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TIME-LAPSE PHOTOGRAPHIC OBSERVATIONS OF MORPHOGENESIS IN ROOT NODULES OF COMPTONIA PEREGRINA (MYRICACEAE)[1]

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ABSTRACT

Seedlings of the sweet fern *Comptonia peregrina* (L.) Coult. were grown aeroponically with their roots bathed in a nutrient mist lacking nitrogen except for 10 ppm N at the outset. The initiation and early development of root nodules capable of fixing atmospheric nitrogen were recorded with time-lapse photography through early development to the establishment of highly branched, roughly spherical nodules. In *Comptonia* multiple primary nodule lobes are formed at or near the site of infection with as many as 10 primary lobes occurring together. On the shoulders of the swollen primary lobes new primordia develop, forming secondary nodule lobes, which may persist without nodule root elongation, giving a coralloid appearance. The tips of the lobes may elongate, forming nodule roots which grow vertically upward, or, if disturbed, in random orientation. Nodule roots occasionally form lateral roots. The root axis upon which the nodule forms undergoes secondary thickening on the proximal side of the nodule attachment; the distal portion of the root shows no secondary thickening and later atrophies. Thus, nodules are perennial structures on a woody root system. The endophyte infects and occupies the basal cortical tissues of the primary nodule lobes and successive nodule lobes as they are formed, being restricted to the swollen bases and not infecting the elongate nodule roots. Development of the nodule is interpreted in terms of complex host-endophyte interactions involving the initiation of multiple primordia forming nodule lobes, the active inhibition of nodule lobes and finally nodule root elongation. Anatomical evidence for the endogenous origin of nodule primordium formation substantiates the view obtained from time-lapse photomacrography.

THE INITIATION and development of nodules on the roots of non-leguminous, symbiotic nitrogen-fixing plants have been little studied. In his report on nodule formation in *Casuarina cunninghamiana* Miq., Torrey (1976) reviewed the earlier literature on this subject and pointed out the confusion as to the origin of the nodules. In different genera nodules range from simple bifurcated and swollen structures to large coralloid spheres several centimeters in diameter. Some nodules form elongate nodule roots, others only swollen nodular lobes.

One of the problems in the analysis of the morphogenesis of these root nodules has been the dependence on field collections and the random sampling of nodules of diverse sizes, shapes, and developmental stages. Such structures are difficult to relate to each other in ontogenetic terms. Induced infection of seedling root systems grown in sand or water culture or in aeroponics with the infective agent prepared in nodule suspensions has made possible more complete morphological and anatomical studies of nodule formation in these systems. *Alnus* species have been most closely studied (see review by Angulo, 1974; Becking, 1975; Lalonde and Fortin, 1973). Other genera studied include *Myrica* (Fletcher, 1955), *Ceanothus*, Furman, 1959), and *Casuarina* (Torrey, 1976). Increasing attention has been paid to the ultrastructural details of the endophyte-host interaction. Some efforts have been directed toward a better understanding of the hormones involved in nodule development (Silver, Bendana, and Powell, 1966; Dullaart, 1970). Yet little is known about the ontogeny of root nodules once infection is initiated.

In *Casuarina* Torrey (1976) showed that the nodules resulted from repeated endogenous lateral root initiations which formed a truncated and complexly branched spherical structure. The actinomycete-like endophyte occupies infected cortical cells of each successive lateral root formed. Nodule roots develop from nodule lobes after escaping from the inhibitory effects of the endophyte. Variation among genera with respect to the occurrence of nodule roots appears to be related to the degree of inhibition imposed on nodule root primordia by the endophyte.

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In the present study of nodule morphogenesis in *Comptonia peregrina* (L.) Coult., individual nodules were followed closely from the onset of initiation until their establishment as large, roughly spherical structures, with the intention of establishing clearly the sequential development of nodule morphology. Infection, nodule initiation and ontogeny at the cellular level in *Comptonia* will be the subject of a separate publication. Few studies of nodulation in *Comptonia* have been published. Ziegler (1960) and Ziegler and Hüser (1963) first studied root nodules of *Comptonia peregrina* for their capacity to fix atmospheric nitrogen. More recently, Fessenden, Knowles, and Brouzes (1973) reported further detailed studies of acetylene-reducing activity of excised nodules of *Comptonia* made from field collections. Laboratory and greenhouse studies of *Comptonia* which had been hampered by difficulties in seed collection and germination have now been resolved (Del Tredici and Torrey, 1976).

MATERIALS AND METHODS—Seeds of *Comptonia peregrina* were scarified, soaked in 500 ppm gibberellic acid for 3 h and sown in sand following the method described by Del Tredici and Torrey (1976). Young seedlings 2–3 wk old were transferred to an aeroponic tank (Zobel, Del Tredici, and Torrey, 1976), provided with ⅛-strength Hoagland's solution minus nitrogen and allowed to develop. At the outset $Ca(NO_3)_2$ and KNO_3 at 10 ppm N were provided to allow the seedlings to become established. Thereafter, no additional nitrogen was provided. About 1 wk after transfer to the tank, each seedling root system was inoculated by thoroughly brushing it with a suspension of nodule tissue taken from older plants growing in aeroponics. The inoculum was prepared by grinding nodule material in distilled water in a glass mortar and pestle in the proportion of 1 g of nodule to 20 ml of water, followed by passing the suspension through a double layer of cheesecloth. Initiated nodules were observed within 2–3 wk of inoculation with the earliest observed at 12 days. Close observation of young seedling roots could be arranged by removing the whole seedling, floating the root system in a dish of nutrient solution and studying it under a dissecting microscope at up to 50 ×.

Seedlings of *Comptonia* were observed in this manner several times weekly in order to obtain early stages of nodulation. Any lateral roots or occasional senescent nodule roots which obscured nodule observation were removed as necessary.

Nodules were photographed at frequent intervals to illustrate progressive changes in nodule size and shape. For photography, the nodules were positioned by using Plasticine modelling clay or small weights so that the same orientation could be achieved in successive photographs. The root system was completely immersed horizontally in solution with the shoot resting on the edge of the dish. Illumination from two sides was achieved with 100-watt bulbs in reflectors. Photographs were made with Kodak Panatomic-X film at one and two times magnification using a tripod-supported Nikon F 35-mm camera fitted with a bellows extension and a normal 50 mm lens. After photographing them, the plants were immediately returned to the aeroponics tank. In the last stage, nodules were excised, fixed, and dissected in phosphate buffer and photographed as before. In some sequences, because of the three-dimensional structure to be shown, photographs were taken of the nodule at more than one depth of focus. Photomontages were prepared in some cases from these paired photographs.

Histological preparations were made from nodules fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in an acetone series and embedded in Araldite resin. Sections were cut at 1–2 μm and stained with toluidine blue O in borax buffer, following the methods of Newcomb and Fowke (1974).

OBSERVATIONS—In *Casuarina* early nodule initiation in seedling roots is easily seen because the infection site is marked by a striking red pigmentation of the earliest nodule lobe (Torrey, 1976). No such marker is found in *Comptonia*, where the earliest stages of nodule development which are visible externally resemble lateral root primordia, except that the nodule primordia are multiple, that is, several are arranged closely together along the root axis (usually a lateral root) in linear rows (Fig. 1, 3, 5) matching the internal protoxylem poles (Fig. 6). As will be described elsewhere, the nodule is initiated by a single root hair infection and invasion of the cortical tissues of the lateral by the endophyte. Root nodule lobes characteristically are initiated both proximal and distal to the infection site.

Figure 1 shows an early nodule with two rows of three nodule lobes arranged close together. In Fig. 3 is another nodule with four swollen nodule lobes already with nodule roots growing from two of the lobes. Such early nodules develop very rapidly. Figure 3 (inset) shows the nodule illustrated in Fig. 3 before it could be designated a nodule with any certainty. Figure 2 shows the same nodule as in Fig. 1 just 4 days later. The six nodule lobes have increased in diameter and four additional lobes have formed. Nodule lobe primordia are indicated by the dark terminal papilla marking each apex. Two of the older lobes show elongating nodule roots. The base of each of these nodule roots is already somewhat swollen by the formation of two new nodule lobe primordia which will develop subsequently as branches arising from the base. The basal branching of successive nodule lobes is a characteristic feature of nodule development in *Comptonia* and

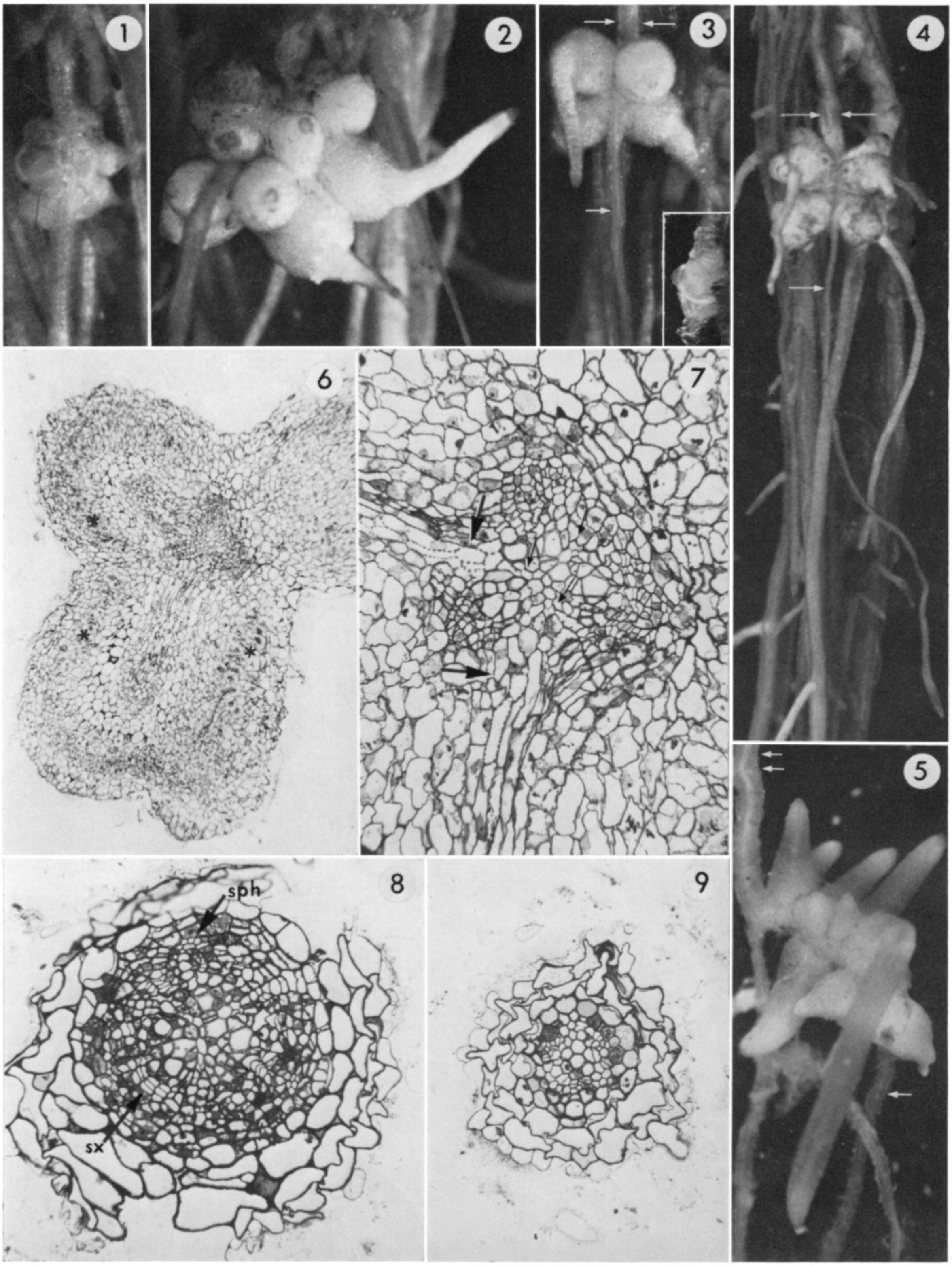

Fig. 1–9. Photographs and photomicrographs of young developing root nodules on seedlings of *Comptonia peregrina* grown in aeroponic culture. **1.** Nodule on lateral root of seedling 22 days after inoculation. Six nodule lobes in two rows of three are evident. × 15. **2.** The same nodule as in Fig. 1 photographed 4 days later, showing ten nodule lobes. Four lobes showing basal swellings due to new nodule lobe primordia are evident. Two of the pri-

soon leads to complex structure in the nodule. Figure 4 illustrates a later stage of development of the nodule shown in Fig. 3. In Fig. 5 is shown a variation in early nodule development with about ten primordia formed closely together, giving almost the appearance of fasciation. Swollen bases infected with the endophyte and the beginning of elongation of nodule roots are apparent.

Nodules are persistent structures in most woody species in which they occur, lasting for a number of years. The early development of the root upon which the nodule forms assures this persistence. The distal portion of the root may show no effect or actually atrophy, as was observed also in *Casuarina* (Torrey, 1976). In *Comptonia* the proximal portion of the root on which the nodule occurs initiates secondary thickening (Fig. 3, 4, 5). A vascular cambium develops and secondary xylem and phloem are formed (Fig. 8). The original cortex sloughs away. On the distal part of the root only primary tissues occur (Fig. 9). At the level of nodule lobes (Fig. 6, 7), the secondary tissues are evident and the attachment of the nodule lobes to the root axis at the protoxylem poles can be seen. Presumably, hormonal stimuli originating in the nodule lobes initiate a response in the proximal tissues of the root but not in the distal portion.

In Fig. 10–14 is shown the development of a single nodule over a period of 28 days using time-lapse photomacrography. Such a sequence allows one to follow with precision the multiplicity of nodule lobe branchings and the succession of nodule root elongations. It is also a dramatic way of assessing nodule growth and morphogenesis. Such an analysis leaves no doubt that nodule formation involves a succession of lateral root branchings under the influence of the endophyte. In Fig. 10 six primary nodule lobes are distinguishable, each with an elongate nodule root. Each of these shows newly developing nodule lobe primordia. In Fig. 11 and 12 the subsequent elongation of nodule lobes as nodule roots can be followed. In Fig. 13 and 14 secondary and tertiary nodule lobes have developed and in some cases have formed nodule roots. By day 28 a large spherical nodule with multiple nodule roots has developed and the origins relating to earlier stages are difficult to assess with certainty.

The primary lobes are given arabic numerical designations in an arbitrary order which does not indicate a developmental sequence. The designation of subsequent nodule lobes and their ordered sequence is also given by arabic numerals. Primary lobes are designated 1, 2, 3, etc. Secondary lobes are designated 11, 12, 13 from primary lobe 1, 21, 22, 23 from primary lobe 2, etc. Tertiary lobes are designated 111, 112, 113 or 121, 122, etc. Thus, each lobe can be associated with its origin by the numerical designation. One could expect the internal vasculature connecting these successive branches to be confusingly complex if cut in section at this stage of development.

Internal nodule structure—The internal structure of primary nodule lobes and their secondary lobes confirms the macroscopic observations made above. Figure 15 illustrates the origin of a nodule lobe as an endogenous branch of an already developed primary lobe. The internal vascular connection is apparent. Evidence of another sub-terminal lobe in another plane is given by the localization of sub-divided meristematic cells in the cortical region in between the tip and the lobe cut in median section. Figure 16 shows part of the three-dimensional structure of a primary nodule. The internal vascular tissues in this whole-mount view occur in several planes, but all attach to the central axis as in an endogenously branched system.

Figures 17 and 18 show two stages in the development of the nodule lobe before nodule root elongation has occurred. In Fig. 17 is seen, in longitudinal tangential section, a nodule lobe cut

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mary nodule lobes have begun to elongate forming nodule roots. × 15. **3.** Nodule with four nodule lobes, two showing elongate nodule roots. The proximal side of the lateral root bearing the nodule is marked by double arrows. The distal side is marked by a single arrow. × 15. **3** (inset). The same root shown in Fig. 3, photographed 4 days earlier. × 15. **4.** The same nodule as in Fig. 3 photographed, at lower magnification, 21 days later. Proximal (double arrows) and distal (single arrow) portions of the lateral root bearing the nodule are indicated. × 7.5. **5.** Young nodule showing multiple nodule lobes (at least 10) along a lateral root axis with several elongate nodule roots. The lateral root proximal to the nodule (double arrow) is about 2 × the diameter of the distal part of the lateral root (single arrow). × 15. **6.** Transection of a lateral root through the region of attachment of nodule lobes in a young developing nodule. The endophyte in the swollen cortex tissue of the nodule lobes is marked (*). × 82. **7.** Transection of a nodule similar to Fig. 6, at higher magnification, showing the central cylinder of the lateral root from which nodule lobes have originated. The vascular connections of two nodule lobes to the vascular tissue of the lateral root are seen in longitudinal section (large arrows). Protoxylem points (3) are marked with small arrows. × 280. **8.** Transection of a lateral root cut proximal to the site of nodule attachment. Note the sloughing root cortex and the thick central cylinder showing a considerable amount of secondary xylem (sx) and secondary phloem (sph). × 280. **9.** Transection of the same lateral root as in Fig. 7 photographed at the same magnification but cut distal to the site of nodule attachment, showing only primary vascular tissues in the central cylinder. The root is triarch. × 280.

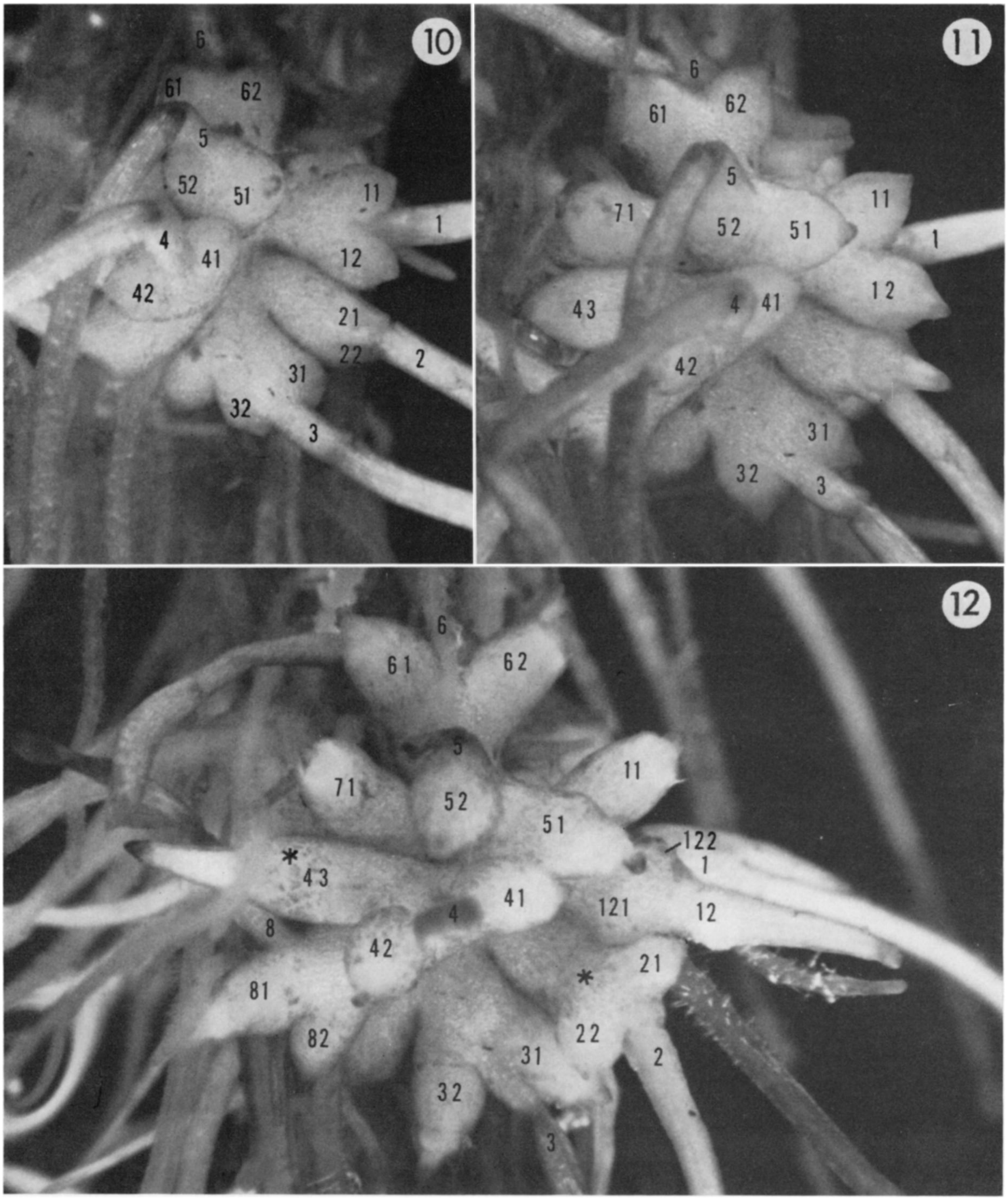

Fig. 10–12. Figures 10–14 show, in a time-lapse sequence, the development of a young nodule into a complexly branched multilobed structure. **10.** Developing nodule showing six primary nodule lobes (1–6) each with elongate nodule roots. At the bases are newly developing lobes (e.g., 11, 12, 31, 32, etc.) which have not yet shown nodule root elongation. **11.** The same nodule 3 days later. Note that several nodule lobes have begun tip elongation (e.g., 12, 21, 31, 32, etc.). Other nodule lobes have become more prominent (e.g., 43). **12.** The same nodule 10 days after Fig. 10. Note that the nodule has about doubled its size, largely by forming new nodule lobes (e.g., 81, 82) and developing elongated nodule roots. The swollen nodule lobes show cortical tissues torn and darkened on their surfaces (*). All × 19.

through part of the central cylinder. Many of the cells are filled with tannins, suggestive of a dormant structure. Figure 18 illustrates a later stage in which the nodule lobe is more elongate and cylindrical with cortical cells showing hypertrophy and endophyte invasion. The papilla at the tip is seen in section and represents cortical derivatives carried along rather than a newly formed cap. Tannins fill the outer subepidermal layers and certain elongate cells of the central cylinder.

Variations in nodule morphology—Two major types of variation in nodule morphology were observed in *Comptonia* roots grown in aeroponics. One concerned variation in the elongation of nodule roots from nodule lobes. The second concerned the direction of nodule root elongation. Some of this variability is illustrated in Fig. 19–22. Figure 19 shows a young nodule in which the mature nodule lobes have given rise to elongate nodule roots which are oriented apparently at random. Newly developed nodule lobes are also present but are too young to show elongate roots (for example, they show no papilla). Figure 20 illustrates a nodule with many fully formed nodule lobes, only about half of which have formed elongate nodule roots. The nodule lobes appear to be inhibited and each shows a clearly marked papilla. Note that the initial direction of nodule root elongation tended to be upward, but this orientation was lost with later growth. Figure 21 shows a nodule with numerous mature lobes, but a number have not yet developed into nodule roots. The latter show no tendency toward vertically upward growth. Note that several of the nodule roots show lateral root branches, an uncommon occurrence which strongly supports the view that the nodule roots are part of a modified lateral root system. Figure 22 illustrates the opposite extreme to that shown in Fig. 19. This nodule is comprised of many nodule lobes, only a few of which have developed into elongate nodule roots. Dark papillae mark long-established nodule lobes which show a persistent inhibition of nodule root formation. It is this type of structure which has usually been referred to by other authors as coralloid in morphology. Sometimes, such structures are observed in material collected in the field. In these cases, it is difficult to determine whether the coralloid structure is caused by lack of nodule root development as in Fig. 22 or by atrophy and sloughing off of nodule roots in situ or during washing off of the adhering soil at the time of collection.

DISCUSSION—The present observations on *Comptonia* confirm the view that nodule development in these systems involves the stimulation of lateral root-like primordia by an invading endophyte to form a primary nodule lobe (or several such lobes). Thereafter, there occur repeated sequential root initiations at the bases of each of the developing nodule roots, forming a complex, compact branched system.

Angulo, Van Dijk and Quispel (1975) discussed the evidence concerning the sequence of events in the initial infection and the problem was addressed as well by Quispel (1975) and by Torrey (1976). The first lateral root-like primordium in *Alnus* and in *Casuarina* has been termed the primary nodule lobe (Becking, 1975). *Comptonia* nodule initiation differs from the typical situation found in *Casuarina* in that multiple primordia appear almost simultaneously instead of

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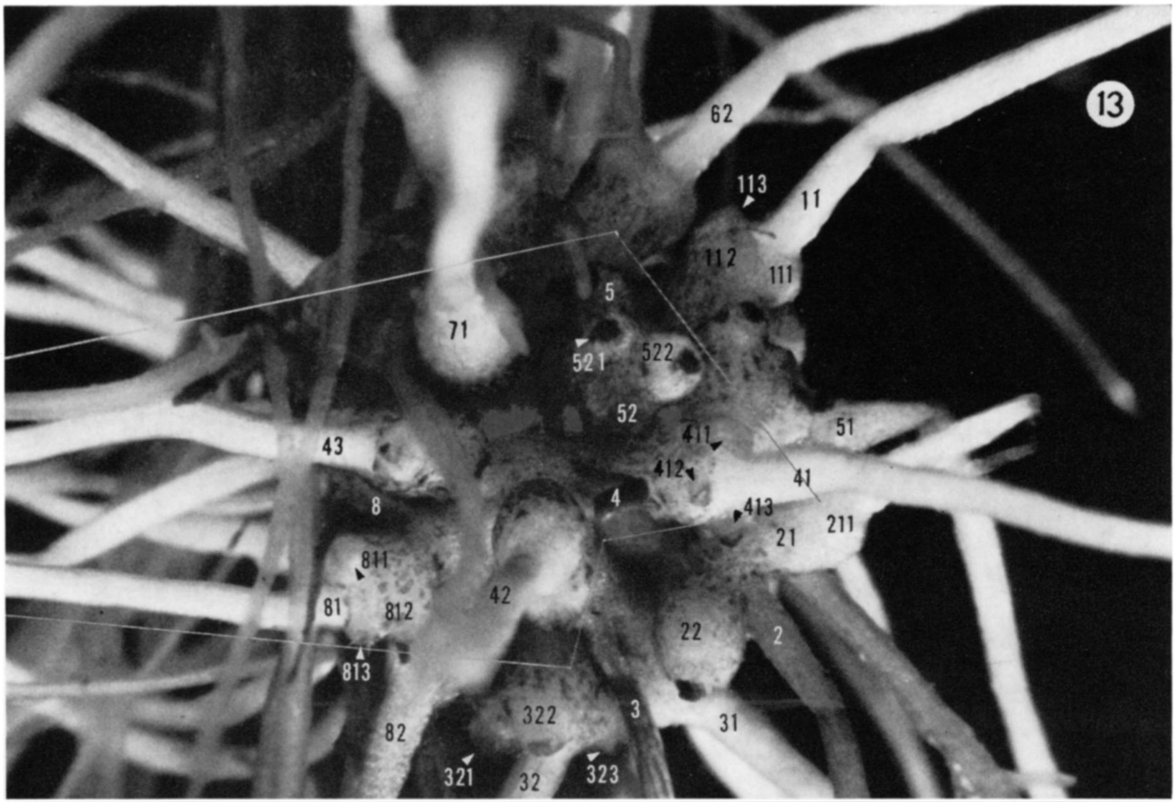
Fig. 13, 14. Continuation of the time-lapse photographic series of the nodule first shown in Fig. 10. Photomontages were used to show structures clearly. **13.** The same nodule as in Fig. 10, 17 days later. Secondary and tertiary nodule lobes show elongate nodule roots (e.g., 41, 42, 43) and still more primordia at their bases (e.g., 111, 112, 113). Some nodule roots show two basal branches (e.g., 21 and 22 at the base of 2) while other nodule roots have three basal branches (e.g., 421, 422 and 423 at the base of 42). **14.** The same nodule as in Fig. 10 after 28 days. Both × 16.

Fig. 15–18. Microscopic views of nodules of *Comptonia* showing the internal vascular structure. **15.** Median longitudinal section of a young nodule lobe (small arrow) which originated from an already developed nodule lobe which is here cut tangential to its main axis (large arrow). The endogenous origin of the primordium is clear from its attachment to the central cylinder (cc) of the main primordium. Note the endophyte (end) occupying cortical cells on either side of the main axis. External cell layers over each primordium are filled with tannin, forming papillae over each apex. × 100. **16.** Cleared nodule of *Comptonia* mounted whole and photographed with polarized light. The birefringence of the secondary walls of the primary xylem strands marks the main vascular pathways of the endogenously originated nodule lobes. Bright refractile spots are calcium oxalate crystals in the cortical cells of the nodule. × 95. **17.** Longitudinal section of a nodule lobe cut tangential to the central axis. The tissues of the central cylinder are clearly distinguished from the tannin-filled cortical cells and epidermis. The terminal cells forming the papilla are filled with tannins and some cells have collapsed and been sloughed away. This lobe has the appearance of a dormant or inhibited apex. × 290. **18.** Median longitudinal sections of a nodule lobe which is columnar in shape but has not begun its elongation as a root. Compare with lobe 11 in Fig. 11. The tip is papillate, the central cylinder is root-like but the cortex is infected with endophyte (end) to within a few hundred microns of the tip. Tannins fill cells of the central cylinder, the outer cortical layers and the apical papilla. × 110.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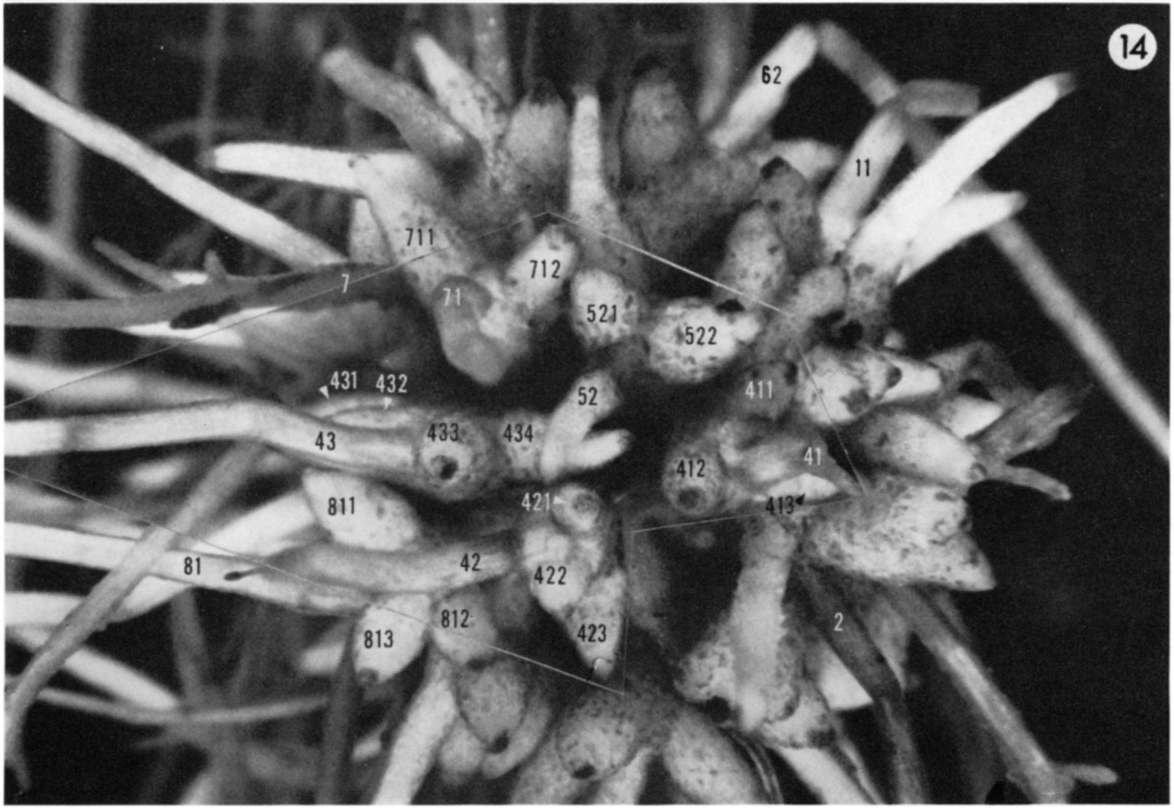
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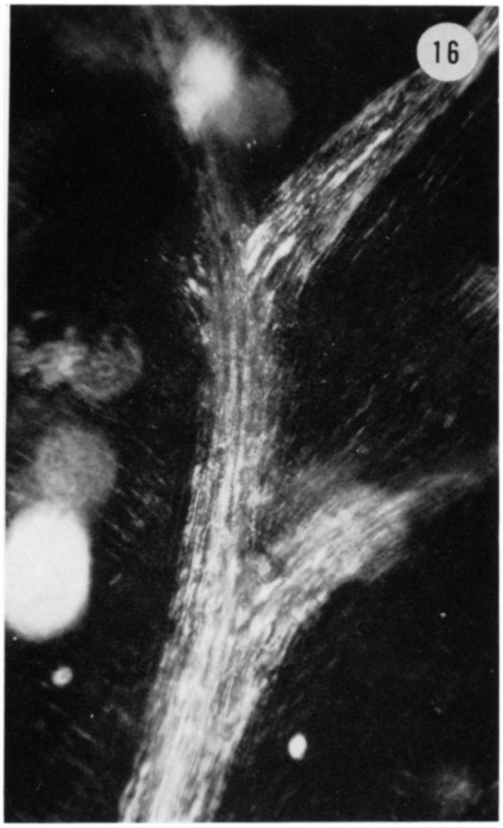
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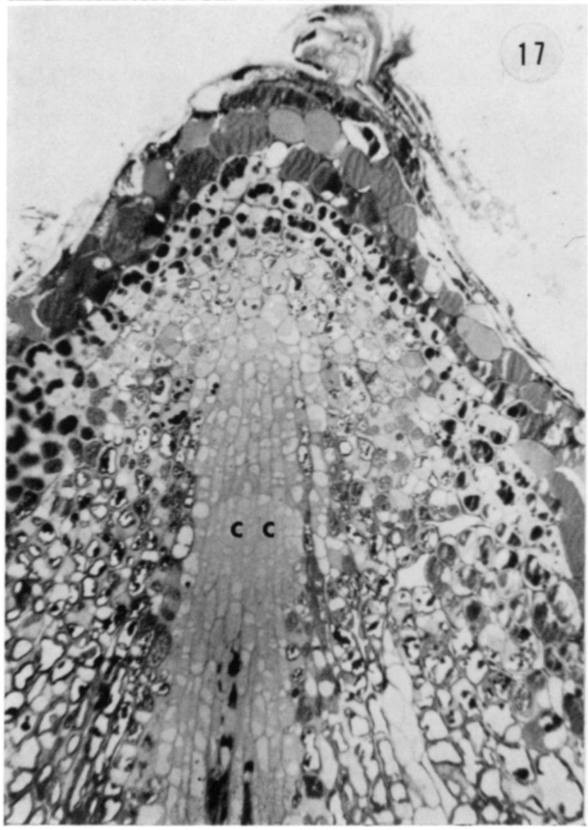
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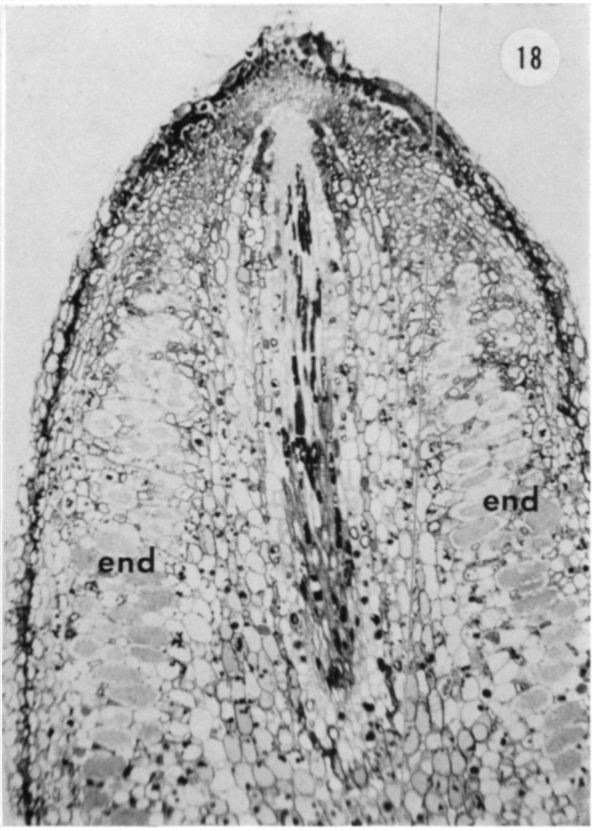
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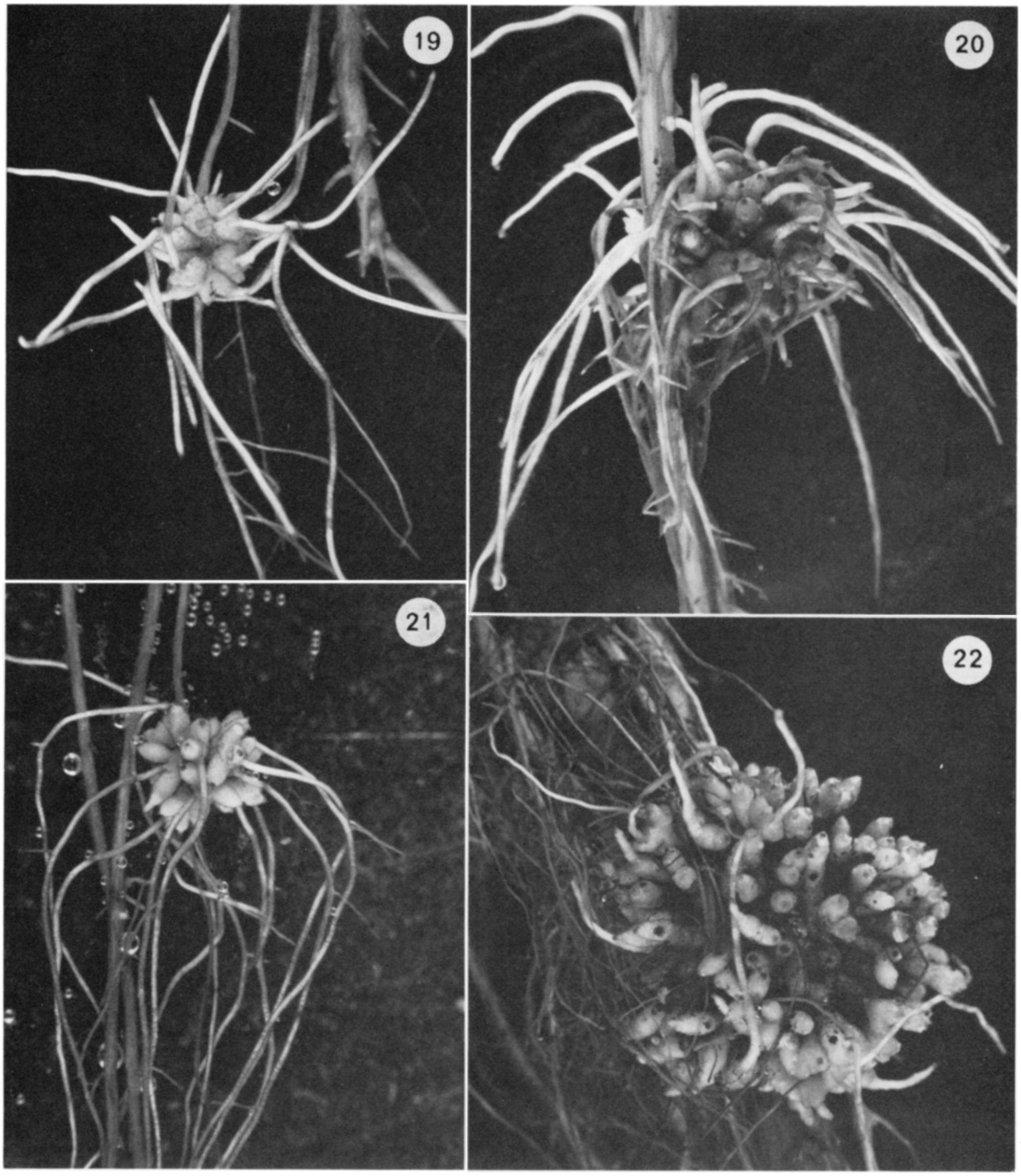

Fig. 19–22. Root nodules of *Comptonia* grown in aeroponic culture showing variations in morphology. **19.** Young nodule with elongating nodule roots oriented at random. **20.** Older root nodule with about half of the nodule lobes showing inhibition (marked by papillae) and the others are elongated nodule roots. **21.** A much lobed nodule with most of the lobes inhibited, showing only a few elongate nodule roots. The basic branching pattern is the same as in Fig. 19. **22.** A multilobed nodule with many elongate nodule roots mostly growing downward. Lateral branching of the nodule roots is very evident. All × 4.

as a single primordium. Multiple primary nodule lobes have also been observed in *Alnus* (Angulo et al., 1975), although the few published illustrations of this early stage (Becking, 1968, Angulo, 1974) usually show only a single primary nodule lobe. In *Comptonia* several to as many as 6–14 such lobes appear almost simultaneously in response to endophyte invasion. Detailed anatomical studies to be published elsewhere describe the infection process, the invasion of the root cortex, and the early development of the primary nodule as it occurs in *Comptonia.*

Early after initiation, the nodule lobe remains as a swollen, partially arrested structure with a terminal rounded meristem covered by an anomalous cap-like papilla. At some stage which is not completely predictable, a nodule lobe begins terminal extension and the nodule root it forms elongates as a cylindrical structure with a terminal cap. The elongation of nodule roots from these nodule lobes is a normal event in *Comptonia* as it is also in *Casuarina* but not in *Alnus.* Frequently, in undisturbed water culture or aeroponics, the nodule roots grow vertically upward, elongating to about 5–6 cm and then stopping further elongation and becoming senescent. The extension of nodule roots is not synchronous but, if it occurs, is usually in a sequence related to order of initiation. On occasion, the nodule roots show lateral endogenous branches, attesting to the fact that these are part of a branching root system. When disturbed or handled, the nodule roots show random orientation. Not all nodule lobes show terminal elongation but may remain in an arrested state for the life of the nodule.

It seems evident from these observations that nodule initiation and development in *Comptonia* involves a complex series of changing host-endophyte interactions which probably have a hormonal basis. The nature of the initial infection process remains unknown. Studies by Lalonde (in press) suggest that a complicated host-soil microorganism interaction precedes the actual invasion. As will be described in detail elsewhere, once inside the root cortex following root hair penetration, the host responds first by cortical cell hypertrophy and proliferation at the infection site, which is then further enlarged by the initiation and partial outgrowth of a meristem initiated as a lateral root primordium which involves the cortical tissues as well. This nodule lobe primordium is then inhibited from immediate further elongation presumably by endophyte action. Finally, the primordium escapes inhibition by the endophyte and extends, forming the endophyte-free nodule root. Only the swollen cortical tissues at the base of the nodule root contain the endophyte. Thus at least four sequential events occur, viz., cortical cell hypertrophy, the initiation of primordia, the active inhibition of primordial development and finally nodule root extension. The last event is especially interesting in hormonal terms because nodule root extension is frequently negatively geotropic, unlike normal lateral root development. The present study helps to define these sequential events and serves as prelude for further exploration of the physiological bases for such morphogenesis.

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