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A Survey of Cell Biology

Editor-in-Chief

G. H. BOURNE

K. W. JEON

M. FRIEDLANDER

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G. H. BOURNE
St. George's University School of Medicine
St. George's, Grenada
West Indies

Editors

K. W. JEON
Department of Zoology
University of Tennessee
Knoxville, Tennessee

M. FRIEDLANDER
Jules Stein Eye Institute
UCLA School of Medicine
Los Angeles, California

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Morphogenesis and Fine Structure of *Frankia* (Actinomycetales): The Microsymbiont of Nitrogen-Fixing Actinorhizal Root Nodules

WILLIAM NEWCOMB AND SUSAN M. WOOD

Department of Biology, Queen's University
Kingston, Ontario, Canada K7L 3N6

I. Introduction

The genus *Frankia* of the order Actinomycetales consists of a diverse group of bacteria often exhibiting hyphal growth. Members of the genus *Frankia* are characterized by the ability to form nitrogen-fixing nodules on the roots of certain woody angiosperms (Becking, 1974) and may be distinguished from other actinomycetes by their morphogenetic patterns *in vivo* and *in vitro;* cell wall chemistry, serology, and DNA homology (Lechevalier, 1984); and surface laminations of the spore cell wall (Berg and Lechevalier, 1985). Both the nodules induced by *Frankia* and the species of plants which bear these nodules are termed actinorhizal (Torrey and Tjepkema, 1979). Previously these nodules were referred to as nonlegume nodules, which is a confusing term because *Rhizobium* can induce nodulation on the nonleguminous angiosperm *Parasponia* (family Ulmaceae) (Trinick, 1973, 1979).

A. Importance of *Frankia* Symbioses

The ability of *Frankia* to induce root nodules, which may provide part or all (the latter occurs usually only under laboratory conditions) of the nitrogen requirements of the actinorhizal host plant is of considerable importance to forestry, land reclamation, natural ecosystems, and plant genetic engineering. In many field situations, low levels of combined nitrogen in the soil may be limiting to plant growth; thus, the presence of root nodules, which chemically reduce (fix) atmospheric (molecular) nitrogen, overcomes deficiencies of ammonium and nitrate in the soil and greatly aids plant growth.

While actinorhizal plants are not important sources of food for people and their domesticated animals, these plants are nevertheless of consider-

able economic importance, particularly for forestry and land reclamation. The red alder, *Alnus rubra*, can be planted as a nurse crop for the lumber species Douglas fir, *Pseudotsuga taxifolia*, which uses the fixed nitrogen released by decaying alder leaf litter; however, plantings using red alder are not as profitable as pure Douglas fir stands which rely upon commercial fertilizer applications (Atkinson *et al.*, 1979). In addition, alder trees may influence the mineralization of soil nitrogen. *Alnus rubra* is suitable for land reclamation, adds nitrogen to the soil, and produces wood suitable for lumber or pulp (Atkinson *et al.*, 1979; National Academy of Science, 1980). The autumn olive (*Elaeagnus umbellata*) may be interplanted with black walnut (*Juglans nigra*) trees. After 7 years under these conditions black walnut was 134% taller than black walnut trees planted alone (Funk *et al.*, 1979). Lumber of mountain mahogany (*Cercocarpus ledifolius*), which is a native plant of northern California, is sold commercially for special uses.

Casuarina, which is widely distributed in tropical regions, is reported to be the world's best firewood (in terms of kcal/unit mass) (National Academy of Science, 1980). *Casuarina* is a valuable tree because firewood is an essential but often limited commodity in developing countries (National Research Council, 1984). In addition, *Casuarina* acts as a windbreak or erosion controller, supplies lumber for building construction, and provides bark for leather tanning in Madagascar (National Academy of Science, 1980). The wood of two species closely related to *Casuarina*, *Allocasuarina torulosa* and *A. fraseriana*, is used in woodturning in Australia (J. G. Torrey, personal communication). The sweet fern, *Comptonia peregrina*, and autumn olive, *Elaeagnus umbellata*, are valuable for the revegetation and landscaping of nitrogen-poor sites such as strip mines and highway roadsides (Carpenter and Hensley, 1979). *Comptonia* frequently recolonizes burned sites. *Myrica gale*, which is in the same family as *Comptonia*, returns to the soil about 70% of the nitrogen it fixes annually and thus is an important source of combined nitrogen (Schwintzer, 1984). Since *Frankia* forms nodules on a diverse group of angiosperms, an improved knowledge of the factors controlling actinorhizal nodule development could lead to advances in plant genetic engineering, extending the range of plants nodulated by *Frankia*.

B. The Genus *Frankia*

Considerable interest in the biology of *Frankia* and actinorhizal species has occurred since 1978, when the first isolation of a *Frankia* strain (HFPCp11), *in vitro* culture and reinfection of the host plant, *Comptonia*,

was reported (Callaham *et al.*, 1978) and verified (Lalonde, 1978). *Frankia* grows *in vitro* predominantly in the form of septate hyphae and under suitable conditions will form *Frankia* vesicles, the presumptive site of nitrogen fixation, and spore-containing sporangia (see Sections IV,D,2 and IV,E,1). Similar structures also form *in vivo* under suitable conditions. Breakthroughs in the isolation and culture of *Frankia* (Callaham *et al.*, 1978; Tjepkema *et al.*, 1986) have permitted the isolation and culture of the endophytes of many other actinorhizal species (Baker, 1982). In turn, the availability of pure cultures of numerous *Frankia* strains has stimulated research on actinorhizal nodules by allowing the routine culture of single strain inocula for either commercial applications or laboratory studies. *In vitro* cultures and dependable supplies of nodules have facilitated cross-inoculation studies, studies of the morphogenesis and ultrastructure of the microsymbiont, and physiological studies which would be either difficult or impossible to conduct using intact or crushed nodules as inocula.

This review discusses the structure and morphogenesis of the microsymbiont *in vivo* and *in vitro,* particularly in relation to the development and physiology of the symbiosis. In this review the *in vivo* and *in vitro* aspects of *Frankia* are treated separately, partly for historical reasons but more importantly, to aid the uninitiated reader. We believe that it is less confusing to treat the microorganism as it exists within the nodule, separately from the cultured microbe, which is more amenable to experimental manipulation. Cross-references to *in vitro* and *in vivo* observations are made where appropriate. Limitations to published observations are pointed out with the aim of stimulating future studies, and comments on possible directions for further research are made throughout the text in an attempt to relate these ideas to published observations and to avoid unnecessary repetition.

Important relevant papers are cited, but the reader should be aware that we have not attempted to provide a complete bibliography in the limited space of this review. The reader is referred to an excellent review on physiological aspects of the actinorhizal symbiosis (Tjepkema *et al.*, 1986), and two other recent reviews have dealt with the infection process (Berry, 1983, 1986), genetics of *Frankia* (Normand and Lalonde, 1986), and taxonomy of *Frankia* (Lechevalier and Lechevalier, 1986). Several earlier reviews (Quispel, 1974; Becking, 1975, 1977; Silvester, 1977; Torrey 1978; Akkermans and van Dijk, 1981) are also useful for information on other aspects of the actinorhizal symbioses. The reader is also directed to the proceedings of the several international meetings on actinorhizal symbioses; these proceedings are listed in Table I.

TABLE I
PROCEEDINGS OF INTERNATIONAL *Frankia*–ACTINORHIZAL CONFERENCES

Meeting	Meeting date	Publication
Symbiotic Nitrogen Fixation in Actinomycete-Nodulated Plants, Harvard Forest, Petersham, Massachusetts	April 1978	*Bot. Gaz.* **140** (Suppl.); S12–S126
International Conference on the Biology of *Frankia*, University of Wisconsin, Madison, Wisconsin	August 1982	*Can. J. Bot.* **61**, 2768–2967
Workshop on *Frankia* Symbioses, Noordwijkerhout and Wageningen, The Netherlands	September 1983	*Plant Soil* **78**, 1–258
Frankia and Actinorhizal Plants, University of Umea, Umea, Sweden	August 1984 August 1986	*Plant Soil* **87**, 1–208 *Physiol. Plant.* **70**, 235–377

II. Host Plants

Frankia has been observed to form nitrogen-fixing symbioses on approximately 220 plant species of dicotyledonous angiosperms belonging to eight families and 23 genera (Torrey and Tjepkema, 1983; Moiroud and Gianinazzi-Pearson, 1984) (Table II). The actinorhizal host species constitute a diverse collection of taxa. Not all species within a genus are necessarily actinorhizal (Table II), although it is important to note that not all species in many genera which have actinorhizal species have been examined carefully for nodulation. Interestingly, no leguminous species or monocotyledonous plants are known to form nitrogen-fixing symbioses with *Frankia*. Most actinorhizal host plants are perennial woody shrubs or trees.

The reports of nodulation in the genus *Rubus* (Rosaceae) (Bond, 1976b; Becking, 1984) have been seriously questioned because subsequent collections by Stowers (1985) revealed *Myrica rubra*, which was nodulated, as the only actinorhizal plant in the Indonesian site from which *Rubus ellipticus* nodules were reported to be collected by Becking (1984). Attempts to find nodulated plants of *R. ellipticus* and ten other *Rubus* species in Pakistan proved futile (Wheeler, 1981). Clearly further studies are necessary to establish whether *Rubus* is nodulated only in particular sites and to elucidate the distinguishing features of these sites.

TABLE II
TAXONS OF ACTINORHIZAL ANGIOSPERMS[a]

Order	Family	Genus
Casuarinales	Casuarinaceae	*Allocasuarina*
Casuarinales	Casuarinaceae	*Casuarina*
Casuarinales	Casuarinaceae	*Gymnostoma*
Coriariales	Coriariaceae	*Coriaria*
Cucurbitales	Datiscaceae	*Datisca*
Fagales	Betulaceae	*Alnus*
Myricales	Myricaceae	*Myrica*
Myricales	Myricaceae	*Comptonia*
Rhamnales	Elaeagnaceae	*Elaeagnus*
Rhamnales	Elaeagnaceae	*Hippophae*
Rhamnales	Elaeagnaceae	*Shepherdia*
Rhamnales	Rhamnaceae	*Ceanothus*
Rhamnales	Rhamnaceae	*Colletia*
Rhamnales	Rhamnaceae	*Discaria*
Rhamnales	Rhamnaceae	*Kentrorhamnus*
Rhamnales	Rhamnaceae	*Retanilla*
Rhamnales	Rhamnaceae	*Talguenea*
Rhamnales	Rhamnaceae	*Trevoa*
Rosales	Rosaceae	*Cercocarpus*
Rosales	Rosaceae	*Chamaebatia*
Rosales	Rosaceae	*Cowania*
Rosales	Rosaceae	*Dryas*
Rosales	Rosaceae	*Purshia*
Rosales	Rosaceae	*Rubus*[b]

[a] Modified and updated from Torrey (1978) and Akkermans and van Dijk (1981).

[b] Despite at least two published reports of nodulation (Bond, 1976b; Becking, 1984), Stowers (1985) was unable to find nodulated members of this genus (see Section IV,D,3).

III. Nodule Morphology and Anatomy

A. Differences between Actinorhizal and Leguminous Nodules

Actinorhizal nodules differ in development, morphology, and anatomy from *Rhizobium*-induced root nodules, despite the basic similarities in the two types of symbiosis. These differences and similarities are summarized in Table III. In many, but not all, actinorhizal and leguminous plants, the first step in nodule initiation involves invasion of a deformed

TABLE III
FEATURES OF ACTINORHIZAL AND LEGUMINOUS ROOT NODULES

Feature	Actinorhizal	Leguminous
Host plant	Certain perennial woody dicots	Certain members of the family Fabaceae
Causal organism	Actinomycetes of genus *Frankia*	Gram-negative bacteria of genera *Rhizobium* and *Bradyrhizobium*
Entry site of causal organism into root	Root hair (usually) or intercellularly through epidermis	Root hair (usually) or intercellularly through epidermis
Origin of nodule cells	Pericycle and endodermis of infected root	Cortex of infected root
Dissemination of bacteria within nodule	Growth of *Frankia* hyphae through host cell walls	Formation and growth of infection threads from which bacteria escape endocytotically; in some species, infected cells may undergo mitotic divisions
Tissue organization of nodule lobes	Similar to lateral roots; central vascular cylinder; infected cells in middle cortex	Vascular bundles in nodule cortex outside central zone of infected cells
Nodule shape	Multilobed; lobes cylindrical-shaped	Usually single-lobed; lobes cylindrical-, spherical-, or collar-shaped
Nodule roots	Present in *Myrica*-type nodules	Usually absent
Ploidy of infected cells	*Coriaria* and *Datisca* multinucleate; others possibly polyploid (on basis of nuclear size, not c values)	Usually polyploid (based on c values and chromosome numbers)
Hemoglobin	Present in several; in low concentration or absent in a few	Present in all effective nodules
Peribacteriod membrane	Absent but continuous profiles of host plasma membrane surround encapsulated endophyte	Present in all effective and many ineffective nodules
Peribacteroid space	Absent	Present between bacteria and peribacteroid membrane
Capsule	Present between *Frankia* cell wall and host plasma membrane	Absent
Site of nitrogenase	Symbiotic vesicles except in *Casuarina*, *Allocasuarina*, and *Gymnostoma*	Differentiated intracellularly released bacteria

root hair by the microsymbiont. During this invasion in developing actinorhizal nodules, the actinomycete becomes encapsulated by a polysaccharide layer, forming the so-called capsule, Presumably, this layer is produced by the host cytoplasm because the capsule does not form *in*

vitro. The origin of the capsule might be confirmed by the use of antibodies specific for *Frankia* cell surface polysaccharides. Thus, the microbe is separated from the host cytoplasm by the capsule and host plasma membrane. This differs from the situation in most leguminous nodules, in which the invading rhizobia initiate the formation of an infection thread. It is from these infection threads that the rhizobia escape endocytotically "into" (see Section IV,C,1) the host (legume) cytoplasm.

While the encapsulation process within the root hair is proceeding, mitosis in the nearby cortical cells is stimulated. The bacteria, whether they be *Rhizobium, Bradyrhizobium* (Jordan, 1982), or *Frankia,* invade the newly divided cortical cells. (Hereafter, for the sake of simplicity both *Rhizobium* and *Bradyrhizobium* are referred to as *Rhizobium.*) In the case of a *Rhizobium*-induced nodule, the patterns of plant cell division and growth of cortical cells determine whether the nodule is spherical-, cylindrical-, or collar-shaped (Newcomb, 1981a). Generally, *Rhizobium*-infected nodules consist of a single lobe, although mutiple-lobed cylindrical-shaped nodules occasionally occur, as in *Pisum sativum* (Syono *et al.*, 1976).

B. Nodule Morphology

Actinorhizal nodules generally consist of numerous conical-shaped lobes (Fig. 1), each of which is a modified lateral root. Thus, in the case of most actinorhizal nodules, a proliferation of cortical cells near the infected deformed root hair leads to the differentiation of modified lateral roots, which become the lobes of the nodules. Actinorhizal nodules are perennial and may be quite large (Fig. 1). In the case of *Myrica gale*, each nodule lobe is probably physiologically active for only one growing season (Schwintzer *et al.*, 1982). It is not known if this is also true for other actinorhizal nodules.

The presence or absence of determinate nodule roots distinguishes the two morphological types of actinorhizal nodules. The *Alnus* type of actinorhizal nodule lacks nodule roots and has a corralloid knobby appearance due in part to the dichotomously branching pattern of the nodule lobes (Figs. 1 and 2). In *Alnus japonica* single-lobed nodules formed when no combined nitrogen was added to the growth medium. Higher levels of combined nitrogen resulted in the formation of few larger multilobed nodules, while coralloid-shaped nodules developed under conditions of low phosphorous nutrition (Burgess and Peterson, 1987b). The *Myrica* type of actinorhizal nodule bears nodule roots which arise from the distal region of each mature nodule lobe. Immature nodule lobes lack nodule roots but sometimes have a caplike structure at the distal end. *Myrica*-type nodules

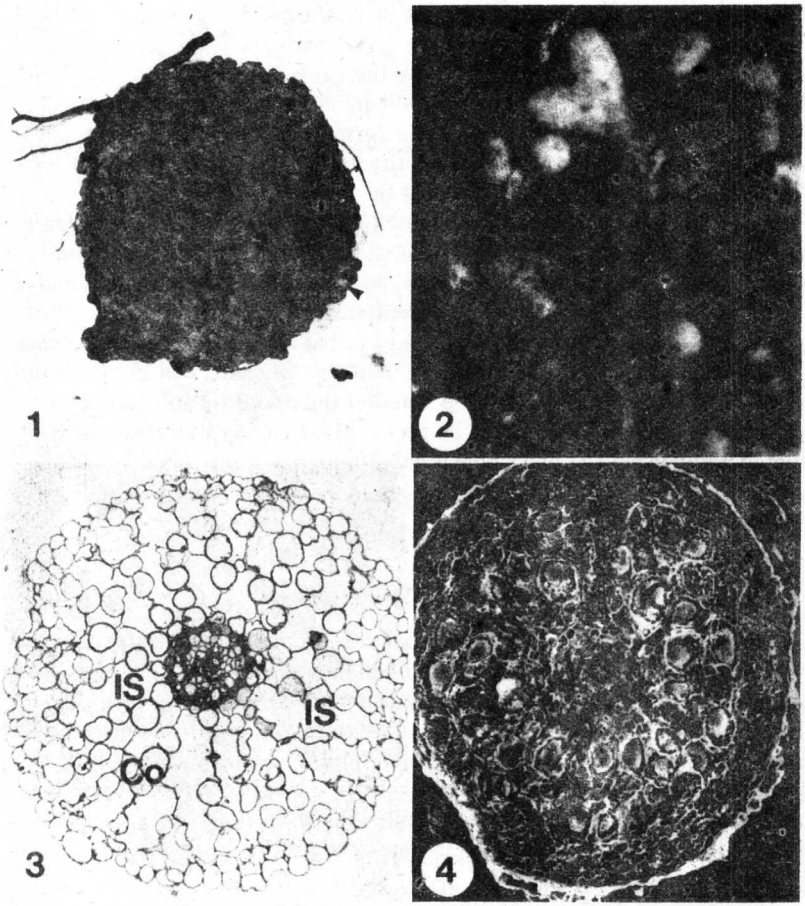


FIG. 1. Whole nodule of *Dryas drummondii* and attached roots. The nodule consists of numerous nodule lobes (arrows) and has a flattened appearance because it was growing next to a flat rock. ×1.7. Reproduced with permission from Newcomb (1981a). *Can. J. Bot.* **59,** 2500.

FIG. 2. Dichotomously branched nodule lobes of *Cercocarpus ledifolius*. ×7.4

FIG. 3. Transverse section of nodule root of *Myrica gale*. Large intercellular spaces (IS) are present among the cortical (Co) cells. The central vascular cylinder (VC) is also shown. ×16. Reproduced with permission from Torrey and Callaham (1978). *Can. J. Bot.* **56,** 1357.

FIG. 4. Scanning electron micrograph of a nodule lobe of *Cercocarpus ledifolius*. Nodule lobe was frozen in liquid N_2, fractured fortuitously in a transverse plane, sputter coated, and examined in SEM. Large infected cells (IC) are present in the cortex. The central vascular cylinder (VC) is also shown. ×60.

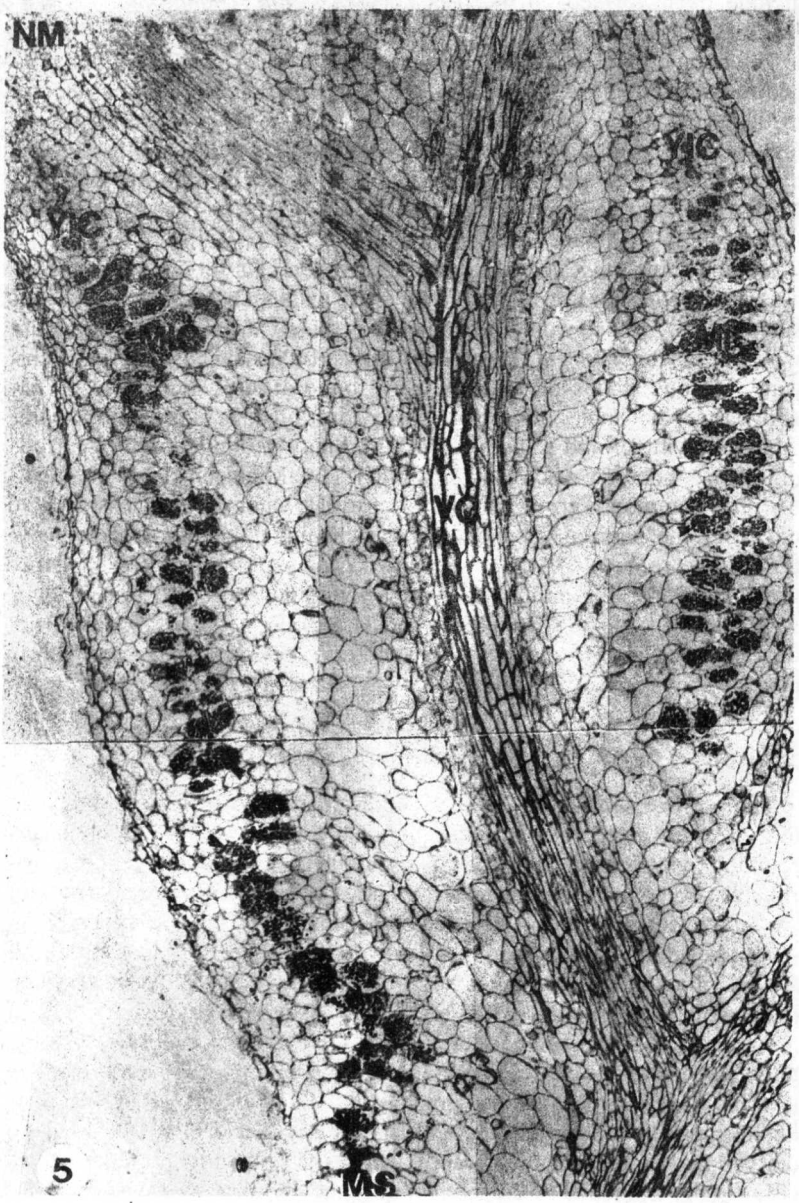
include those formed on the roots of *Casuarina, Comptonia, Myrica, Datisca,* and *Gymnostoma.*

The nodule roots of these genera may show either positive or negative geotropism, but most commonly grow upward. There is experimental evidence to suggest that these upwardly growing roots may play an important role in facilitating gas exchange between the nodule and atmosphere, by providing a greater surface area for oxygen absorption (Tjepkema, 1978, 1979) and large intercellular spaces (Fig. 3) for oxygen transport (Bond, 1952; Callaham and Torrey, 1977).

Nodule roots only affect the rate of nitrogen fixation (as measured by acetylene reduction) at relatively low pO_2 values (Tjepkema, 1979), but such low pO_2 values would be found immediately around the roots in the wet sites in which *M. gale* commonly grows. In Massachusetts and Michigan, vigorous stands of *M. gale* were found at sites more than 10 cm above the water table, but growth was poorer on soils closer to the water table (Schwintzer and Lancelle, 1983) and may be limited during periods of spring flooding (Schwintzer, 1985). In Michigan and Scotland, *M. gale* commonly grows at sites where the water table is less than 10 cm from the surface (Spence, 1964; Schwintzer, 1978). Interestingly, the nodule roots of *M. gale* seedlings, grown on a sand soil moisture gradient, were long and thick in wet soils and short and thin in drier well-aerated soils (Schwintzer and Lancelle, 1983). Nodule root development in *M. gale* was reported to be greater in wet poorly aerated soils than in drier sites in Scotland (Sprent and Scott, 1979).

C. Nodule Anatomy

The arrangement of tissues in an actinorhizal nodule lobe is similar to that of lateral roots. Actinorhizal roots have a central stele which is surrounded by a centrifugal sequence of endodermis, several layers of cortical cells, and epidermis or periderm. Only certain layers, usually those of the middle cortex, become invaded by hyphae of the microsymbiont (Fig. 4). However, this point merits further attention because most structural studies of actinorhizal nodules have concentrated on the cytology of the infected cells and the fine structure of the prokaryotic microsymbiont and not the arrangement of nodular tissues. It has been reported that the infected cells only occur on one side of the stele in *Coriaria* and *Datisca* nodules (Akkermans and van Dijk, 1981). Whether this occurs in other actinorhizal nodules is unknown. Most of the cells of each nodule lobe are derived from a meristem (correctly called the nodule lobe meristem) located at the distal end of the lobe. Within the nodule lobe are gra ents of developing cells, with the youngest infected cells located near the nodule


NM
VC
VC
VC
MS
5
MS

lobe meristem and the mature infected cells located more proximally (Fig. 5). Similarly, different stages of developing vascular tissue cells may be observed in distal and proximal regions of the central vascular cylinder of sctinorhizal nodules.

IV. Nodule Development

A. Infection Process

The infection process involves the invasion of a deformed root hair by *Frankia* in *Alnus glutinosa* and *A. rubra* (Pommer, 1956; Becking, 1968; Angulo Carmona, 1974; Angulo Carmona *et al.*, 1976; Lalonde, 1977a,b; Berry and Torrey, 1983; Berry *et al.*, 1986), *Casuarina cunninghamiana* (Callaham and Torrey, 1977; Callaham *et al.*, 1979), *Comptonia peregrina*, *Myrica cerifera*, and *M. gale* (Callaham *et al.*, 1979). This event often involves only a few deformed root hairs on a root and thus is particularly difficult to document at the ultrastructural level. The lack of similar reports for other actinorhizal species may in part represent this difficulty.

It appears that only one infected deformed root hair is necessary to initiate nodule development in *A. rubra* (Berry *et al.*, 1986). However, it is interesting that the number of infected root hairs may be related to the amount of inoculum. Under inoculum-limiting conditions only a few nodules were produced on *Comptonia* and just one infected root hair was associated with each nodule (Callaham and Torrey, 1977). Use of heavier inocula in the form of nodule suspensions or cultures of *Frankia* HFPCpI1 (Torrey and Callaham, 1979) resulted in the formation of abundant nodules on seedlings of *M. gale*; root hair infections were very numerous and more than one could sometimes be observed within a single 1-μm section (Callaham *et al.*, 1979). While the use of large concentrations of inoculum may produce more than one infected root hair per nodule, only one of the infections per nodule produced a cortical infection, and the other infections developed later or aborted (Callaham *et al.*, 1979).

FIG. 5. Light micrograph montage of a near-median longitudinal section of nodule lobes from a 6-week-old *Comptonia peregrina* nodule showing the nodule lobe meristem (NM), young infected cells (YIC), mature infected cells (MIC) containing symbiotic vesicles (arrows), infected cells containing mature sporangium (MS), and the vascular cylinder (VC). ×125. Micrograph courtesy of K. A. VandenBosch. Reproduced with permission from VandenBosch and Torrey (1985). *Am. J. Bot.* **72**, 99.