of the new nodule lobes.

One can conclude from the above discussion and summary of the evidence that nodules in *Casuarina* arise by the successive endogenous initiation of modified lateral roots whose bases are infected by an actinomycete-like endophyte. Nodule clusters of alders, and possibly those of other genera, arise in basically the same way as described for *Casuarina*, the main difference being that in *Alnus*, the apical growing point of the nodule lobe rarely escapes to form a nodule root. Clearly, more rigorous developmental studies of the nodules of alders and other non-legumes will be necessary to establish whether branching in them is also endogenous, as described for *Casuarina*.

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