Determinate development of nodule roots in actinomycete-induced root nodules of *Myrica gale*

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Young seedlings of *Myrica gale* L. grown in water culture were inoculated with a nodule suspension containing the effective actinomycete which induced root nodule formation. Nodule development was followed from initiation to nodule lobe formation and nodule root development using living materials and fixed nodules sectioned for light microscopy. After root hair infection and prenodule formation, three stages were observed: nodule lobe formation, a transition or arrested state, and nodule root development. The primary nodule lobe meristem originates endogenously and its formation involves pericycle, endodermis, and cortical cell derivatives. The lobe develops slowly to about 2 mm in length while the cortical cells are invaded by the actinomycete endophyte. After a period of arrest of variable duration, from a few days to several weeks, the nodule lobe meristem begins altered development, forming the elongate nodule root which undergoes slow but continuous growth to about 3- to 4-cm final length. New nodule lobe primordia are initiated endogenously at the base of existing nodules lobes, ultimately forming a cluster of nodule roots. Each nodule root, which elongates at about 0.1–1.0 mm per day, has a terminal apical meristem with reduced root cap formation and produces a modified root structure possessing an elaborate cortical intercellular space system and a reduced central cylinder. Nodule root growth is distinctive in that it shows strong negative geotropism. The endophyte is restricted to cortical cells of the nodule lobe and is totally absent from tissues of the nodule root. A probable role for nodule roots is to facilitate gas diffusion to the nitrogen-fixing endophyte site in the nodule lobe when nodules occur under conditions of low oxygen tension.

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De jeunes plantules du *Myrica gale* L. cultivées en milieu aqueux et on leur a inoculé une suspension de nodules contenant l'actinomycète qui induit effectivement la formation de nodules racinaires. Les auteurs ont suivi, chez du matériel vivant et chez des nodules fixés et sectionnés pour étude au microscope photonique, le développement des nodules à partir des premières étapes jusqu'à la formation du lobe nodulaire et au développement de la racine nodulaire. Après l'infection des poils racinaires et la formation de pré-nodules, trois stades ont été observés: la formation du lobe nodulaire, un stade de transition ou d'arrêt et le développement de la racine nodulaire. Le méristème primaire du lobe nodulaire a une origine endogène et sa formation implique le péricycle, l'endoderme et des cellules corticales. Le lobe se développe lentement jusqu'à une longueur d'environ 2 mm, alors que les cellules corticales sont envahies par l'actinomycète endophyte. Après une période d'arrêt de durée variable (de quelques jours à plusieurs semaines), le méristème du lobe nodulaire amorce un développement modifié, formant la racine nodulaire allongée qui croît lentement mais sans arrêt jusqu'à une longueur finale de 3 à 4 cm. De nouveaux primordiums de lobes nodulaires sont amorcés de manière endogène à la base des lobes nodulaires déjà présents, ce qui produit finalement une touffe de racines nodulaires. Chaque racine nodulaire, qui s'allonge d'à peu près 0.1–1.0 mm par jour, a un méristème apical terminal avec une formation réduite de la calotte racinaire et produit une structure racinaire modifiée possédant un système cortical complexe d'espaces intercellulaires et un cylindre central réduit. La croissance de la racine nodulaire a cette particularité de présenter un fort géotropisme négatif. L'endophyte est restreint aux cellules corticales du lobe nodulaire et est complètement absent des tissus de la racine nodulaire. Les racines nodulaires ont probablement le rôle de faciliter la diffusion des gaz vers l'endophyte fixateur d'azote dans le lobe nodulaire, lorsque les nodules sont dans des conditions de faibles tensions d'oxygène.

[Traduit par le journal]

Introduction

Actinomycete infection of the roots of certain nonleguminous woody angiosperms leads to the initiation and development of root nodules which are capable of fixing atmospheric dinitrogen. Such nodules develop from a local stimulation by the endophyte of multiple modified lateral roots whose repeated branchings form a perennial structure which may become several centimetres in diameter. Two morphological types of actinomycetous nodules are known: the *Alnus* type in which the lobes of the nodule are arrested in growth, forming a roughly spherical coralloid mass, and the *Myrica* type which, while basically similar, differs in that each nodule lobe gives rise to an elongate root which is negatively geotropic. In 1952, Bond called attention to the peculiar and striking nature of these root structures which he termed "nodule roots." In earlier descriptions of nodules, they had been simply described as "rootlets" (e.g., McLuckie 1923). Among the genera forming nodules of the *Myrica* type are included all species of Myrica, the closely related *Comptonia peregrina*, and all species of *Casuarina* which have been studied. More recent reports include that of *Myrica* by Fletcher (1955), of *Casuarina* by Bond (1957) and Torrey (1976), and of *Comptonia* by Bowes *et al.* (1977) and Callaham and Torrey (1977). Little attention was paid in these studies to the development of the nodule roots, which are interesting structures in themselves. They are determinate organs, reaching 3–4 cm in length when they cease elongation. They grow vertically upward, showing strong negative geotropism, and they possess reduced and modified internal structure. The present study was undertaken to explore these peculiar features.

Materials and Methods

Seeds of *Myrica gale* L. were germinated in sand after cold treatment and young seedlings were transferred either to water culture in foil-covered glass jars or to aeroponic tanks (Zobel *et al.* 1976) and inoculated with nodule suspensions prepared from mature nodules taken from older, greenhouse-grown plants (Bowes *et al.* 1977). Observations were made of the root systems at frequent intervals and the pattern of nodule development was followed in detail in living material. Nodules taken at different stages were fixed in 3% glutaraldehyde, postfixed in osmium tetroxide, embedded in araldite resin, and sectioned and stained for microscopic observation according to methods described earlier (Callaham and Torrey 1977).

Observations

Morphological

Stages in the initiation and early development of root nodules in *Comptonia peregrina* have been described and illustrated in some detail (Bowes *et al.* 1977; Callaham and Torrey 1977). Nodule development in *M. gale* is similar in many details; after the prenodule stage, each nodule consists initially of only the 1- to 2-mm-long primary nodule lobes, giving the coralloid appearance of nodules of the *Alnus* type. From time-lapse studies of *Comptonia*, it is clear that nodule roots develop from continued but altered development from the apical meristem of the nodule lobe. The nodule root begins elongation and by continued growth produces a cylindrical, tapering structure which is white and rootlike in appearance but exhibits marked negative geotropism in contrast with neighboring normal lateral roots. In *M. gale*, the average final length of nodule roots is about 3.0 cm (Fig. 1).

Nodule roots may show branching along their lengths, but lateral branches were observed infrequently in both *Comptonia* and *M. gale*. Root hairs were usually not present on nodule roots in either species, although under special environments root hairs may develop. Under optimal conditions, nodule roots persist for a growing season and then tend to dry up and drop off with new nodule lobes and roots developing in the subsequent season. Thus, nodule roots are transient structures functioning on a seasonal basis.

Rates of Nodule Development

Repeated close observations at 2-day intervals were made of nodule lobe development and nodule root elongation on individual nodules of plants of *M. gale* grown in water culture to determine the rates of development. Three morphological stages could be distinguished following nodule initiation: viz., nodule lobe formation, a transition state, and nodule root elongation. Once a nodule lobe was initiated, its development continued until the lobe was ~2 mm in length; then a transition stage followed when the nodule lobe remained in an arrested state as a swollen lobe with a terminal pointed meristem but no elongation. Nodule lobe formation and the transition state together usually occupied about 3 weeks in *M. gale*. In some cases, the transition period was very short, with nodule root elongation beginning as soon as the lobe was fully formed. In other cases, the arrested state of the lobe persisted for many days or up to several weeks.

FIGS. 1–4. Stages in nodule root development in *M. gale*. Fig. 1. A lateral view of a root nodule of a seedling grown continuously in aeroponics showing a number of upward-growing nodule roots. Normal lateral roots have grown downwards and formed multiple plagiotropic or somewhat positively geotropic lateral branches. Droplets are from condensation of the nutrient mist. × 3. Fig. 2. A median longitudinal section of a nodule lobe primordium initiated at the base of an obliquely cut earlier-formed nodule lobe already invaded by endophyte (*en*). A second younger nodule lobe primordium is seen at lower right cut obliquely. Note the central elongate cells of the central cylinder (*cc*) and the enlarged cells of the future cortex (*c*). × 130. Fig. 3. An enlarged view of the smaller primordium in Fig. 2 shows at lower right the future cortical cells of this nodule lobe being invaded by the actinomycetous endophyte. × 250. Fig. 4. Future cortical cells of a nodule lobe primordium similar to Fig. 3 with early invasion by the actinomycetous endophyte. Note the density of cytoplasm and organelles in the immediate proximity of the endophyte filaments (*en*); nucleus (*n*), vacuole (*v*). × 1200.

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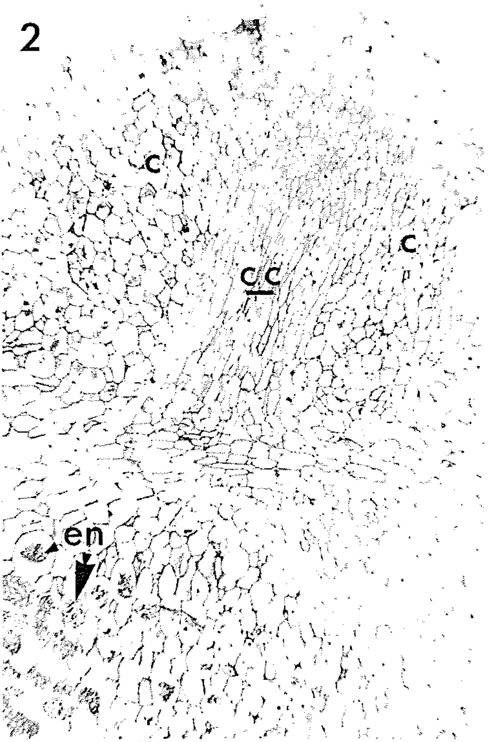

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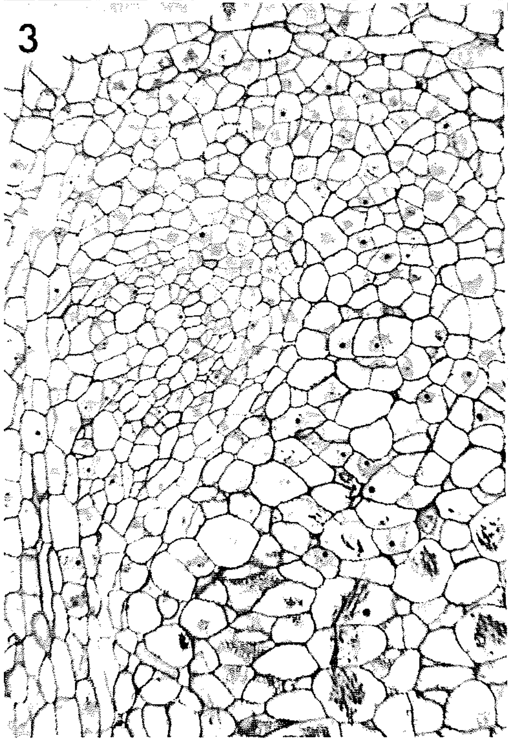

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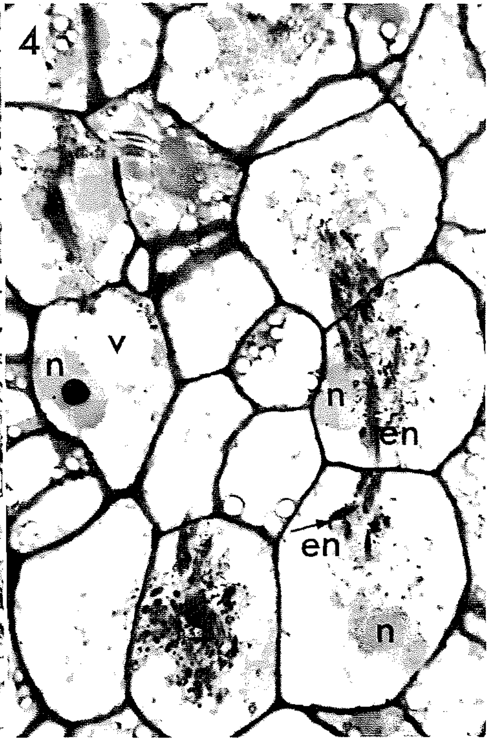


After a period of arrest, the apex of the nodule lobe gave rise to a smaller diameter root apical meristem which grew from the center of the lobe, curving from the original orientation until it was pointed more or less vertically upward. Once nodule root elongation began, growth was continuous until the final length of 3–4 cm was reached. Rates of elongation observed were different in different nodule roots, varying from 0.1 mm/day to 1.0 mm/day. Once they began to elongate, nodule roots of *M. gale* grew slowly, elongating more or less continuously for a month or more. In some cases, new nodule lobes developed at the base of a nodule root when the existing nodule root was less than 1.0 mm in length. In other nodules, no new nodule lobes developed even after the nodule root was midlength or greater. In some nodules, all of several nodule lobes produced elongate nodule roots; in others, only one nodule root elongated while other lobes remained arrested.

Anatomical Observations

Primordium Formation

The initiation of a nodule primordium in *Comptonia* was described earlier (Callaham and Torrey 1977). Essentially the same sequence of endogenous origin was observed in *M. gale*, including initial proliferation of pericycle and endodermal cells of the root in which initiation occurred, the incorporation into the nodule meristem of subdivided root cortical cells, and the delimitation of a central procambial cylinder and surrounding vacuolated enlarged cells of the nodule cortex. When the nodule lobe is still less than 1.0 mm long, these tissue arrangements are already clear. This same sequence of endogenous origin and involvement of the pericycle, endodermis, and cortex also occurs in the formation of the higher-order nodule lobes (Fig. 2). Cells at the distal end of the primordium become enlarged and vacuolated but no sloughing of cells occurs at this stage. The cortical tissues are compact, showing little evidence of intercellular spaces. Invasion of the endophyte into the nodule lobe cortex occurs at this early stage (Figs. 3, 4) and nodule root elongation has not yet begun, the root appearing to be arrested. A corklike periderm develops over the surface of the nodule lobe (Fig. 5). The nodule lobes at this stage form structures morphologically not unlike those observed in the *Alnus*-type coralloid nodules.

Nodule Root Elongation

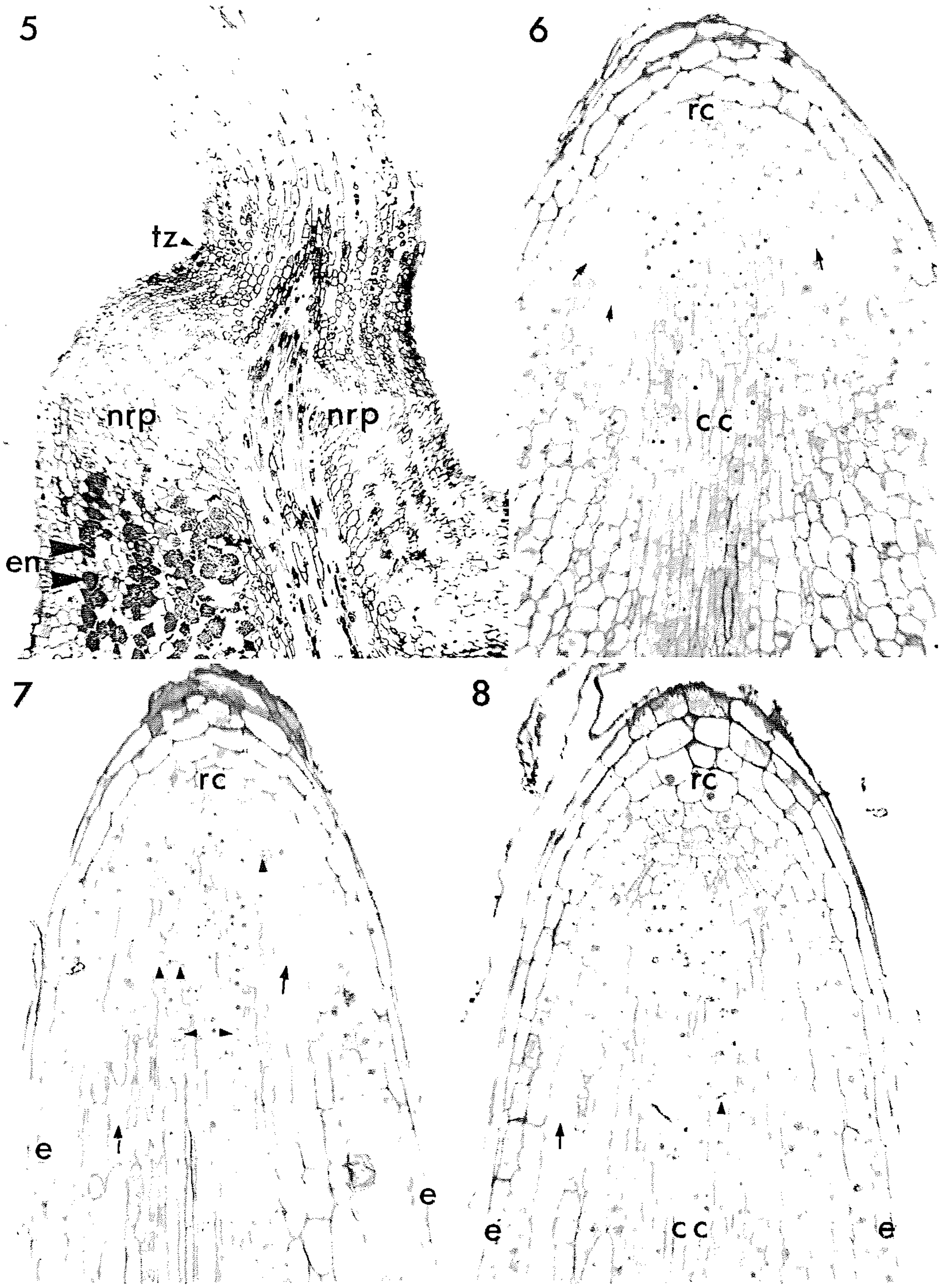
Primary nodule primordia are initiated more or less simultaneously at an infection site (Callaham and Torrey 1977). In *Comptonia*, many more primary nodule primordia are formed initially than in either *Casuarina* (Torrey 1976) or in *M. gale*, where one to three usually develop in response to each infection. In all of these species, nodule primordia form and develop sequentially, one lobe developing to the transitional state before the next nodule primordia are initiated just below the transitional zone (Fig. 5), which marks the region where the swollen nodule lobe changes to elongating nodule root.

In *M. gale*, cell elongation begins in the nodule root after a period of arrest and then continues uninterrupted until the nodule root reaches its final length. Throughout its elongation, the root-meristem-like terminal organization of a root is maintained, which is most clearly evident in longitudinal section. The terminal structures from nodule roots of *M. gale* 1.8, 2.0 mm, and 8.0 mm in overall length are shown in Figs. 6–8, respectively. Relatively little change in the terminal structure is evident during nodule root development.

Figure 5 is a near-median longitudinal section of the basal portion of an elongate nodule root showing the nodule lobe from which it developed and the transition region between the swollen lobe and the narrower nodule root. Further primordial nodule lobes at the base of the nodule root are cut obliquely. The endophyte which occupies the cortical cells of the nodule lobe is not seen in the nodule root itself. In the zone of transition of lobe to root, the cell walls in this transition zone stain darkly and many cells contain tannins. No clear anatomical barrier to endophyte invasion of the root itself is evident. In this figure, the curvature of the nodule root resulting from a geotropic response is seen. Extensive intercellular air passages which occur in the nodule root abruptly terminate at the transition zone.

Since nodule roots formed in water culture are not subject to abrasion from contact with soil, little or no sloughing off of cells occurs and terminal root cap formation appears to be slight (Figs. 6–8). The most distal cell layers show thickened walls with typical metachromatic staining with toluidine blue. About six cell layers are easily distinguished as part of the root cap by their distinctive starch deposits. Lateral displacement of the outermost cap cells along the periphery of the root has occurred but only slightly. In nodule roots, there is a notable absence of the continuous root cap formation which characterizes normal root apical development.

Also striking is the early development of intercellular spaces in the nodule root cortex. As soon as the nodule root begins elongation, intercellular spaces form by what appears to be active dissolution of intercellular cementing material in inner and middle cortical cell layers as close as 200–300 μm



FIGS. 5–8. Sections of nodule roots of *M. gale*. Fig. 5. A longitudinal section at the base of a developed nodule root showing the swollen nodule lobe with two nodule root primordia (*nrp*) of different ages cut in oblique section. At lower left, cortical cells of the nodule lobe show well established endophyte (*en*). The central cylinder of the nodule lobe is near median but becomes oblique at the transition zone (*tz*) and is not seen in the nodule root because of its marked geotropic curvature. × 80. Figs. 6–8. Median longitudinal sections of the nodule root apex at different stages of development. Intercellular spaces (small arrows), root cap cells (*rc*), central cylinder (*cc*), mitosis (small arrowheads), epidermis (*e*). Fig. 6. A nodule root 1.8 mm in length. × 225. Fig. 7. A nodule root 2 mm in length. × 225. Fig. 8. A nodule root 8 mm in length. × 240.

from the root apex (Fig. 6). Even at levels where active mitosis occurs in the central cylinder, intercellular spaces have formed in the cortex (Fig. 7). Intercellular space formation remains active in this differentiating region just proximal to the apex and gives rise to air channels along the full length of the nodule root. The integrity of the root structure is maintained by the continuous epidermis and by one or two intact outer cortical cell layers abutting the epidermis (Figs. 8, 11, 12).

Proximal to the root cap, the central procambial cylinder is clearly marked by smaller cell size and dense cytoplasm. Mitotic activity can be observed scattered throughout the ground meristem and procambium with cell divisions oriented largely so as to form new cell walls perpendicular to the long axis of the root (Fig. 7). Cells of the central cylinder elongate markedly but show relatively little radial enlargement. Mature differentiated elements of xylem and phloem occur most prominently at the base of the root but can be traced to within a few millimetres of the tip in roots which have stopped elongation (Figs. 9–12).

Figures 9–12 show transections of a 3.0-cm-long nodule root of *M. gale* cut at different levels from base to apex. The nodule root cut in transection near its base (Fig. 9) is composed of primary tissues, a continuous intact single-cell-thick epidermal layer and a wide cortex with ramifying intercellular spaces (cf. Fig. 5). The innermost cortical layer is a ring of dark-staining cells comprising the endodermis. No intercellular spaces are evident in tissues within this layer. Figure 10 is an enlarged view of the central cylinder at the same level as Fig. 9, showing the simplified arrangement of vascular tissues with a roughly radial pattern of primary xylem alternating with primary phloem in a triarch arrangement. The endodermis and many of the cells of the central cylinder show tannin deposits.

The most distinctive tissue of the nodule root is the aerenchymatous cortex, referred to by Fletcher (1955) as trabeculate. At midlength (Fig. 11), radial columns of cortical cells, some highly distorted, maintain the cylindrical shape of the root and the elaborate intercellular space system. A similar structure was observed by Fletcher in normal lateral roots of *M. gale* grown in water culture. Even near the nodule root tip (Fig. 12), these air passages are extensive, having developed immediately below the apex (Fig. 8).

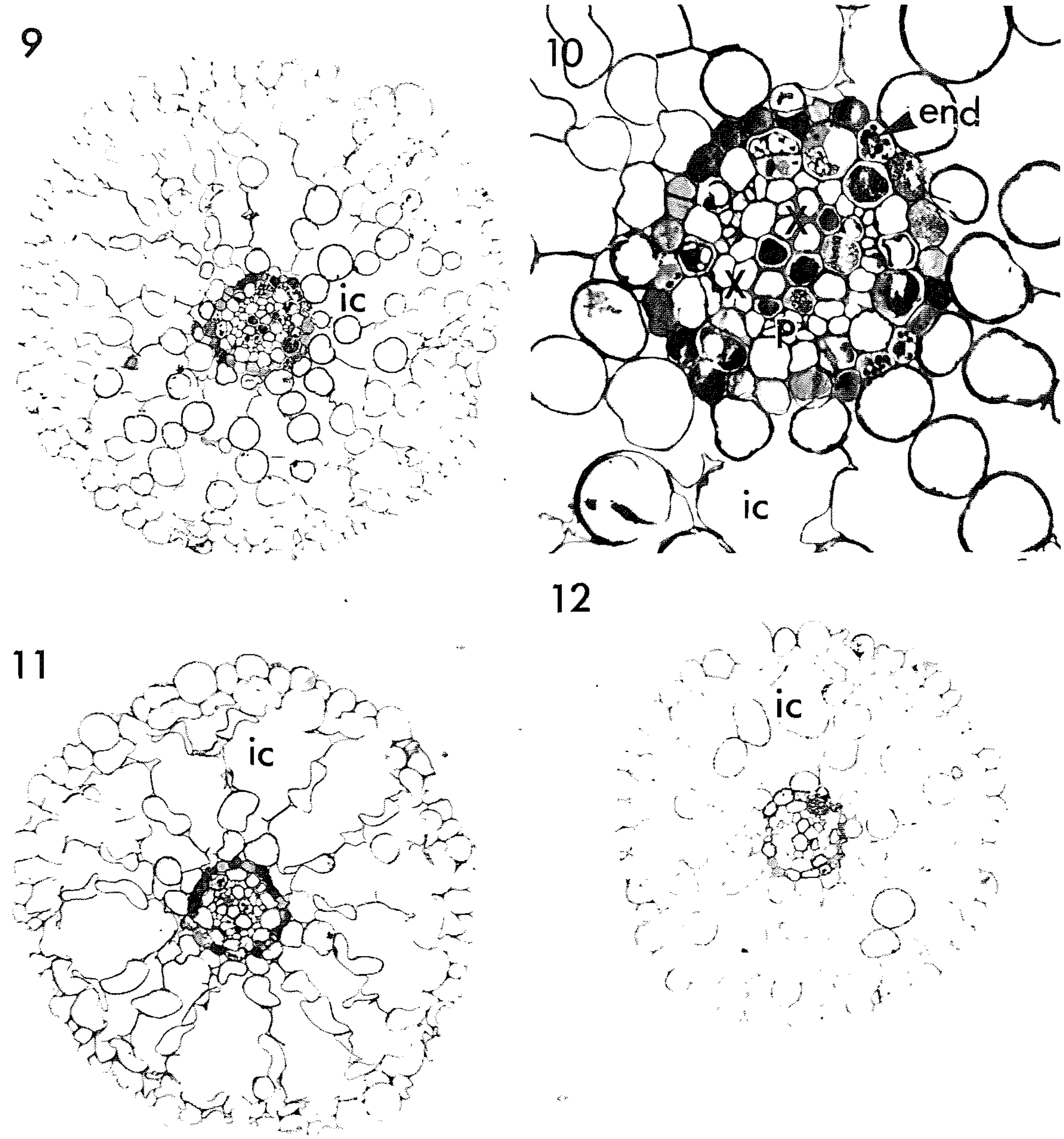
Although a detailed anatomical study of normal lateral roots has not been made, observations indicate that root cap formation is much more extensive in lateral roots, that they elongate in an indeterminate manner, sometimes matching the main axis in length, and that they grow downward or plagiotropically.

Discussion

Nodule roots are highly modified lateral branches both in their origin and in their development. The nodule lobe apex originates from cell derivatives of the pericycle, endodermis, and cortex of the root axis from which it develops. At an early stage, the cortical tissues are invaded by the actinomycetous endophyte whose presence appears to induce a state of temporary growth arrest. A swollen nodule lobe with hypertrophied cortical cells filled with filamentous and vesiculate endophyte is formed. Thereafter, from the terminal papillalike apex of the nodule lobe develops the nodule root proper, which elongates slowly and continuously in a vertically upward direction until it reaches its final length and stops growth. The transition from nodule lobe to nodule root is abrupt. The former structure shows limited intercellular spaces and contains infected cortical layers and an outer corklike periderm; the nodule root is highly aerenchymatous, contains no endophyte, and while rootlike in appearance, shows no root hairs, no well developed root cap, and exhibits a simplified primary tissue structure and strong negative geotropism.

Deduction of the ontogeny of the root apex from static histological sections is not possible (Torrey and Feldman 1977). Although the nodule root apex is structurally similar to a normal root apex, we could not determine whether the pattern of cellular activity was the same. Mitotic activity with almost random distribution is seen in the terminal millimetre of the nodule root apex sampled at different ages. Mitoses were infrequently observed in the region of the root cap initials and seemed to predominate in apical areas destined to form cortex and pericycle of the mature structure. Analyses of nodule root apical activity using [^{3}H]thymidine incorporation and autoradiography will be reported elsewhere.

Several unusual features of these nodule roots have been studied in the past and have led to interesting speculations as to cause or function. A clearly convincing demonstration of the physiological basis for the negative geotropism of these roots is lacking. Silver *et al.* (1966) attributed this response to nondetectable low indoleacetic acid content of the nodules associated with a high indoleacetic acid oxidase activity. Negative geotropism in roots can be induced experimentally by treatment with synthetic growth regulators such as naphthylphthalamic acid, claimed to block auxin



FIGS. 9–12. Transverse sections cut from a 3.0-cm-long nodule root of *M. gale*. Intercellular spaces (*ic*). Fig. 9. Section cut near nodule root base showing central cylinder with aerenchymatous cortex. × 230. Fig. 10. Enlarged view of central cylinder of nodule root in Fig. 9 showing intercellular spaces (*ic*) of the cortex, the central cylinder with endodermis (*end*), primary xylem (*x*), and primary phloem (*p*). Note the extent of tannin deposits. × 690. Fig. 11. Section cut 14 mm proximal to nodule root apex. × 230. Fig. 12. Section cut 7 mm proximal to nodule root apex. × 230.

transport (Mentzer and Nétien 1950) or with presumed auxin antagonists (Rufelt 1957). The peculiar nature of the very reduced root cap in nodule roots suggests a possible explanation related to the idea that the root cap may function in the normal geotropic response by inhibition of root cell elongation (cf. Wilkins (1975) and Audus (1975)). These possibilities need further research.

It has been suggested (Bond 1957) in plants such as *M. gale* which can grow with its nodulated roots totally immersed in water, that the nodule roots serve to transport gases to the region at the base occupied by the N_2-fixing endophyte. Oxygen is essential for the fixation process as well as a supply of dinitrogen. Gas diffusion to the nodule lobe would be markedly facilitated by the presence of the highly elaborated intercellular space system which occupies a good portion of the volume of the nodule root. This possible role has been explored by Tjepkema and will be discussed elsewhere (Tjepkema 1978).

However, the endophyte is severely restricted in its distribution, being totally excluded from the nodule root itself and occupying only specific cell layers in the cortex of the nodule lobes. The basis for these restrictions is not understood. The actinomycete appears to be microaerophilic (Callaham *et al.* 1978) and may be unable to grow under the more aerobic state of the nodule root cortex, or it may be that oxidized cellular products in specific cell wall layers serve as barriers to its penetration. Such an explanation might be the basis for the effectiveness of the barrier to inward radial invasion at the level of the endodermis. Further work on this restriction in penetration of cells is needed as it may provide clues to the nature of susceptibility to infection and the basis of infectivity in general.

Acknowledgments

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