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Developmental Fate of *Rhizobium meliloti* Bacteroids in Alfalfa Nodules

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Nitrogen-fixing bacteroids are degraded during nodule senescence. This is in contrast to recent implications that viable bacteroids can be released into soil from legume nodules. Rhizobia originating from persistent infection threads in senescing nodule plant cells seem to be the source of viable cells required for perpetuation of the *Rhizobium* spp. population in the soil. Our conclusions were derived from electron microscopic examination of stages of development and senescence of alfalfa root nodules.

Ultrastructural studies on the development of legume nodules and their microsymbionts are well documented. Most of these studies, however, focused on the initial steps in the symbiotic development (3, 5, 7, 8, 10, 11, 17-20, 22, 24, 26, 39). Although morphology during nodule senescence has been examined in some *Rhizobium*-legume associations (2, 4, 9, 12, 29, 35, 36), only the *R. leguminosarum*-pea symbiosis has been studied in detail with respect to nodule and bacteroid senescence (14, 21, 33). During clover nodule senescence, bacteroids are described to undergo transformations from rods to various spherical and polyhedric bacteroids (12). In pea nodule senescence, bacteroids are also described to transform from rods to vacuolated spherical forms which eventually deteriorate (21). Plant cells in the senescent region of nodules from postharvest alfalfa are greatly disorganized and structurally empty (37). The fate of the rhizobia in these nodules remains unknown.

Classical work with micromanipulation (1) suggested that fully transformed bacteroids are nonviable. Bacteroids from nodules show no multiplication in a variety of media and also fail to renodulate the host legume. Recent reports, however, claim that up to 90% of the bacteroids are viable (13, 34). These conflicting results raise questions concerning the actual fate of bacteroids in the *Rhizobium*-legume symbiosis. In this communication, we describe the developmental fate of bacteroids in senescent alfalfa nodules.

The *R. meliloti*-alfalfa association, similar to the clover and pea systems, has the advantage that the rhizobia go through distinct morphological transformations during establishment of the symbiosis (17, 19, 22, 25, 36). Furthermore, the nodule propagates by elongation with nodule meristem(s) at the distal end(s) of the nodule (nodule tip). This anatomical arrangement provides an excellent developmental gradient of the symbiotic process along the length of a nodule (25). The youngest stage of symbiotic development is localized at the nodule tip, and the oldest nodule tissue (having the oldest stages in symbiotic development) is represented in the part of the nodule that attaches to the root. We took advantage of this special arrangement to conduct a systematic study of the ultrastructure of rhizobia in five different zones of the nodules, focusing on the senescent tissues.

MATERIALS AND METHODS

Rhizobium meliloti 102F51 was provided by J. C. Burton, The Nitragin Co., Milwaukee, Wis. Alfalfa seeds (*Medicago sativa* Vernal) were purchased from The Olds Seed Co., Madison, Wis. The inoculum was grown in yeast extract-mannitol broth (38) at 30°C. Plants were grown in a greenhouse at 23°C with supplemental light. The plants were given a nitrogen-free nutrient solution (W. Leps, unpublished data) containing 2 mM $CaSO_4$, 5 mM K_2SO_4, 5 mM NaH_2PO_4, 40 μM EDTA (ferric salt), 0.5 μM $CoCl_2$, 0.5 mM $MgSO_4$ and 1 ml of micronutrients per liter (38). The pH of the nutrient solution was adjusted to 7 with 4 N NaOH after autoclaving. Plants were inoculated 10 days after germination. Nodules, about 8 mm in length, were harvested 14 weeks after inoculation and were sectioned into five equal-length portions. Zone I contained the nodule meristems, whereas Zone V contained the senescent nodule tissues.

Nodule segments were fixed separately in 2.5% glutaraldehyde-0.025 M potassium phosphate (pH 6.8) overnight at room temperature. The sections were postfixed in 1% OsO_4-0.025 M potassium phosphate overnight in the cold and dehydrated in a graded series of ethanol. The samples were rinsed twice in propylene oxide and infiltrated overnight with two changes of Spurr standard low-viscosity medium (31). Polymerization was at 70°C for 8 h. Light gold and silver sections were stained with 2% uranyl acetate and lead

citrate (28) and examined with a Carl-Zeiss EM9 electron microscope. The term "bacteroid" in this article refers only to enlarged rhizobia that are enclosed by membrane envelopes in the nodule cytoplasm.

RESULTS

Bacteroid development. Zone I, which contained the nodule meristem, consisted mostly of uninfected plant cells. Infection threads were common in this region. Rhizobia inside the infection threads (Fig. 1A) retained the morphology of vegetative bacteria (19, 25). Occasionally, a few rhizobia were observed in the plant cytoplasm (Fig. 1B). These rhizobia, although enclosed in envelopes and devoid of poly-β-hydroxybutyrate granules, were morphologically similar to those in infection threads (Fig. 1B) and cultured rhizobia. They were small (1 to 1.5 μm in length) and had a distinct central nucleoid (25). Zone II consisted of plant cells with more rhizobia released in the plant cytoplasm. These newly released bacteria were morphologically similar to those described in zone I (Fig. 2). Zone III consisted mostly of plant cells completely filled with enlarged bacteroids (Fig. 3). Typical bacteroids were elongated rods (36) ranging from 4 to 7 μm in length and were totally devoid of poly-β-hydroxybutyrate granules and distinct central nucleoids. They were individually enclosed in tight membrane envelopes (36). This stage is very active in nitrogen fixation (25). Zone IV contained some plant cells which showed initial signs of senescence. The envelopes enclosing some bacteroids were loosened, leaving an electron-empty space between the envelopes and the bacteroid outer membranes (Fig. 4A). A few bacteroids at this stage of development also accumulated electron-dense deposits (Fig. 4B), possibly of polyphosphate (6). Our observations on early bacteroid development from zone I to IV were consistent with an earlier study on younger (6 to 8 weeks) alfalfa nodules (25). Infection threads, although most abundant in zone I, were seen in sections prepared from all zones (Fig. 5C).

Nodule senescence. With sections prepared from zone V, senescent bacteroids were easily observed. Except for a few infected cells that retained the morphology as described for zones III and IV, most of the infected plant cells were in various stages of disorganization. Uninfected cells had very large central vacuoles, and the cytoplasm, appearing as thin layers, was confined to the cell periphery. Few prominent organelles were present. In the infected cells, electron-dense cytoplasm (Fig. 5B) and large membraneous vesicles containing one or more disorganized bacteroids (36) were common (Fig. 5A and B). The mechanism for vesicle formation in these cells was unknown, but was probably due to a breakdown in structural integrity of the bacteroid envelopes which originally surrounded the individual bacteroids or to a fusion of envelopes as suggested by Gunning (15). Bacteroids inside these vesicles remained enlarged but highly distorted (Fig. 5A). Except for the presence of a few electron-dense deposits, bacteroids were structurally empty and appeared like "ghost" cells (Fig. 5A and B). At later stages of senescence, the plant cytoplasm, the vesicles, and the bacteroid "ghosts" collapsed (Fig. 7A) and lined the plant cell wall as electron-dense layers (Fig. 6A and B) which eventually disintegrated. The degeneration of bacteroids, as well as the host cytoplasm, was probably complete at this stage. Intracellular and intercellular infection threads in zone V remained structurally intact and showed no signs of disorganization (Fig. 5C).

Origin of vegetative rhizobia. In many disorganized plant cells, populations of vegetative rhizobia, morphologically similar to those in infection threads and in cultures, were seen free in the cytoplasm (Fig. 7A and B), very often close to the persistent infection threads (Fig. 8A to C). These rhizobia were small, had poly-β-hydroxybutyrate granules and distinct central nucleoids, and were not enclosed in envelopes. They were not artifacts from fixation or sectioning procedures, since similar rhizobia were also observed by Tu (36) and Kijne (21) in alfalfa and pea nodules, respectively.

DISCUSSION

Tu suggested that the rhizobia which resembled vegetative bacteria seen in disorganized plant cells are senescent bacteroids after dissolution of the membrane envelopes (36). Bacteroids in zone V were greatly distorted and structurally empty. Transformation of such highly disorganized bacteroid "ghosts" into rhizobial cells certainly seems unlikely. Furthermore, transformation of bacteroids to rhizobial cells would imply that the bacteroids either underwent two or more divisions or shrunk to account for the tremendous size difference (1 to 1.5 versus 4 to 7 μm). Shrinkage of bacteroids was not substantiated, since intermediate phenotypes have not been observed or reported. Division of bacteroids would necessarily increase the number of microsymbionts in the senescent region, but we did not observe such an increase. In fact, the number of microorganisms in the senescent part of nodules decreases significantly (37).

We suggest that these vegetative rhizobia were released directly from the persistent infec-

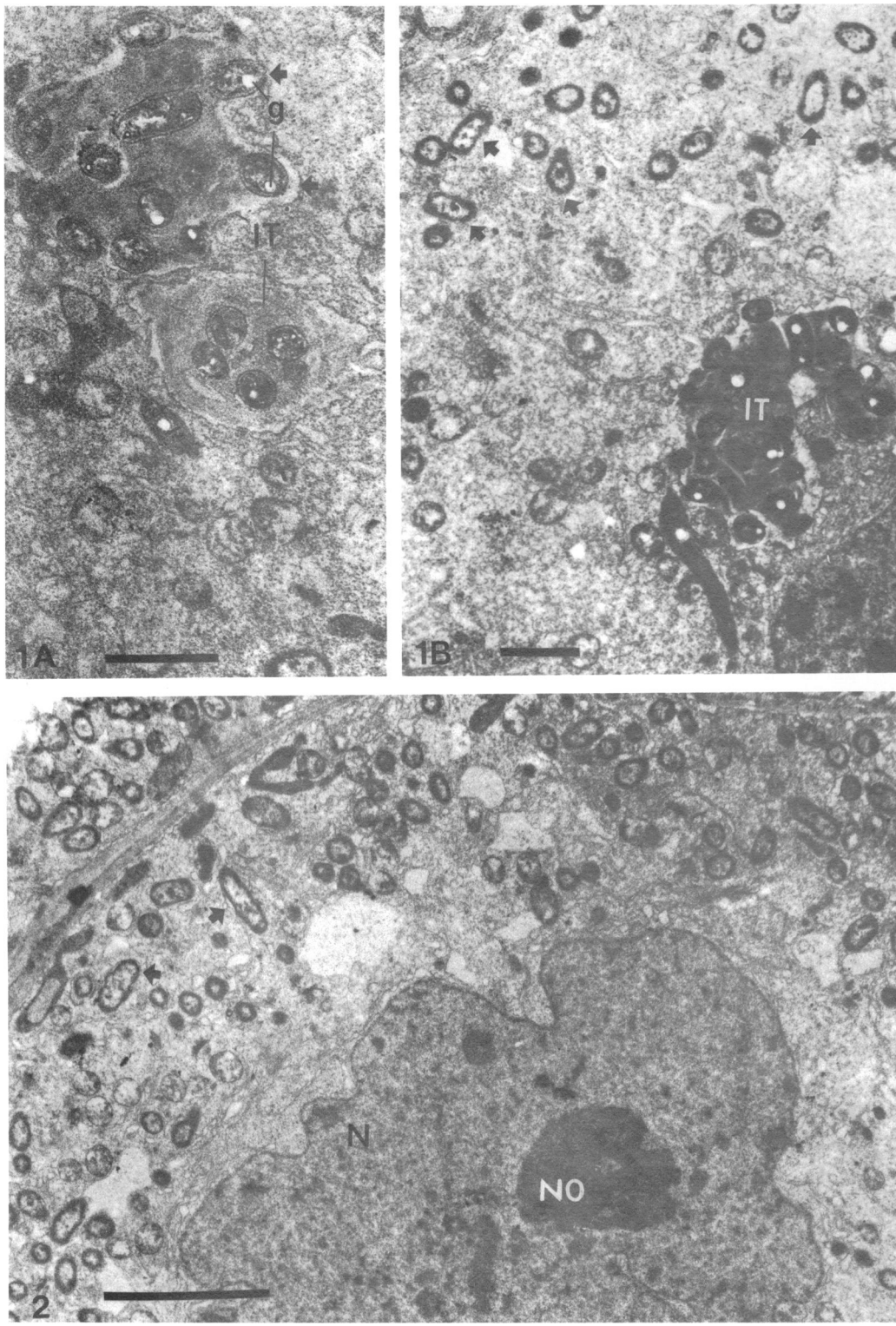


FIG. 1. *Cross-section of infection threads in zone I. (A) Rhizobia (arrows) being released into plant cytoplasm. Note the presence of poly-β-hydroxybutyrate granules in these rhizobia. (B) Rhizobia (arrows) newly released from infection thread into plant cytoplasm. Note the absence of poly-β-hydroxybutyrate granules in the released rhizobia. IT, infection threads; g, poly-hydroxybutyrate. Bars = 2 μm.*

FIG. 2. *Section of zone II showing the abundance of rhizobia (arrows) newly released from infection threads into plant cytoplasm. Note the small size of these rhizobia. N, Nucleus; NO, nucleolus. Bar = 5 μm.*

tion threads in zone V into the disorganized plant cells which, after losing structural integrity (especially the membrane systems), were unable to enclose the rhizobia in envelopes and provide the necessary interactions to induce bacteroid transformation. Kijne (21) also reported the presence of vegetative rhizobia in senescent pea nodules. Alternatively, he described that this may be due to an invasion, made possible by the cell wall degeneration of disorganized plant cells, of rhizobia from old parts of infection thread. Kijne's suggestion implies a new passive mechanism which is different from the mechanism by which rhizobia are released into young, healthy plant cells, since young host cells are not disorganized and are infected without degeneration of senescent cell walls. Our suggestion here requires no such additional mechanism. Vegetative rhizobia can be released into both the disorganized senescent cells and the young healthy host cells by the same mechanism. This mechanism, however, is still unknown. Nonetheless, both of our suggestions are supported by the fact that vegetative rhizobia were not found in all senescent plant cells which contained degrading bacteroids (Fig. 6B), but very often were close to infection threads (Fig. 8A to C).

We have recently isolated a mutant of *R. meliloti* which forms ineffective nodules in alfalfa (A. S. Paau, W. T. Leps, and W. J. Brill, manuscript in preparation). In such ineffective nodules, infection threads were formed and rhizobia were released into the host cytoplasm. These mutants, however, failed to transform into enlarged bacteroids and were degraded. Examination of the senescent region of these ineffective nodules revealed the presence of vegetative rhizobia in disorganized plant cells. Similar observations have also been reported with ineffective mutants of *R. trifolii* in clover nodules (23). These results suggest that the presence of vegetative rhizobia in disorganized plant cells does not require prior existence of enlarged bacteroids. This supports the suggested infection thread origin of the bacteria.

Figure 9 summarizes the developmental fate of rhizobia in alfalfa nodules. After successful development into nitrogen-fixing forms, the bacteroids and the infected plant cells were degraded upon senescence. Rhizobia that persist in the infection threads are then released into disorganized plant cells and eventually into soil after complete dissolution of the nodules.

A recent study reported that up to 90% of the bacteroids released from isolated nodule protoplasts were viable (13). This seems to contradict the long-accepted dogma of bacteroid nonviability (1) and our own observations that senescent bacteroids degenerated in nodule tissues. We argue that the transformed bacteroids, after passing a certain critical developmental stage in the nodule, were indeed nonviable. This critical developmental stage probably is at the initiation of nodule and bacteroid senescence. The disorganized senescent plant cells might have lysed during protoplast isolation. The degenerating bacteroids were lost and not considered in total bacterial counts. Likewise, distorted bacteroids could have easily lysed once they were removed from the plant cells. Tsien et al (34), using a low osmolarity buffer, reported that up to 90% of rhizobia isolated from nodule homogenate were viable. Light-scattering analyses of rhizobia isolated from the senescent region in alfalfa nodules with a similar buffer also revealed only the presence of small bacteria (J. R. Cowles, personal communication), although enlarged senescent bacteroids were detected by electron microscopy. Apparently, highly degraded bacteroids were lost during nodule homogenization in low osmolarity buffer and consequently were not included for viability consideration. Indeed, Sutton et al. (32), using a high osmolarity homogenization buffer (containing 0.5 M mannitol) to isolate bacteroids from whole nodules, reported that less than 50% of the isolated microorgan-

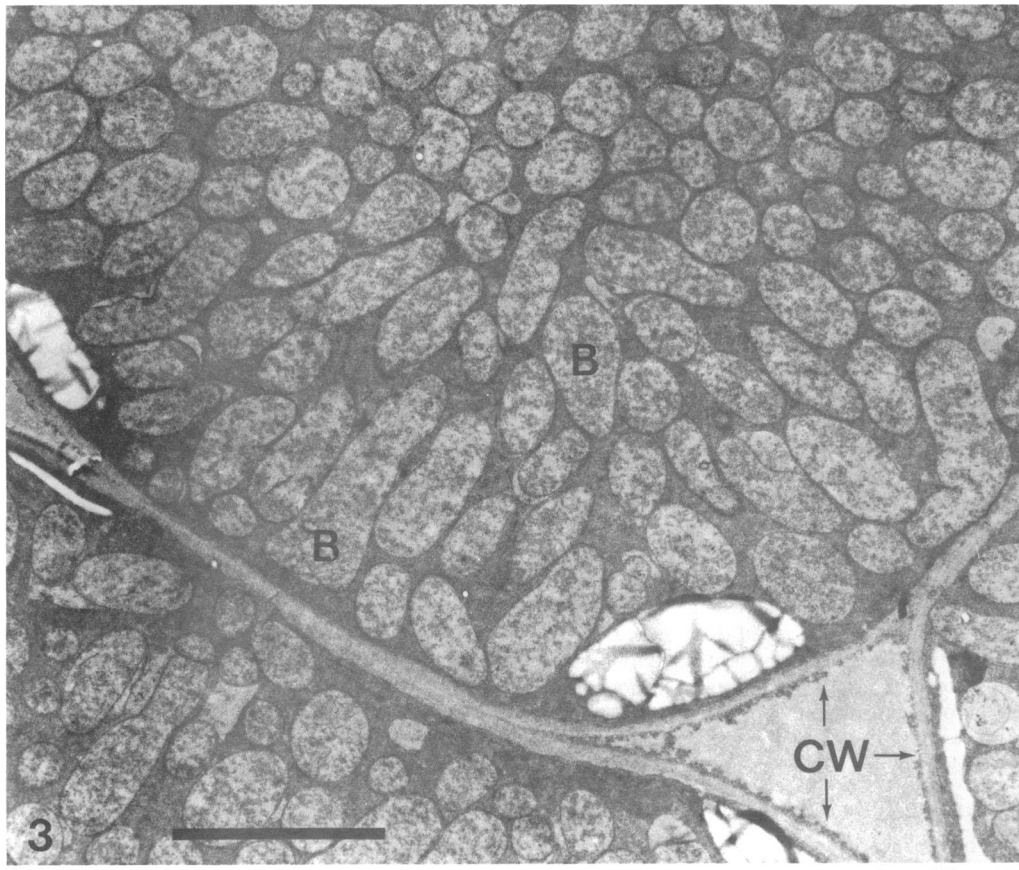
FIG. 3. *Section of zone III showing mature bacteroids. Note the enlarged size and the absence of poly-β-hydroxybutyrate granules and distinct central nucleoid in these bacteroids. B, bacteroid; CW, cell wall; S, starch granules. Bar = 5 μm.*

FIG. 4. *Section of zone IV bacteroids with (A) loosened envelopes (arrows) signifying initiation of senescence and (B) electron-dense deposits (double arrows), possibly of polyphosphate. B, bacteroid. Bars = 2 μm.*

FIG. 5. *Section of zone V showing (A) vesicles containing bacteroid ghosts, (B) large vesicles each containing more than one bacteroid ghost, and (C) an infection thread showing structural integrity of the rhizobia contained within. B represents a later stage of senescence than A. IT, Infection thread; g, poly-β-hydroxybutyrate; V, vesicle. Arrows point to bacteroid ghosts. Bars = 2 μm.*

FIG. 6. *Section of zone V showing (A) layers of degraded bacteroids and plant cytoplasm and (B) layers of collapsed bacteroids and plant cytoplasm attached to the cell wall of a degenerated cell. An adjacent completely disintegrated cell contained a few vegetative rhizobia (arrows). Bars = 1 μm.*

FIG. 7. *Section of zone V showing (A) a disorganized plant cell containing both vegetative rhizobia (arrows) and remains of degraded bacteroids (double arrows) and plant cytoplasm and (B) vegetative rhizobia (arrows) and remains of degraded bacteroids (double arrows) at a higher magnification. Note the structural similarity of these vegetative rhizobia and the rhizobia inside the infection thread in Fig. 5C. Bars = 1 μm.*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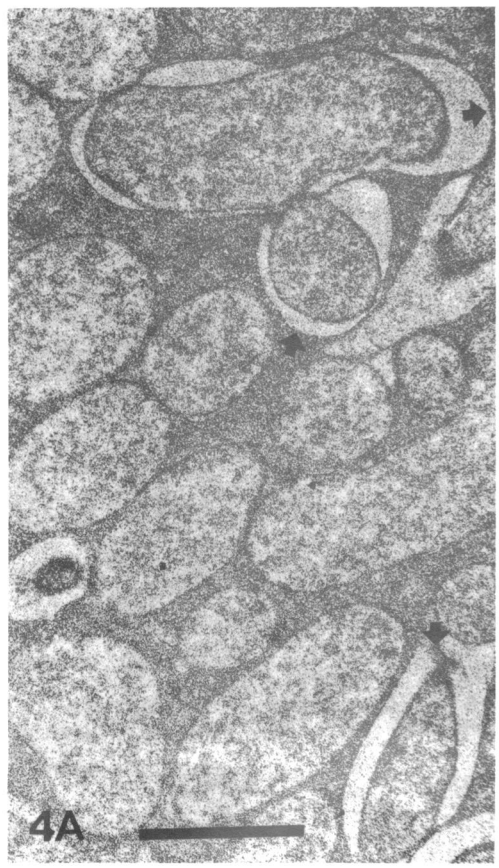
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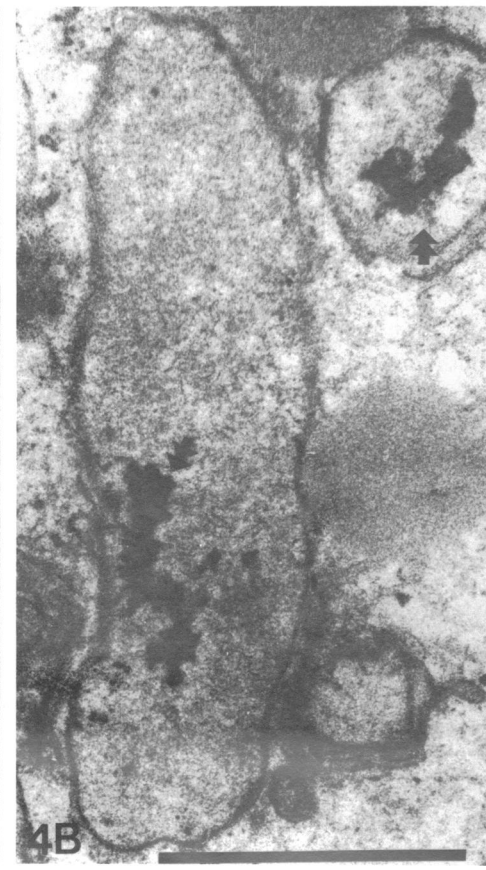
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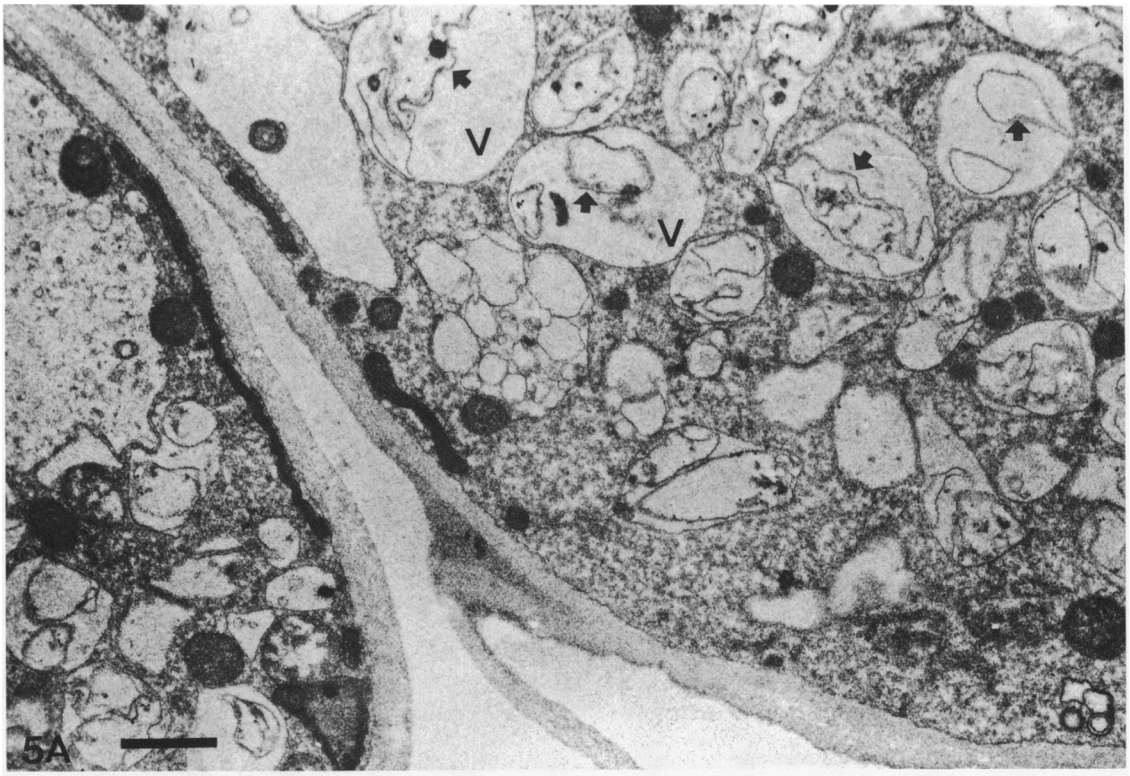
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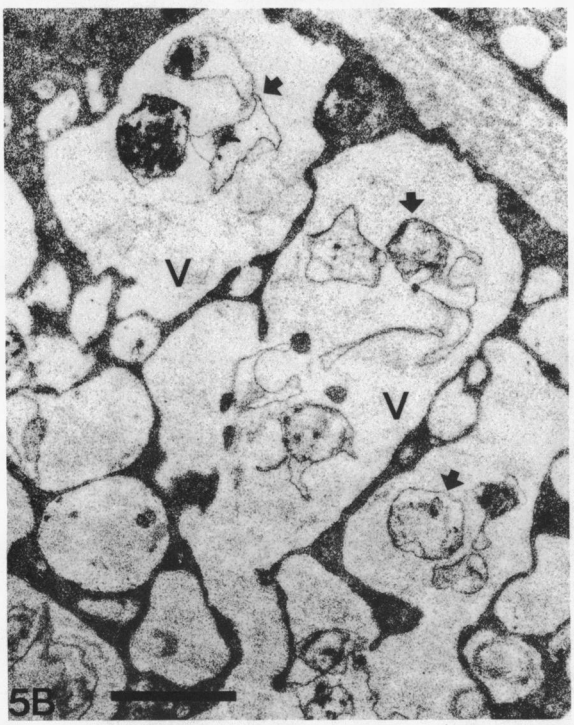
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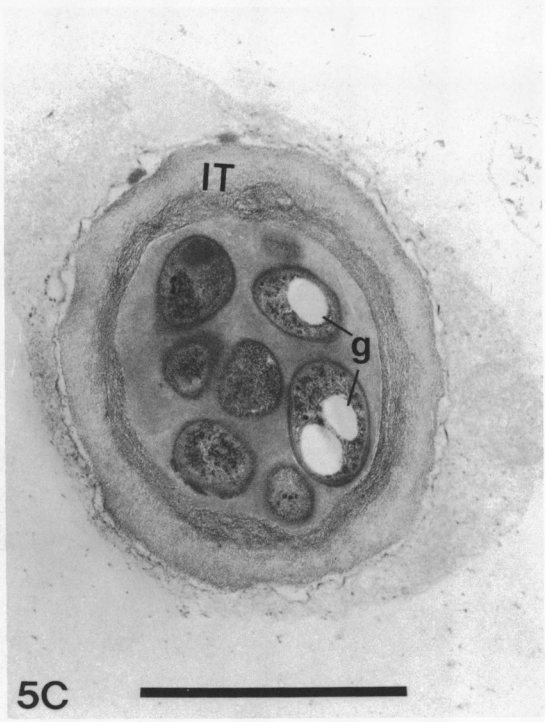
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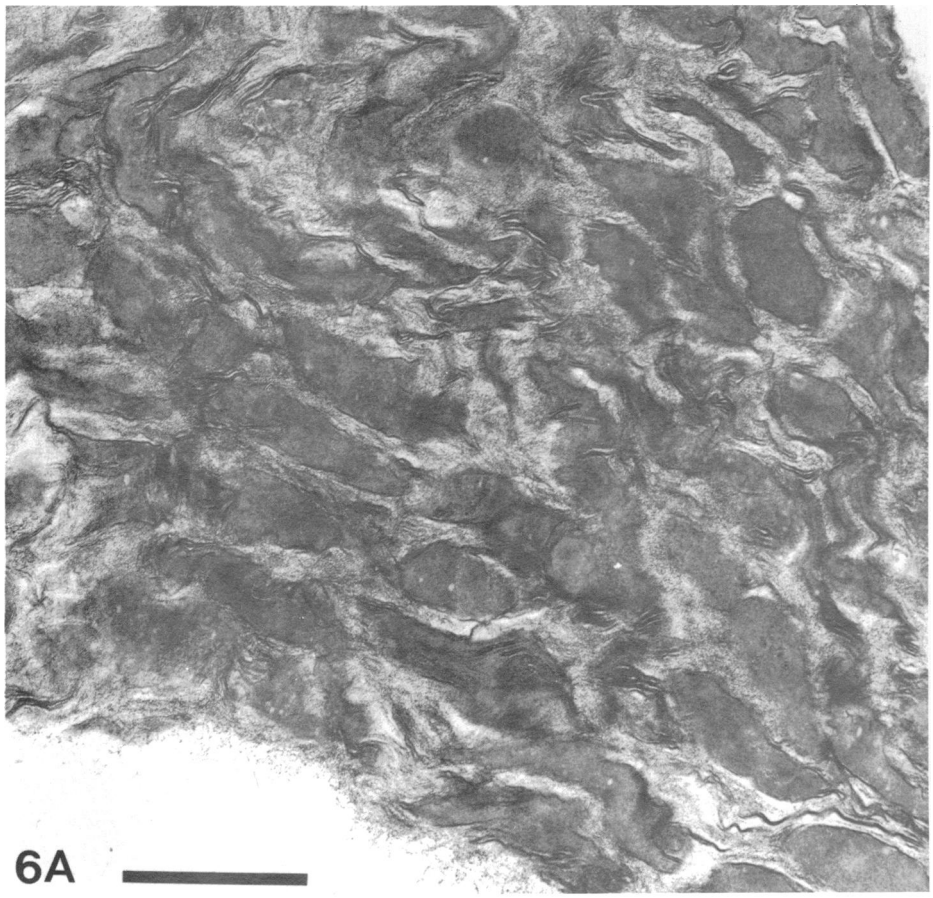
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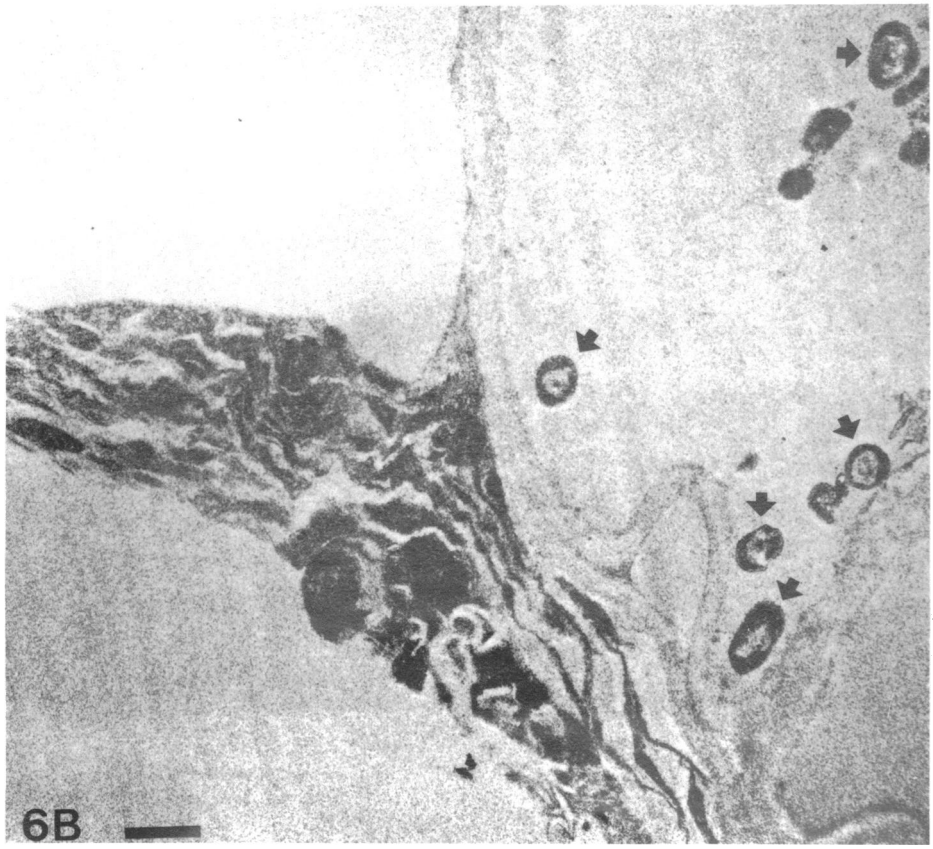
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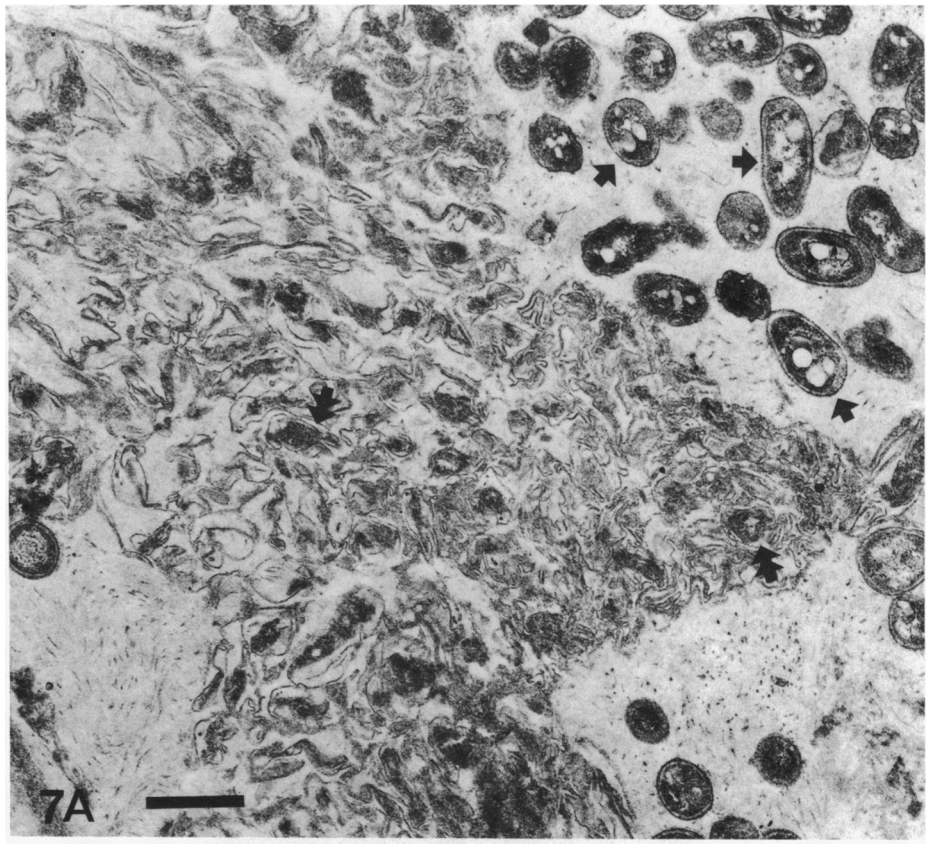
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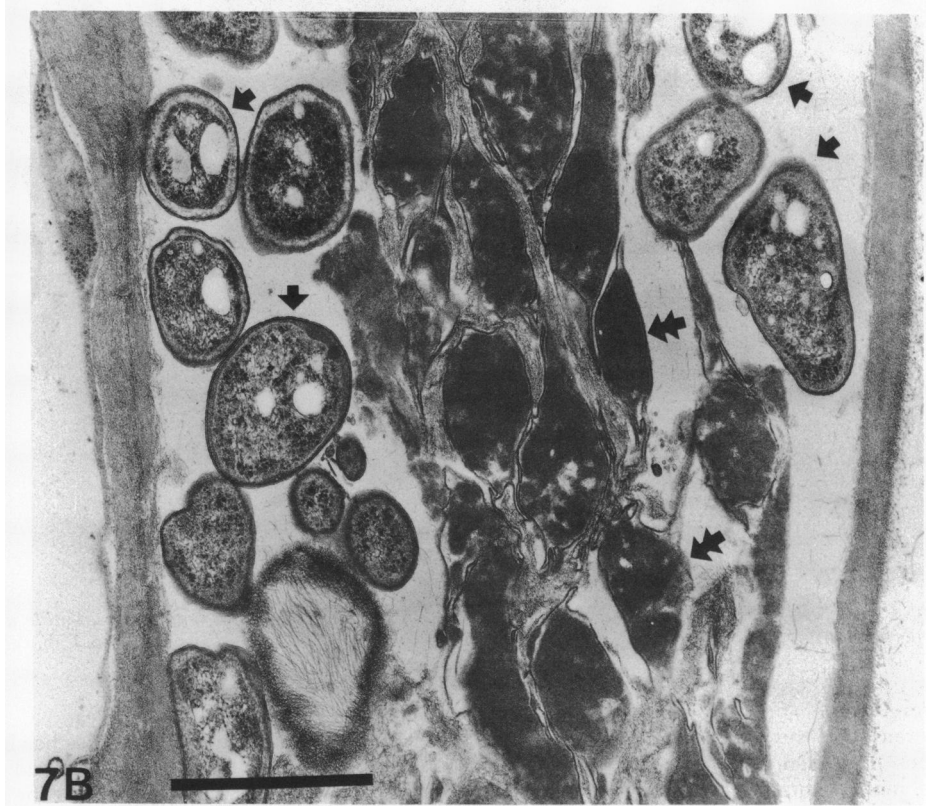
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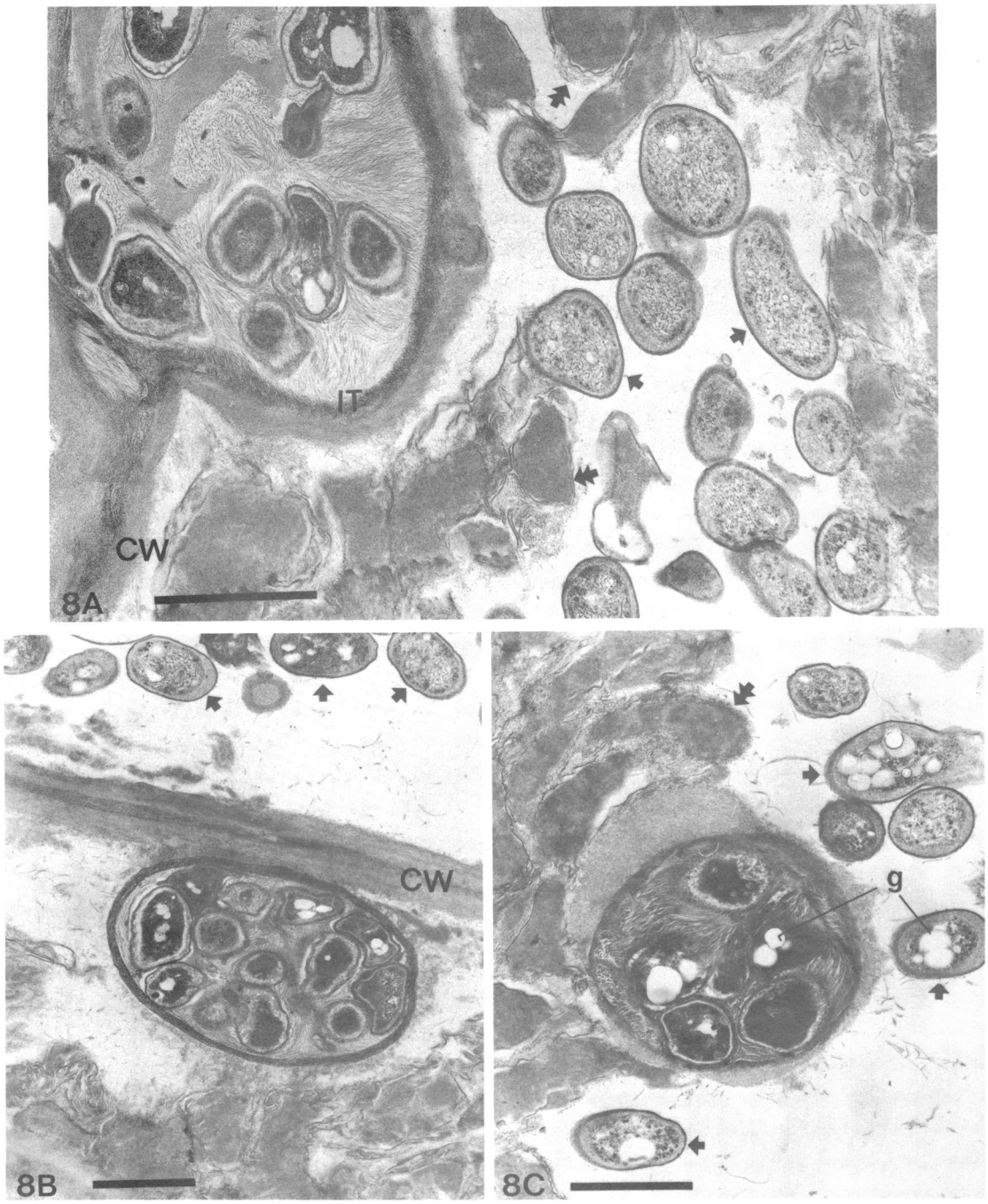

FIG. 8. *Populations of vegetative rhizobia (arrows) and remains of degraded bacteroids (double arrows) close to persistent infection threads in zone V. IT, Infection thread; g, poly-β-hydroxybutyrate; CW, cell wall. Bars = 1 μm.*

isms were viable. The percentage of viability also decreased as the age of the nodules used for extraction increased. This apparently reflects a relative increase in the number of degrading senescent bacteroids in the nodules.

Bacteroid deterioration in nodules has important ecological implications. In natural plant growth (especially in annual legumes) the whole

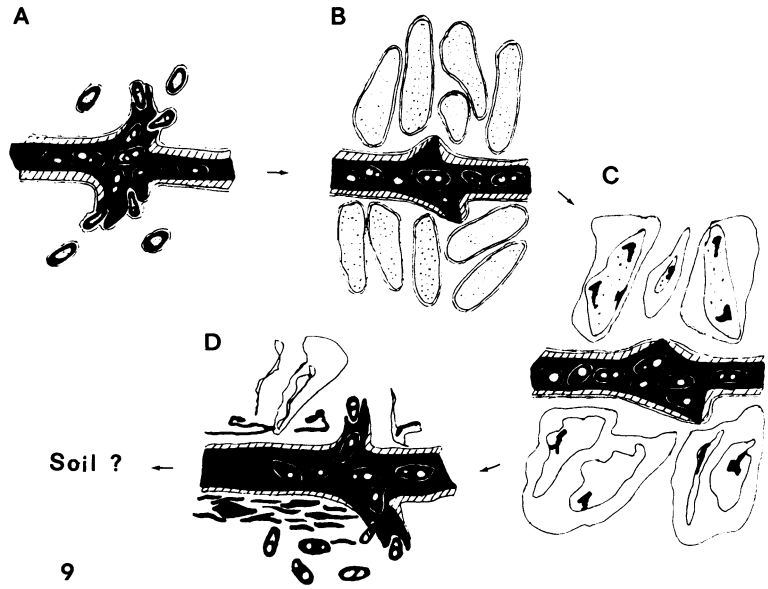

FIG. 9. *Diagramatic representation of the developmental fate of R. meliloti in alfalfa nodules. (A) Initial development of symbiosis: release of rhizobia from infection threads into plant cytoplasm. (B) Mature symbiosis: bacteroids fully transformed and active in nitrogen fixation. (C) Beginning of senescence: beginning of bacteroid and plant cytoplasm disorganization and formation of vesicles containing bacteroid ghosts. (D) Late senescence: total disintegration of bacteroids and plant cytoplasm and release of vegetative rhizobia from infection threads.*

nodule eventually senesces, and all transformed or enlarged bacteroids in it deteriorate. Scott (30) defined that a symbiotic association should result in "an amelioration of environmental status" for the species involved. The number of rhizobia in soil has been estimated to range from 10 to 10^3 per g of soil (16). Despite the degeneration of bacteroids during nodule senescence, the number of vegetative rhizobia which persisted in infection threads and in disorganized plant cells probably exceeds such densities. Judging from their structural integrity, these rhizobia are most likely viable and can be released to the soil after dissolution of the senescent nodules. This is supported by results reported by Reyes and Schmidt (27) that the rhizobial population density in the rhizosphere of disintegrating roots from mature nodulated legumes at harvest was two to three orders of magnitude higher than the rhizobial population densities in nonrhizosphere soil and in rhizosphere of young nodulated legumes. A similar increase was not observed in disintegrating root rhizosphere soil of mature corn at harvest. Release of rhizobia from senescent nodules certainly could account for most, if not all, of this sudden specific increase in rhizobial population. In spite of bacteroid degeneration, the association of rhizobia with legumes, therefore, increases the ecological amplitude of *Rhizobium* even after nodule disintegration and hence constitutes a truly symbiotic relationship.

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