C. P. Vance and L. E. B. Johnson
Agricultural Research, SEA, USDA, and University of Minnesota, St. Paul

Nodulation: *A* Plant Disease Perspective

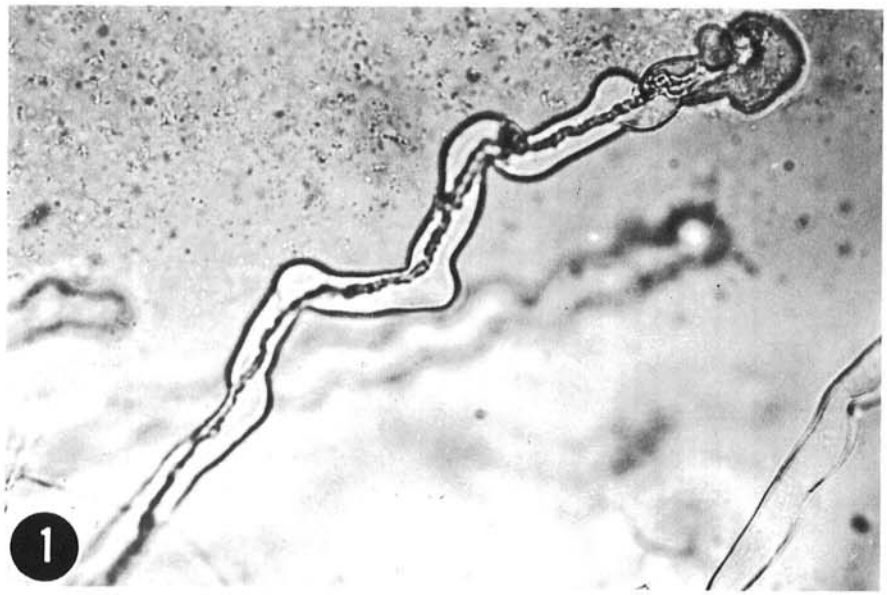


Fig. 1. Infection thread and curled root hair of alfalfa 5 days after inoculation. (×624)

Biological nitrogen fixation is second only to photosynthesis as the most important biochemical process on earth. Biological fixation contributes an estimated 140 million metric tons of nitrogen annually to the earth, of which 80% is fixed via symbiotic associations and 20% by free living organisms. The biological cost of fixed nitrogen is substantial, about 2.5% of the primary photosynthesis on land (6). To produce an equivalent amount of nitrogen through the Haber-Bosch process would require about 9.25×10^{12} cubic feet of natural gas. The high cost of nitrogen via biological fixation and chemical fixation makes it imperative that we understand and improve biological nitrogen fixation.

Legumes are a major source of protein, oil, and forage for human and animal consumption and are among the world's most important crops. About 85% of nitrogen fixed in agricultural soils comes from pulse and pasture legumes. Because of great diversity in plant characteristics, legumes are widely adapted. One of the primary reasons for this wide adaptation is the association of legumes with the *Rhizobium* bacteria. When effective, this association enables the plant to fix gaseous N_2 and depend less on soil nitrogen. In numerous instances, however, the association is ineffective and becomes a detriment to the plant.

Joint contribution from the Minnesota Agricultural Experiment Station and the ARS-USDA. Paper No. 11,494, Scientific Journal Series, Minnesota Agricultural Experiment Station.

How an Effective Plant-Bacteria Association Is Established

Before an effective N_2-fixing association can occur, legume roots must become associated with *Rhizobium* bacteria. When this happens, the plant provides an energy source and an ecological niche for the bacteria and the bacteria provide a source of fixed nitrogen for the plant. The effective plant-*Rhizobium* association is established by stimulation of bacterial growth or bacterial attraction in the vicinity of root hairs, followed by curling of root hairs and bacterial penetration (5,8). Infection threads then develop, and the bacteria migrate into the root cortex through the threads (Fig. 1). Stimulation of cell division in the root cortex by the bacteria and enlargement of cells result in nodule formation. The bacteria multiply in the nodule tissue and develop the capacity to fix N_2.

Rhizobium numbers increase markedly near the root surface, particularly in association with legumes (5,12). Clover plants have been shown to stimulate *R. trifolii*. Although generally most pronounced in infertile soils, stimulation of *Rhizobium* growth may not be specific. *R. japonicum* is stimulated by soybean but is outnumbered 500–1,000-fold by other soil microbes. Bacterial growth is probably stimulated by exudation of such organic compounds as sugars, amino acids, and vitamins from roots. These compounds can be utilized as a source of energy or as growth stimulators.

Studies with legume root exudates indicate that plants can attract *Rhizobium* spp. to their roots. Currier and Strobel (4) found that birdsfoot trefoil exuded a glycoprotein that could specifically attract *Rhizobium* bacteria. Although attraction of bacteria and other organisms to plant roots (chemotaxis) has been noted in several legumes, the specificity of such attractions is not clear. If legume plants do exude proteins or other compounds that attract specific *Rhizobium* bacteria, plant scientists would have a useful tool for selecting plants that specifically interact with superior N_2-fixing strains of bacteria.

Root hairs often curl before being penetrated by the *Rhizobium*. The amount of curling is a function of both the *Rhizobium* strain and the plant cultivar (5,8,12). Nodulation apparently is not limited by curling, because chemicals that increase root hair curling do not increase infection or nodule number. Curling is not always required for infection; many infections occur through straight root hairs. Growth hormones produced by both the bacteria and the plant and the extracellular polysaccharide-protein coat of the bacterium are thought to be responsible

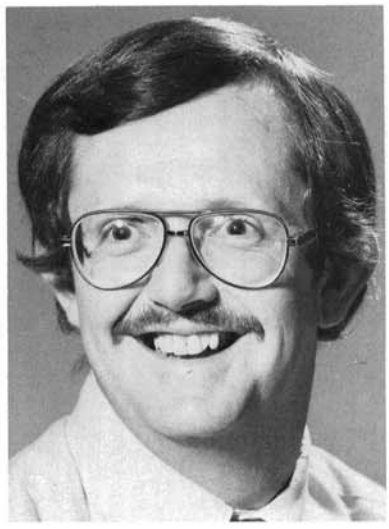
for curling (5,12).

The mechanisms of specificity involved in *Rhizobium* attachment and penetration into legume root hairs are not clear. The binding of a *Rhizobium* sp. to its particular host plant is thought to be mediated through the attachment of root hair glycoproteins (lectins) to specific sugars located on the bacterium (3,10). Whether this binding is specific or general remains in question. Polygalacturonases have been implicated in specificity of alfalfa and clover. *Rhizobium* may either produce or induce in the host plant pectolytic enzymes that are active in degrading the root hair tip to allow bacteria to enter (3,8). These suggestions, however, are not well substantiated. Another postulation is that the root hair invaginates around the bacteria, enclosing them in host tissue. These mechanisms may all interact or be involved sequentially and should not be considered mutually exclusive. Only 1–5% of root hairs become infected, and a low proportion (30%) of those infected result in nodules.

The first microscopically visible sign of infection is swelling and formation of a bright spot in the root hair wall. Cytoplasmic streaming increases near the infection site and the infection thread becomes visible. The infection thread then grows down the root hair toward the root cortex at a rate of 7–10 μm/hr. Studies with alfalfa have shown that the infection thread is similar in chemistry to the root hair wall. The infection thread may be analogous to a papilla response in which the epidermal wall enlarges in reaction to attempted penetration or to mechanical damage.

The Process of Nodulation

Nodules were noted and included in drawings of legume roots as early as the 16th century. The concept that nodules were the result of pathological interactions was widely accepted until the late 19th century. Woronin described the anatomy of a legume nodule in 1867, noted the rod-shaped structures within the nodule, and suggested that nodules were growths caused by bacteria (5). In the late 1880s, Hellriegel and Wilfarth demonstrated that legumes inoculated with microorganisms from soil extracts became nodulated and fixed N_2. Beijerinck demonstrated, through Koch's postulates, that *Vicia faba* nodules were formed by *R. leguminosarum* (5,8). The infection of root hairs and the subsequent development of the nodule were described in detail by Ward and Prazmowski (5). The symbiotic specificity concept—that certain strains of bacteria incite nodules only on certain legumes—was demonstrated in the 1890s by Nobbe and Hiltner. Ineffective nodulation in legumes was described by Beijerinck in 1888 (7) and later by Nobbe and Hiltner (5,7). Major research contributions from plant pathologists accentuate the interest in nodules resulting from the interaction of a bacterium with a plant.



Carroll P. Vance

Dr. Vance is a research plant physiologist with USDA/SEA-AR and the University of Minnesota. His primary interests are physiology and biochemistry of host microbial interactions. He received his Ph.D. from the Plant Pathology Department at Ohio State University, Columbus.

Lois E. B. Johnson

Dr. Johnson is a postdoctoral research fellow in the Department of Agronomy at the University of Minnesota. She has been associated with the USDA Forage Project for the last 10 years. She received her Ph.D. from the Department of Plant Pathology at the University of Minnesota, St. Paul.

Nodules develop after root hair infection. The infection thread can be found in the root cortex about 24 hours after penetration. As the infection thread passes through the cortex, the nuclei of the cortical cells enlarge and adjacent cells may divide.

Whether cortical cells must be polyploid for infection to occur or infection induces polyploidy is not evident. Meristematic activity is initiated in proximity to infection threads. The bacteria within the infection thread may produce or stimulate plant production of auxins and cytokinins in the infected area (8). Studies of pea, lupine, and alfalfa have shown that auxin and cytokinin concentrations are higher in nodule tissue than in root tissue. The transfer of *Rhizobium* nuclear material into plant cells, analogous to infection by *Agrobacterium tumefaciens*, has also been implicated in nodule growth and development. Analysis of base ratios between *Rhizobium* and *Agrobacterium* suggests a close relationship between the two species.

The meristematic tissue in the root cortex divides, giving rise to the nodule (8,12). Infection threads penetrate the nodule cells, then release bacteria; the release mechanism is thought to involve pectolytic and cellulolytic enzymes. After bacteria multiply and fill the cells, nodules synthesize a red protein called leghemoglobin that is important in regulating oxygen tension in the nodule (5,8). Bacteria then show nitrogenase activity and may enlarge appreciably. Enlarged *Rhizobium* bacteria actively fixing N_2 are termed bacteroids. Bacteroids were believed unable to divide, but recent evidence suggests that some may be able to do so. As nodules senesce, bacteroids are lysed but bacteria appear to remain intact.

The similarities between infection by *Rhizobium* and infection by many disease agents are apparent. Can studies of *Rhizobium* infection in legumes offer useful information about disease resistance or other aspects of host-pathogen interaction? Can the contribution of the host and that of the bacterium to infection and development be separated? Answers to these questions will require studies of diverse genetic material. We now have bacterial strains that either are noninvasive or initiate ineffective nodules and plant genotypes that either are resistant to invasion or form ineffective nodules with normally effective strains of *Rhizobium*. These materials show considerable promise in helping to understand infection and nodule development and function.

Concept of Ineffective Nodules

Development of an effective association between the legume host and *Rhizobium* involves a number of specific interactions. Failure at any one of these specific interactions can result in an ineffective

association. Alterations in the environment and either the host or *Rhizobium* genome can cause failure of these specific interactions (2). To evaluate when and how ineffectiveness occurs, we are using strains of *R. meliloti* that form ineffective nodules on most alfalfa genotypes and clones of alfalfa that form ineffective nodules with most effective strains of *R. meliloti*. This diversity of genetic material is enabling us to seek answers to questions relevant to both those interested in plant diseases and those interested in nodule development and function.

Structure of Effective Nodules

Some concept of the structure of effective nodules is needed to evaluate how and why ineffectiveness occurs and how it may be a useful tool. Effective nodules are elongate and cylindrical and have apical meristems (Fig. 2). The nodules have two colored regions: 1) a white one that includes the nodule meristem, cortex, and zone of infection thread invasion and 2) a pink one with cells containing bacteroids in various stages of development; older nodules have a green area indicative of senescence. Vascular bundles that anastomose with the stele of the root are found at the nodule's periphery. Hemispherically shaped meristems are localized at the distal end of the nodule. Infection threads originating from epidermal root hair cells are adjacent to the meristem. Rhizobia released from infection threads enter the host cytoplasm in the thread invasion zone, then increase in number and size and sometimes become pleomorphic (Fig. 3). Cells containing bacteroids grow larger while progressing from early to late symbiotic development (Fig. 2). Ultimately, bacteroids fill the cytoplasm of invaded nodule cells (Fig. 3).

Bacteria-Induced Ineffectiveness

Initially, ineffective nodules induced by the ineffective *R. meliloti* strain appeared similar in structure and development to effective nodules; no apparent differences were observed in meristem, thread invasion, and early symbiotic development. *Rhizobium*-induced ineffective nodules, however,

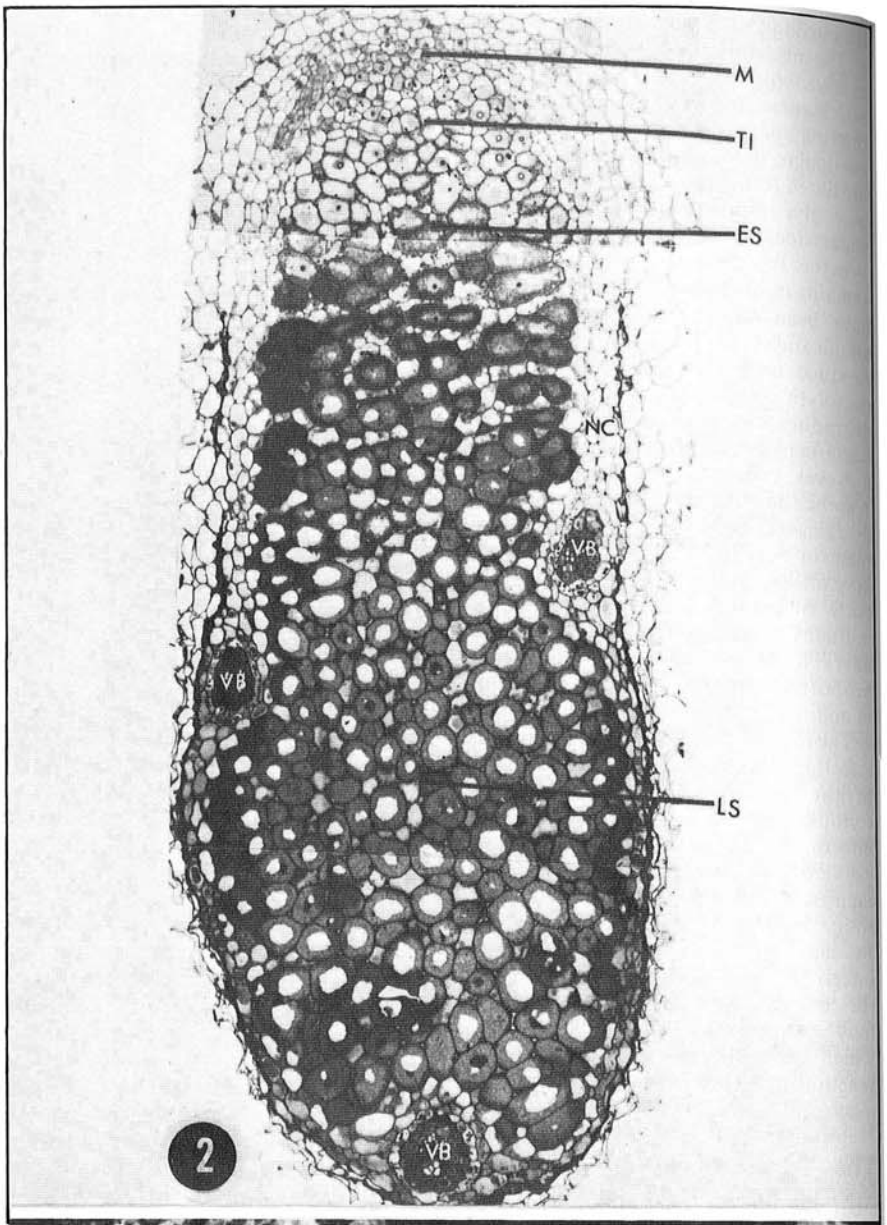


Fig. 2. Longitudinal section of an effective nodule 17–22 days after inoculation. Zones illustrated are: meristem (M), thread invasion (TI), early symbiotic (ES), and late symbiotic (LS). Nodule vascular bundles (VB) are outside the central dark staining mass of cells containing bacteroids and are enclosed by nodule cortex (NC) cells. (×104)

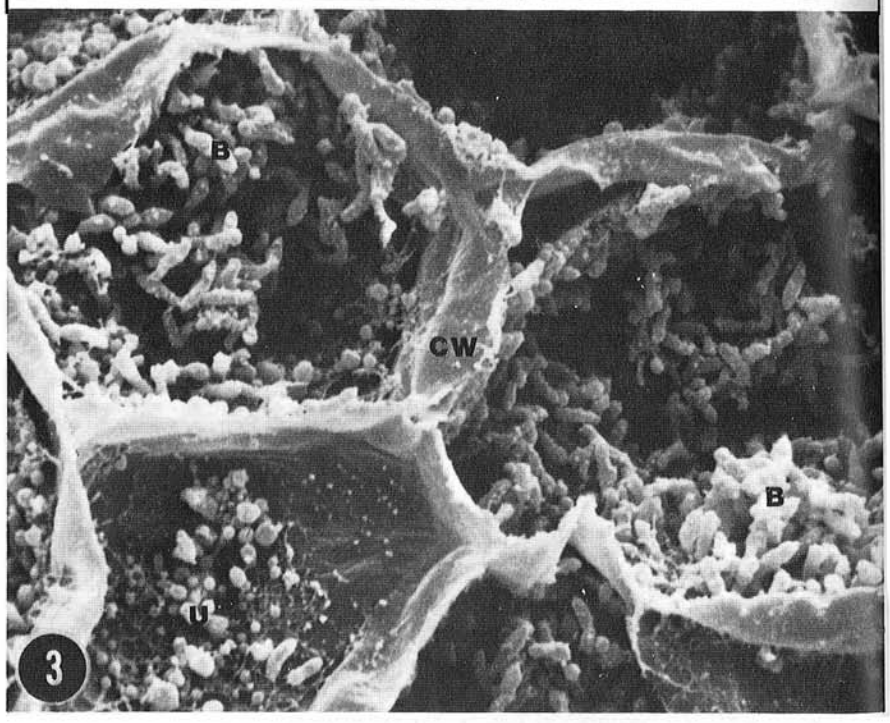
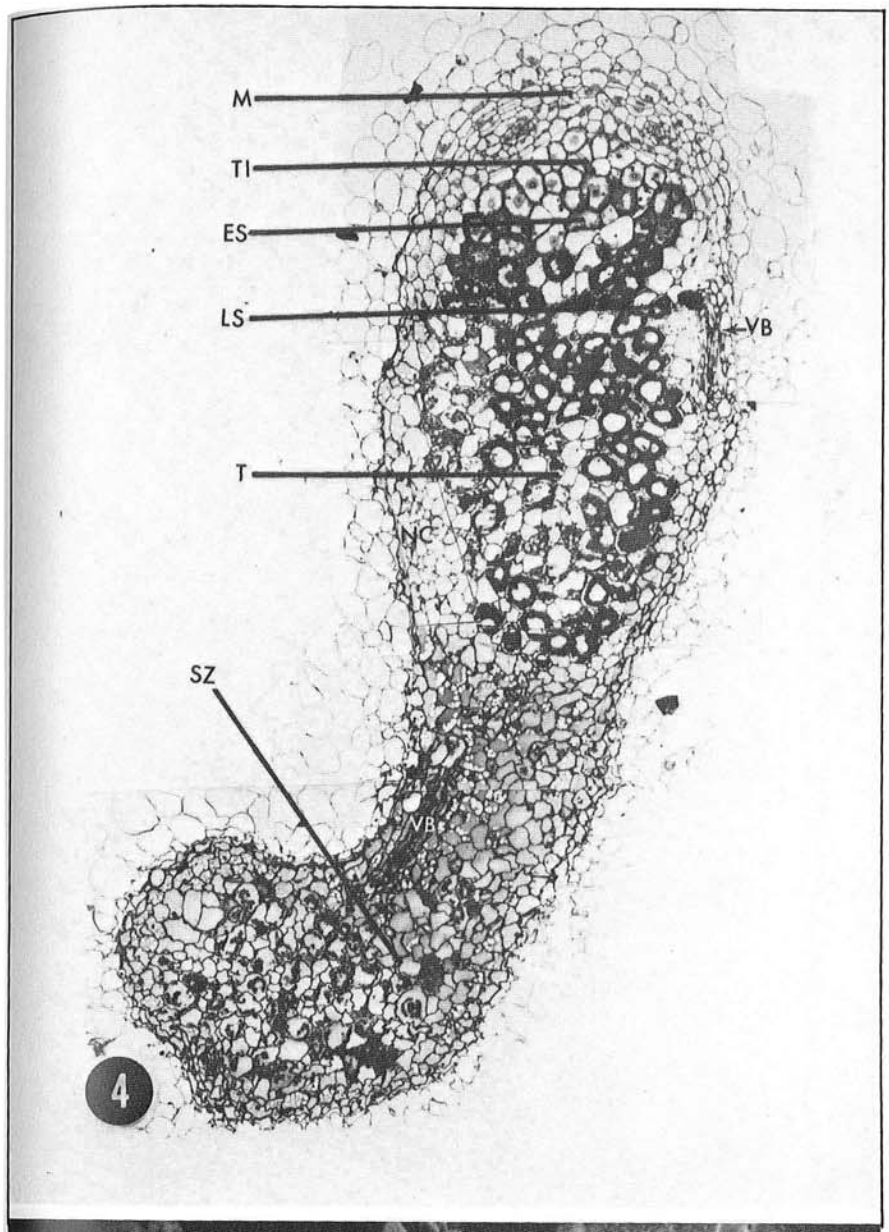
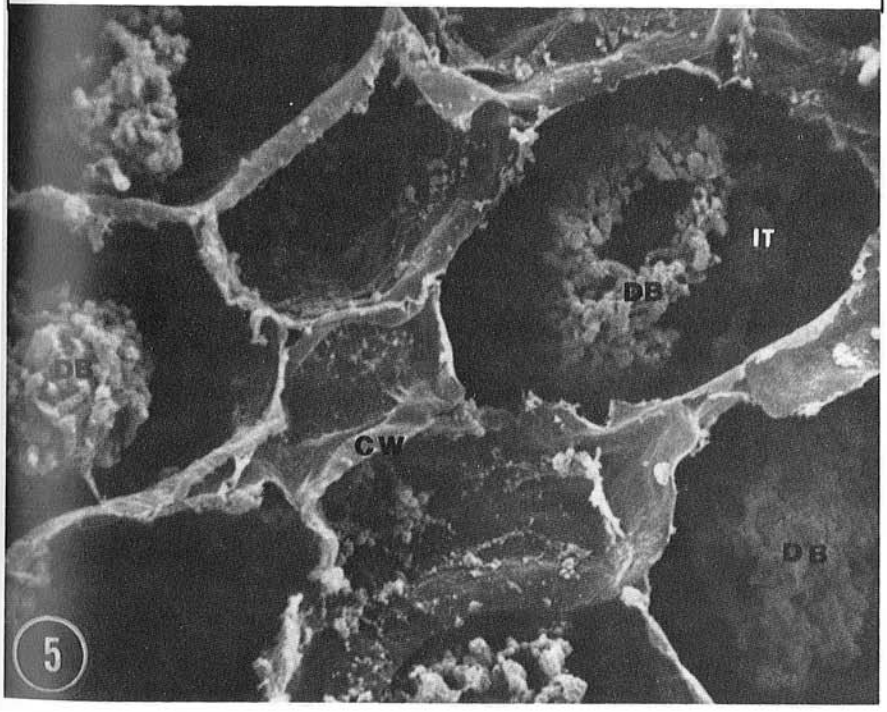


Fig. 3. Scanning electron micrograph of cells containing bacteroids in the late symbiotic zone of an effective nodule 19 days after inoculation. Bacteroids (B) fill most nodule cells, with occasional uninvaded (U) cells. CW = cell wall. (×1,924)





senesce much more rapidly (Figs. 4 and 5) than effective nodules of the same age and size. Macroscopic evidence of senescence is a green region extending over a large portion of the nodule. Cells in this region either are empty or contain bacteroids in various stages of deterioration (Fig. 5).

Major differences in bacteroid development are observed between *Rhizobium*-induced effective and ineffective nodules. Bacteroids in ineffective nodules are smaller (0.5×4.5 μm) than bacteroids (2.0×4.3 μm) in effective nodules and pleomorphism is less evident (Figs. 3 and 5). No differences in size and shape are observed, however, between effective and ineffective bacteria grown on nutrient agar. Bacteroids in *Rhizobium*-induced ineffective nodules senesce and deteriorate much sooner than bacteroids in effective nodules. Bacterial-induced ineffective nodules occasionally have cells in which bacteria released into the cytoplasm multiply but do not develop into bacteroids. These bacteria, in contrast to bacteroids, are surrounded by a matrix appearing to be polysaccharide. This accumulation of polysaccharide could impair recognition events required for further development into bacteroids.

The alterations in bacteroid size and shape in *Rhizobium*-induced ineffectiveness may reflect incomplete development of bacteroids. Ineffectiveness has been associated with incomplete development of bacteria into bacteroids in several legume nodules. Many factors, including defects in bacterial wall synthesis, incomplete formation of membrane envelopes, altered wall antigens, and toxic plant products, may impair bacteroid development.

The premature senescence in *Rhizobium*-induced ineffective nodules may be the result of selective autolysis of bacteroids by the host plant. Bassett et al (1) suggested selective autolysis as a mechanism of ineffectiveness in soybean. They suggested selective autolysis may result from altered bacterial wall and soluble antigens and could be regarded as an attempt by the cell to destroy an incompatible intracellular invader. Such autolysis resembles the digestion of endomycorrhizae by host cells.

Fig. 4. Longitudinal section of a *Rhizobium*-induced ineffective nodule 17–22 days after inoculation. Zones illustrated are: meristem (M), thread invasion (TI), early symbiotic (ES), late symbiotic (LS), transition (T), and senescent (SZ). Bacterial-induced ineffective nodules show extensive senescent and transition zones compared to effective nodules of the same age (compare Fig. 2). (×104)

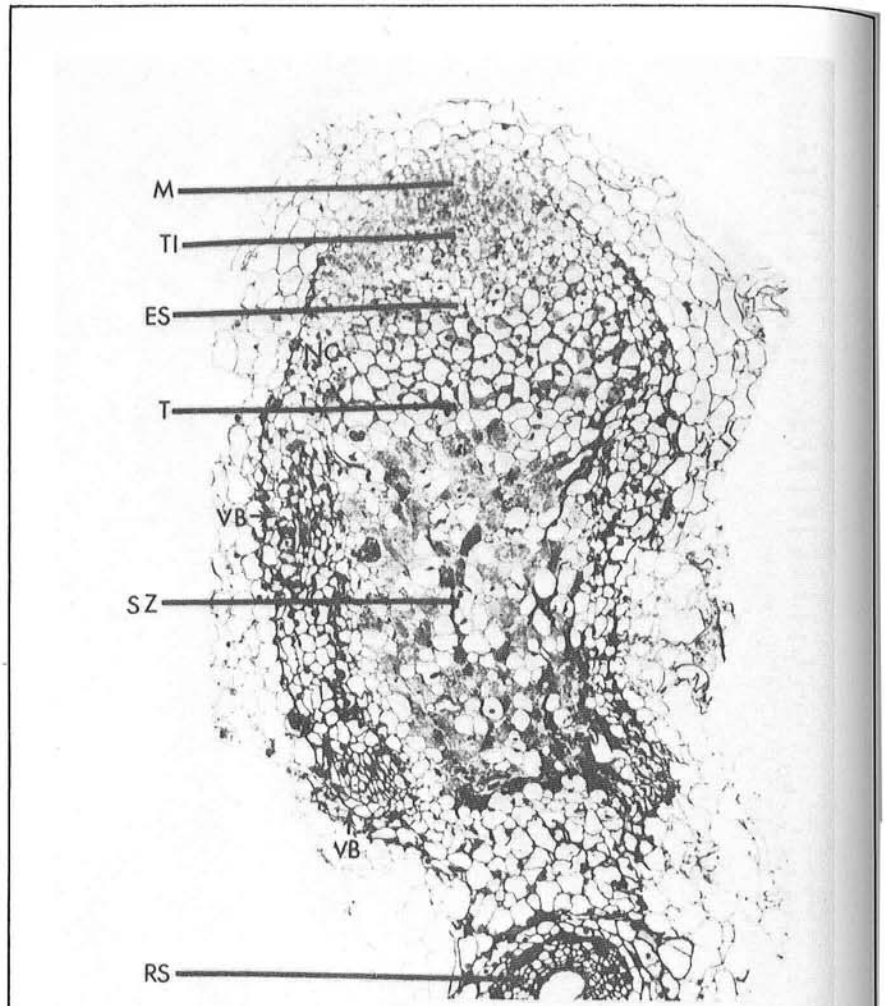
Fig. 5. Scanning electron micrograph of bacteroid containing cells in the senescent zone of bacterial-induced ineffective nodule. Deteriorating bacteroids (DB) aggregate and lyse as premature senescence occurs. IT = infection thread, CW = cell wall. (×1,924)

Plant-Induced Ineffectiveness

Alfalfa genotypes MnAg(In) and MnP1-480 cause ineffective nodule formation with a number of *R. meliloti* strains that normally form effective nodules on other alfalfa genotypes. Ineffective nodules induced by MnAg(In) are similar in structure and development to bacterial-induced ineffective nodules (Figs. 6 and 7). The major differences between MnAg(In)-induced nodules and bacterial-induced ineffective nodules involve bacteroid development. Bacteria in MnAg(In) nodules undergo initial changes to bacteroids that remain small, aggregate, and senesce. The transition zone and late symbiotic zone in MnAg(In) ineffective nodules are either very small or missing (Fig. 6).

The similarity of MnAg(In) ineffective nodules and bacterial-induced ineffective nodules may reflect shared mechanisms. The earlier aggregation and senescence of bacteroids in MnAg(In) could result from either a more rapid response by the plant or early alterations in bacteroid antigenic components that allow for more rapid recognition. Aggregation of bacteroids in both MnAg(In)-induced and bacterial-induced ineffective nodules resembles agglutination caused by bacterial-lectin binding systems (3,10). Comparative studies of lectins in these two types of ineffective nodules may help clarify the role of lectins in host-microbial interactions.

Nodules produced on MnP1-480 differ strikingly from *Rhizobium*-induced effective or ineffective nodules and MnAg(In) nodules (Figs. 8 and 9). MnP1-480 nodules are tumorlike; have multiple meristems, few infection threads, and few cells containing bacteroids; and are white throughout. Most MnP1-480 nodule cells are small and filled with starch (Fig. 9). In contrast to all other nodules we studied, MnP1-480 nodule cells adjacent to the meristem have large starch grains, no infection threads, and no bacterial release. Infection threads are found in a few nodule cells adjacent to the pericycle and occasionally in nodule epidermal cells. Bacteria appear to be released into a



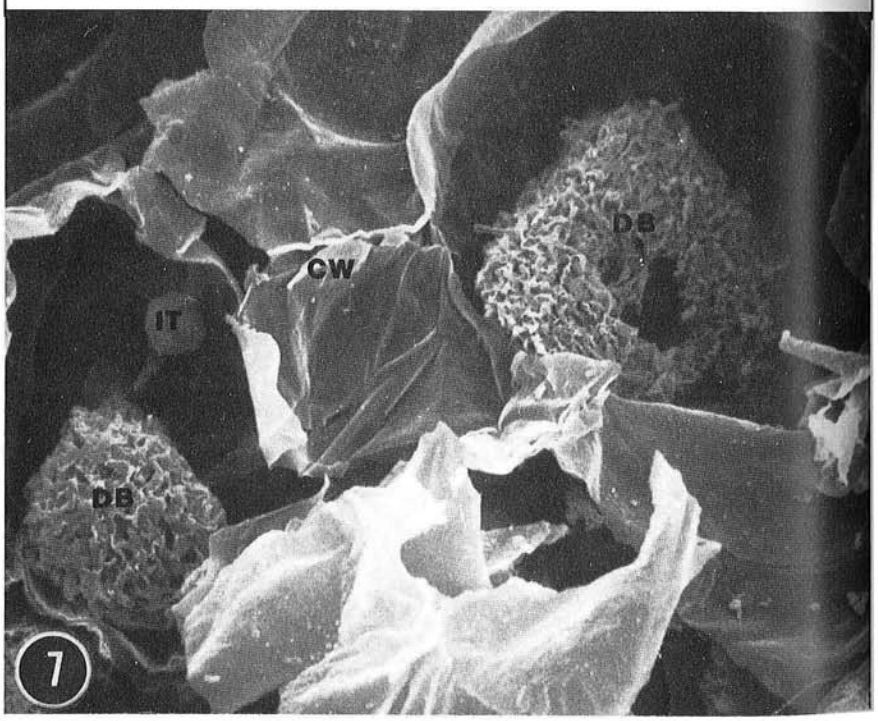
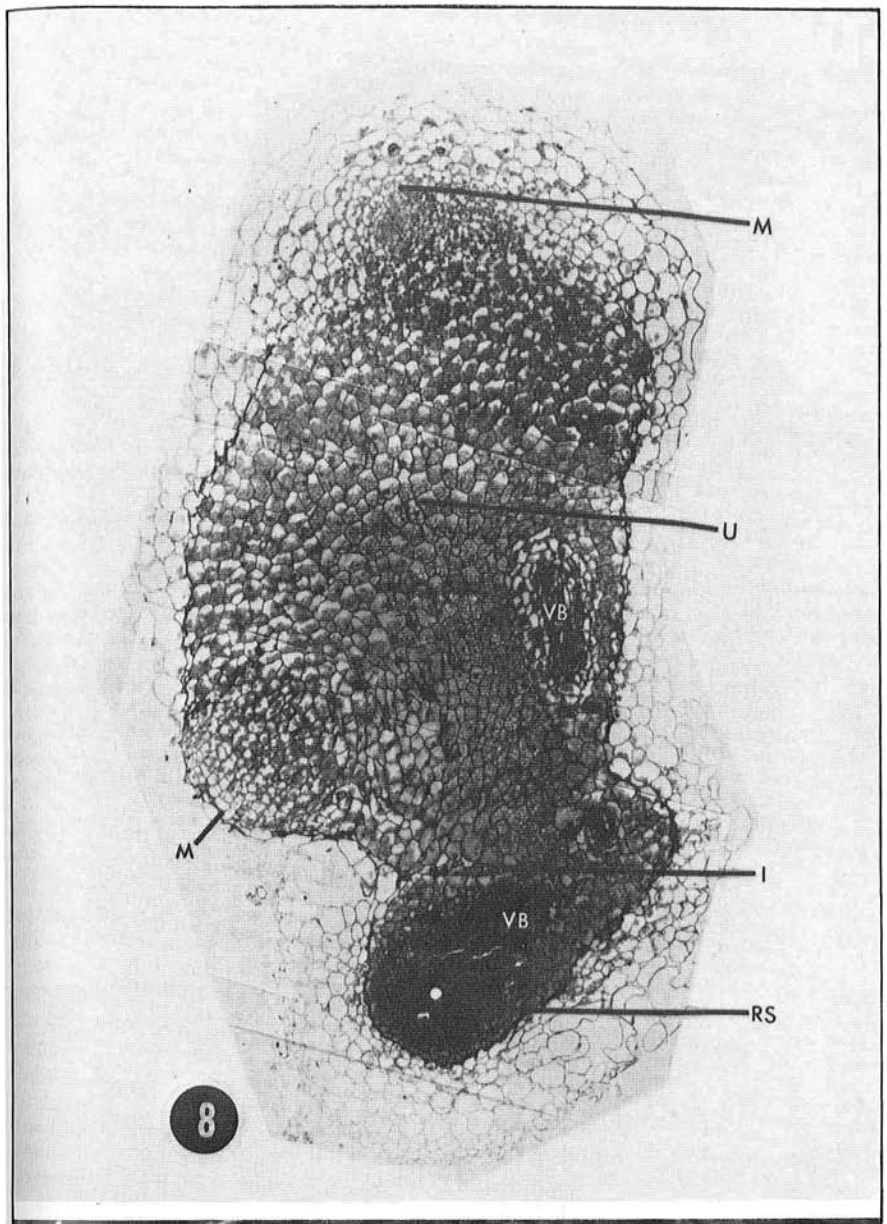
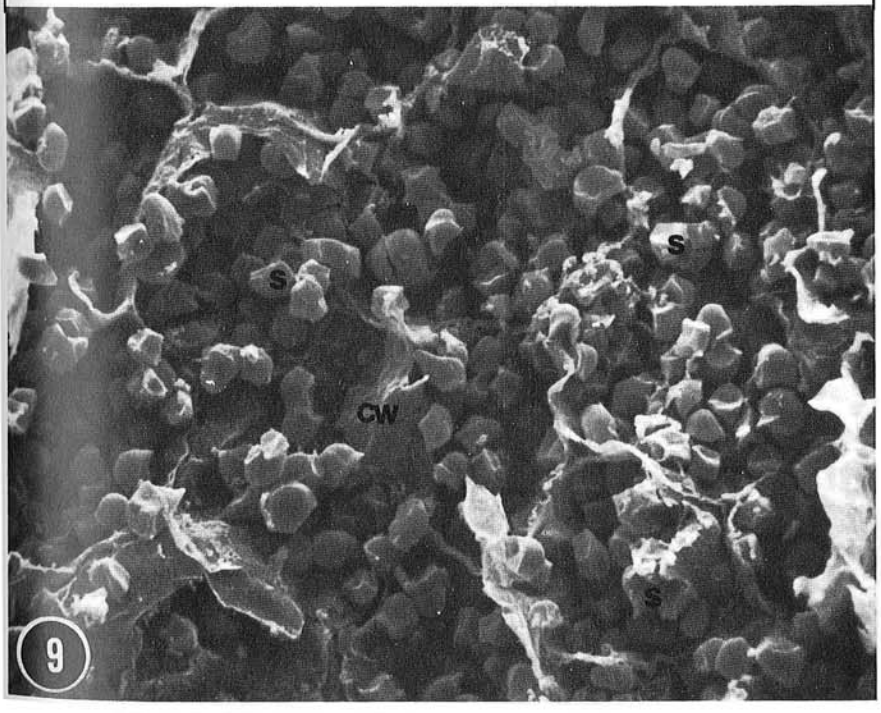


Fig. 6. Longitudinal section of a 24-day-old ineffective nodule induced by alfalfa genotype MnAg(In). Zones illustrated are: meristem (M), thread invasion (TI), early symbiotic (ES), transition (T), and senescent (SZ). This plant-induced ineffective nodule, in contrast to bacterial-induced ineffective nodule, contains no late symbiotic zone. Senescence is observed shortly after the early symbiotic stage. (×104)

Fig. 7. Scanning electron micrograph of bacteroid containing cells in the senescent zone of MnAg(In)-induced ineffective nodules. Deteriorating bacteroids (DB) aggregate and lyse similar to those in bacterial-induced ineffective nodules (compare Fig. 5). IT = infection thread, CW = cell wall. (×1,924)





few cells adjacent to the root pericycle, and some undergo incomplete development into bacteroids. No large increases occur in bacterial populations in the nodule. Released bacteria aggregate and eventually lyse.

The scarcity of bacteria and bacteroids in MnP1-480 nodules could result from toxic products formed within the nodule in response to infection. Evaluation of ineffective nodules for phytoalexinlike compounds may reveal a role for this classic disease-resistance response in nodule development. Pankhurst and Biggs (9) suggested that phytoalexinlike compounds may play a role in *Rhizobium*-legume compatibility and nodule effectiveness. They have shown *Rhizobium* strains to be selectively sensitive to isoflavonoids.

Nodule tissue contines to grow and develop in MnP1-480 in the absence of infection thread proliferation and of numerous cells containing bacteroids within the nodule. This suggests that once nodule meristematic tissue has been initiated, autonomous cell division may occur without direct influence of infection threads or bacteria.

We have recently been able to initiate tissue cultures of MnP1-480 nodule meristems and have had limited success in obtaining proliferation on hormone-free media. These tissue cultures were bacteria-free. This could indicate that the tumorlike MnP1-480 nodules are analogous to tumors induced by *A. tumefaciens* (13). Our observations support the concept that the mechanism controlling nodule growth and development may not be associated with infection threads or with large populations of *Rhizobium* within nodule cells but may result from incorporation of stable bacterial factors into the host plant genome similar to crown gall induction. Recently, Truchet et al (11) used another *Rhizobium*-induced ineffective system to support this concept.

Resolution of Concepts Awaits Studies on Individual Species

Studies of legume nodule initiation, growth, and development in effective and plant-induced and bacterial-induced ineffective associations offer useful information in understanding both

Fig. 8. Longitudinal section of a 28-day-old ineffective nodule induced by alfalfa genotype MnP1-480. Zones illustrated are: meristem (M), uninvaded cells (U), infected cells (I), vascular bundles (VB), and root stele (RS). MnP1-480 nodules have few infected cells, more than one meristem, and an extensive zone of uninvaded cells that are filled with starch. (×104)

Fig. 9. Uninfected cells make up the major portion of the ineffective MnP1-480 nodules. Massive starch (S) accumulations, but neither bacteria nor bacteroids, are evident. CW = cell wall. (×1,924)

disease resistance and developmental biology. Resolution of concepts common to both disciplines awaits the use of molecular, biochemical, histological, and genetic techniques on individual legume species.

Acknowledgments

We thank R. J. Zeyen and the Minnesota Agricultural Experiment Station's Electron Optical Facility for aid in obtaining electron micrographs, J. Burton for ineffective *Rhizobium meliloti*, and D. K. Barnes for ineffective alfalfa genotypes.

Literature Cited

1. BASSETT, B., R. N. GOODMAN, and A. NOVACKY. 1977. Ultrastructure of soybean nodules. II. Deterioration of the symbiosis in ineffective nodules. Can. J. Microbiol. 23:873-883.
2. BERGERSEN, F. J. 1957. The structure of ineffective root nodules of legumes: An unusual new type of ineffectiveness, and an appraisal of present knowledge. Aust. J. Biol. Sci. 10:233-242.
3. BROUGHTON, W. J. 1978. Control of specificity in legume-*Rhizobium* associations. J. Appl. Bacteriol. 45:165-194.
4. CURRIER, W. W., and G. A. STROBEL. 1977. Chemotaxis of *Rhizobia* spp. to a glycoprotein produced by birdsfoot trefoil roots. Science 196:434-435.
5. DART, P. 1977. Infection and development of leguminous nodules. Pages 367-472 in: R. W. F. Hardy and W. Silver, eds. A Treatise on Dinitrogen Fixation. Sect. III. Wiley-Interscience, New York.
6. GUTSCHICK, V. P. 1980. Energy flow in the nitrogen cycle, especially in fixation. Pages 17-27 in: W. E. Newton and W. H. Orme-Johnson, eds. Nitrogen Fixation. Vol. I. University Park Press, Baltimore, MD.
7. JORDAN, D. C. 1974. Ineffectiveness in the *Rhizobium*-leguminous plant association. Proc. Indian Nat. Sci. Acad. 40. Part 13, No. 6, pp. 713-740.
8. LIBBENGA, K. R., and R. J. BOGERS. 1974. Root nodule morphogenesis. Pages 431-471 in: A. Quispel, ed. The Biology of Nitrogen Fixation. North-Holland Publishing Co., Amsterdam.
9. PANKHURST, C. E., and D. R. BIGGS. 1980. Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Microbiol. 26:542-545.
10. SEQUEIRA, L. 1978. Lectins and their role in host-pathogen specificity. Annu. Rev. Phytopathol. 16:453-481.
11. TRUCHET, G., M. MICHEL, and J. DENARIE. 1980. Sequential analysis of the organo-genesis of lucerne (*Medicago sativa*) root nodules using symbiotically defective mutants of *Rhizobium meliloti*. Differentiation 16:163-172.
12. VANCE, C. P. 1978. Nitrogen fixation in alfalfa: An overview. Pages 34-41 in: Proc. Annu. Alfalfa Symp., 8th.
13. VANCE, C. P., L. E. B. JOHNSON, and G. HARDARSON. 1980. Histological comparisons of plant and *Rhizobium*-induced ineffective nodules. Physiol. Plant Pathol. 17:167-173.