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An Ultrastructural Investigation of Infection Threads in *Sesbania rostrata* Stem Nodules Induced by *Sinorhizobium* sp. Strain MUS10

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ABSTRACT : *Sinorhizobium* sp. strain MUS10 forms nitrogen-fixing stem nodules on *Sesbania rostrata*, a tropical green manure crop. In this study, the ultrastructural events associated with the formation of stem nodules were investigated. *Sinorhizobium* sp. strain MUS10 entered the host tissue through cracks created by the emerging adventitious root primordia and multiplied within the intercellular spaces. During early phases of infection, host cells adjacent to invading bacteria revealed cellular damage that is typical of hypersensitive reactions, while the cells at the inner cortex exhibited meristematic activity. Infection threads were numerous in 5-day-old nodules and often were associated with the host cell wall. In several cases, more than one infection thread was found in individual cells. The junction at which the host cell walls converged was often enlarged due to fusion of intracellular branches of infection threads resulting in large infection pockets. The infection threads were made up of a homogeneous, amorphous matrix that enclosed the bacteria. Several finger-like projections were seen radiating from these enlarged infection threads and were delineated from the host cytoplasm by the plasma membrane. As in *Azorhizobium caulinodans* induced root nodules, the release of *Sinorhizobia* from the infection threads into the plant cells appears to be mediated by "infection droplets". A 15-day-old *Sesbania* stem nodule revealed typical ultrastructure features of a determinate nodule, containing several bacteriods within symbiosomes.

Keywords: Crack-entry infection, green manure crop, infection thread

Soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium*, collectively known as rhizobia, have the unique property of forming symbiotic partnerships with leguminous plants. This association results in the formation of specialized structures called nodules. Within the nodules, the bacteria reduce atmospheric nitrogen to ammonia, which is readily utilized by the host plant for growth and development. Although nodules primarily are formed on roots, legumes such as *Neptunia oleraceae*, *Discolobium pulchellum*, certain species of *Aeschynomene* and *Sesbania* can also form stem nodules (Olsson & Rolfe, 1985; James *et al.*, 1992; Boivin *et al.*, 1997). Most of these plants are found in waterlogged conditions and the nodules are usually found on the submerged parts of the stem. Nitrogen-fixing stem nodules of *Sesbania rostrata* have been examined in detail and shown to have several unique features. The nodules occur at specific sites distributed vertically along the stem. These sites, which represent incipient adventitious root primordia, differentiate into nodules when infected with rhizobia. Unlike the root nodules of leguminous plants, formation of stem nodules is not repressed by available soil nitrogen. Stem nodules exhibit substantial nitrogen-fixing activity and as a consequence, stem-nodulated *S. rostrata* has great potential as a green manure crop (Boivin *et al.*, 1997)

Some strains of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium* can induce stem nodules on their appropriate legume hosts. One strain of *A. caulinodans*, ORS571, is particularly interesting because it can fix atmospheric nitrogen both symbiotically and *ex planta* (Dreyfus & Dommergues, 1981). This bacteria forms nodules on the roots and stems of its host, *S. rostrata*. The morphogenesis and structure of stem nodules initiated by *A. caulinodans* on *S. rostrata* have also been examined in detail (Dreyfus & Dommergues, 1981; Tsien *et al.*, 1983; Duhoux, 1984; Dreyfus *et al.*, 1988). Based on these studies, stem nodule development can be briefly summarized as follows: *Azorhizobia* enter the root primordia by a crack-entry mechanism at the point of emerging lateral roots, colonize the intercellular spaces, and extend toward the meristematic zone by means of infection threads. After release of *Azorhizobia* into plant cells from the infection thread, they differentiate into nitrogen-fixing bacteroids and are enclosed by peribacteroid membrane (symbiosome). In contrast to stem nodules, an ultrastructural study has revealed some unusual aspects in the *A. caulinodans*-induced organogenesis of root nodules on *S rostrata* (Ndoye *et al.*, 1994). Bacteria in this interaction were released from infection threads into the host cell by "infection droplets" a mechanism not observed in stem nod-

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ules containing the same bacterium (Ndoye *et al.* 1994). In this study, anatomical and morphological changes leading to the formation of stem nodules on *S. rostrata* by *Sinorhizobium* sp. strain MUS10 (hereafter called MUS10) is reported. This organism is geographically and phylogenetically distinct from *A. caulinodans*. The early stages of infection process by MUS10 closely resemble the developmental sequence in *Rhizobium*-induced root and stem nodules Some developmental events in the stem nodulation by MUS10 also show similarity to the nodule development in the non-leguminous tropical tree *Parasponia rigida*.

MATERIALS AND METHODS

Seeds of *S. rostrata* were obtained from Dr. Catherine Boivin (Institut Francais de Recherche Scientifique pour le Développement en Cooperation, Sénégal, West Africa). The seeds were treated with concentrated sulfuric acid for 30 min and washed extensively in running water prior to germination on 1% water agar at 30°C. When the roots were 4 cm in length, seedlings were transferred to Magenta-type Leonard jars containing Jensens' N-free solution (Vincent, 1970) and grown in a controlled-environment chamber as described (Krishnan & Pueppke, 1991). Root primordia present on the stems of 20-day-old seedlings were inoculated with MUS10 with an artist's brush. Nodules were harvested two days after inoculation (DAI), 4 DAI, 6 DAI, 10 DAI, and 15 DAI and processed for ultrastructural analysis.

Tissue preparation for light and transmission electron microscopy

Stem nodules harvested at different developmental time points were dissected into small pieces (1 to 2 mm) with a razor blade and fixed at room temperature for 4 h in buffered 2.5% glutaraldehyde (pH 7.2, 50 mM sodium phosphate). The tissue samples were washed four times at 15 min intervals with 50 mM phosphate buffer (pH 7.2) and post-fixed with 2% aqueous osmium tetroxide for 1 h at room temperature. After extensive rinses in distilled water, the samples were dehydrated in a graded acetone series and infiltrated with Spurr's resin (Krishnan *et al.*, 1986). Free-hand sections were obtained from freshly harvested nodules with a razor blade and stained with 0.05% methylene blue. Materials fixed in Spurr's resin were cut with a glass knife and stained with 1% (w/v) toluidine blue for 2 min and examined with bright-field optics. Thin sections were cut with a diamond knife and collected on uncoated copper grids, stained with 0.5% aqueous uranyl acetate and 0 4% aqueous lead citrate, and viewed with a JEOL JEM 100B (Tokyo, Japan) electron microscope at 100 kV.

Scanning electron microscopy

Nodules were cut in half with a razor blade and fixed immediately in glutaraldehyde as described earlier. The tissue was post-fixed with 2% aqueous osmium tetroxide for 2 h at room temperature. Fixed nodule tissue was dehydrated in a graded ethanol series and subjected to critical-point drying. The nodules were mounted on specimen stubs with copper tape and sputter coated with gold-palladium. Specimens were examined with a JEOL JSM-35 (Tokyo, Japan) scanning electron microscope at 20 kV.

RESULTS

Sinorhizobium sp. strain MUS10 forms both root and stem nodules on *S. rostrata*. Two-week-old *S. rostrata* seedlings had prominent adventitious root primordia on their stems. These primordia were arranged in vertical rows and measured 0.3 to 0.5 mm in diameter (Fig. 1) When MUS10 was brushed onto the stems, some of these primordia enlarged and formed nodules (Fig 1, larger arrows), while others failed to respond to inoculation (Fig. 1, smaller arrows). A transverse section of a developing stem nodule at 5 DAI revealed a pronounced change in the size and shape of the

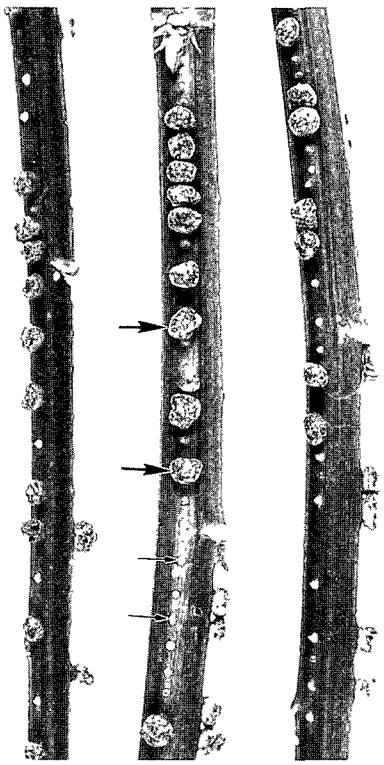

Fig. 1. *Sinorhizobium* sp strain MUS10-induced nodules on the stems of *S rostrata* (large arrows) The nodules are arranged in vertical ranks Uninfected root primordia are also present (small arrows)

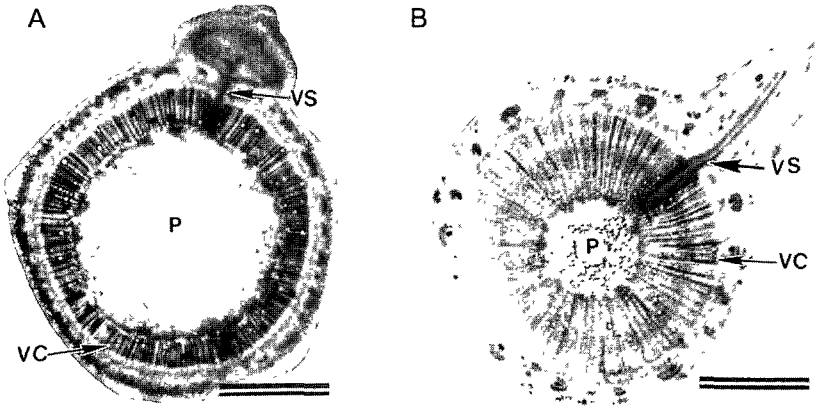

Fig. 2. (A) A free-hand transverse section through a 5-day-old stem nodule reveals swelling of the basal portion of the former root primordium Note the direct vascular connection (arrow) Bar = 1 mm (B) A free-hand section through a root primordium from a plant that had been immersed in water The vascular strand (larger arrow) can be seen extending into the root Bar = 1 mm. P, pith; VS, vascular strand, VC, vascular cylinder

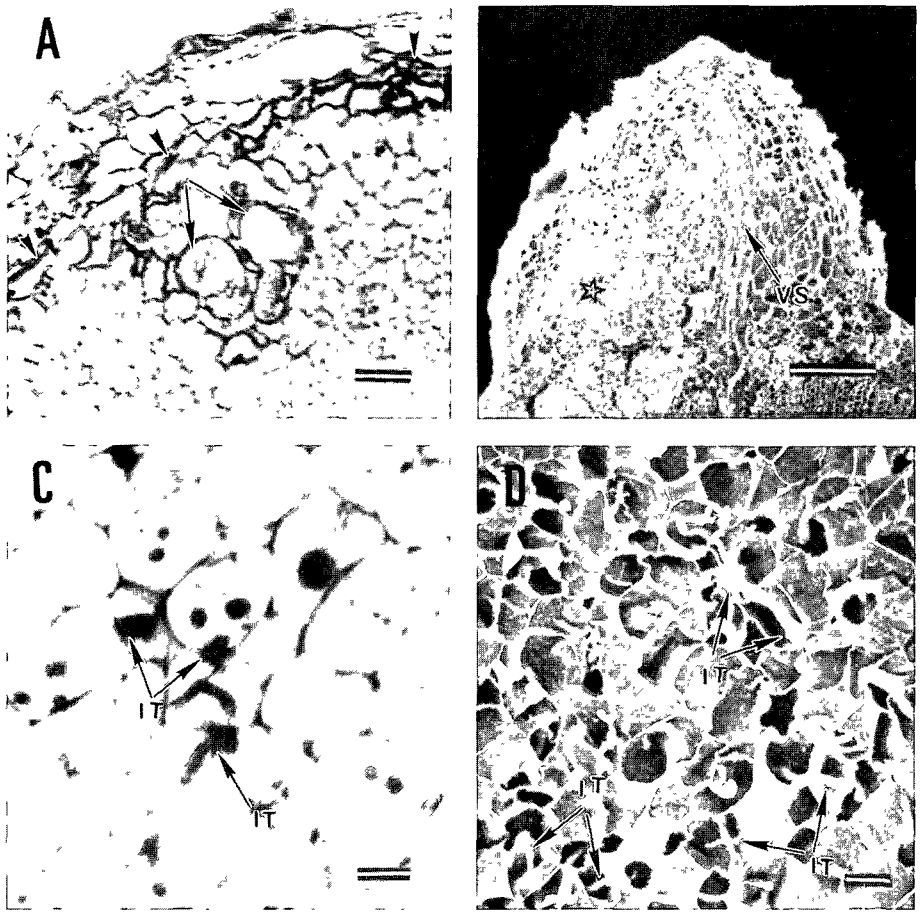

Fig. 3. (A to D) Light and scanning micrograph of a developing stem nodule. (A) Cells showing actively proliferating population of rhizobia (arrows). Note that a band of cells just under the epidermis accumulate dark-staining materials (arrowheads) Bar = 20 μm (B) Scanning electron micrograph showing the localized meristematic activity induced by inoculation (star). A prominent vascular strand (VS) running through the center of the root primordia is also seen (arrow) Bar = 200 μm (C) Light micrograph of a section through a meristematic region of a 5-day-old stem nodule reveals numerous infection threads (IT, arrows) Bar = 10 μm (D) Scanning electron micrograph of 5-day-old nodule showing several infection threads (arrows) in the meristematic region. Bar = 10 μm

adventitious root primordium (Fig. 2A). It had grown to three times larger in diameter than non-responsive root primordia. A prominent vascular strand, within the swollen root primordium, was directly connected to the vascular system of the stem (Fig. 2A, arrow). When *S. rostrata* stems were immersed in water, roots developed from the adventitious root primordia. A transverse section through one such root clearly shows a central vascular strand extending from the vascular cylinder of the stem (Fig. 2B, arrow).

During nodule morphogenesis, the base of the root primordial doubled in size two to three days after inoculation. A cross section of a nodule at 2 DAI showed that cells near the apex of the cone shaped organ had enlarged and were filled with actively dividing rhizobia (Fig. 3A, arrows). Some of the host cells near this area appear to have been damaged. The outer layers of the nodules consisted of prominent, dark-staining cell walls (Fig. 3A, arrow heads). At 5 DAI, nodule morphology had changed from a conical to spherical shape apparently due to meristematic activity of localized regions within the organ (Fig. 3B, asterisk). A central vascular strand can also be observed (Fig. 3B). The cells in the meristematic region revealed widespread infection threads (Fig. 3C, arrows) which were intensely stained. Scanning electron microscopy of cross sections from 4- and 6-DAI nodules revealed several tubular structures traversing from cell to cell (Fig. 3D, arrows). Usually, one infection thread was found in each cell, but in some instances, multiple threads were noted in individual cells (Fig. 4A). Dissec-

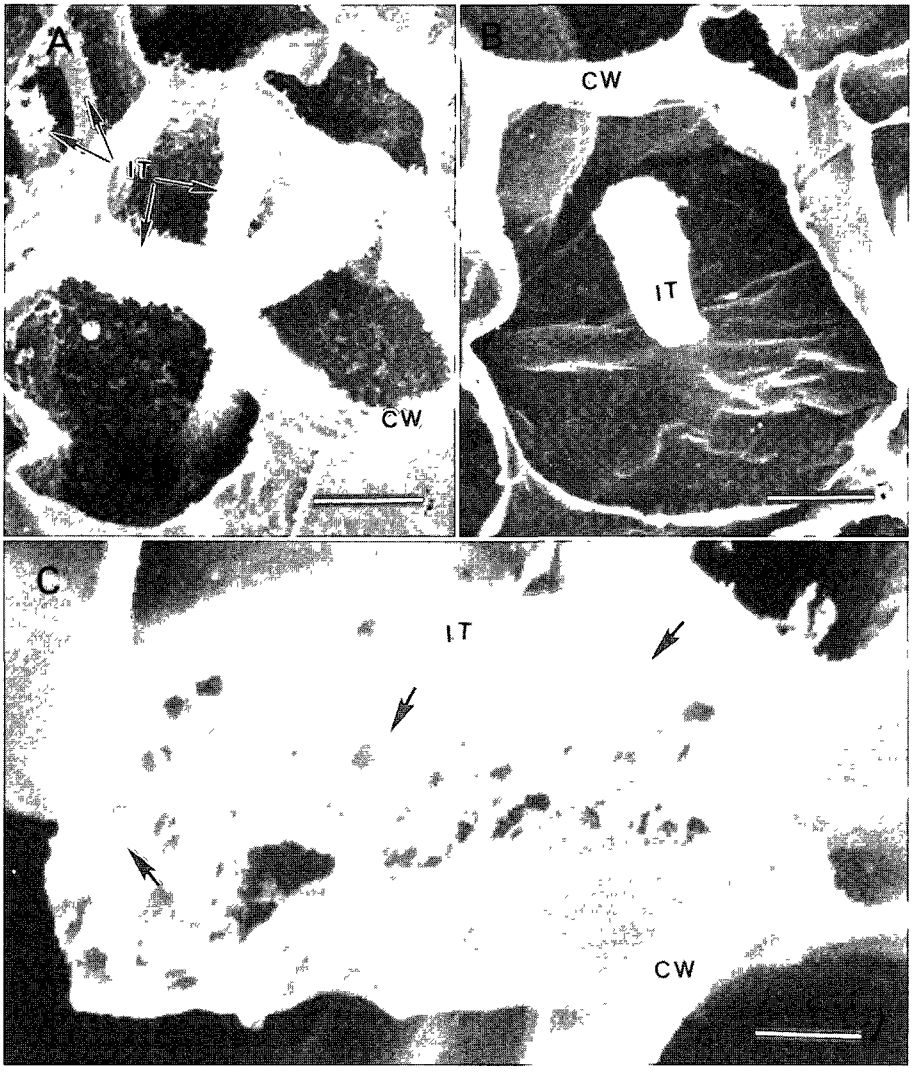

Fig. 4. (A-C) Scanning electron micrograph of infection threads in stem nodules (A) Infection thread can be seen traversing from one cell to another More than one infection thread can also be seen in one cell (arrows) Bar = 4 μm (B-C) Cross-sections of the infection threads revealing the presence of numerous cavities. These cavities apparently represent the location of rhizobia which have dislodged during the processing of the specimen The infection thread shown in Fig 4C represents the swollen junction near the cell walls (CW) Bar = 1 4 μm

tion of the infection threads revealed several cavities within these tubes that presumably harbored rhizobia (Figs. 4B and 4C, arrows).

Transmission electron microscopic examination of 4-day-old nodules revealed free-living rhizobia in the region close to the fissure in epidermal tissue created by the rupture of the swelling root primordia (Fig. 5A). Many of these bacteria stored a large quantity of poly-β-hydroxybutyrate granules (Fig. 5A). Rhizobia proliferate within these intercellular spaces and presumably invade the host cell without being ensconced by plant cell membranes. The host cell cytoplasm is very dense and has the characteristic appearance of disintegration often seen in plant-pathogen interactions (Fig. 5B). The walls of such cells are highly osmiophilic (Fig. 5B, arrow). Numerous infection threads are often seen in 4-day-old nodules and are filled with a homogeneous matrix material (Figs. 5B, 5C, and 5D). Infection threads were surrounded by fibrillar material putatively derived from the host (Fig. 5C, arrow) and often contained dark-staining inclusions scattered around the fibrillar material (Fig 5C, arrow heads). Host cell cytoplasm and organelles are confined to the cells periphery (Fig. 5C). As the infection thread enlarges, finger-like projections radiate from the host fibrillar material surrounding the infection thread (Fig. 5D, arrow). The fibrillar

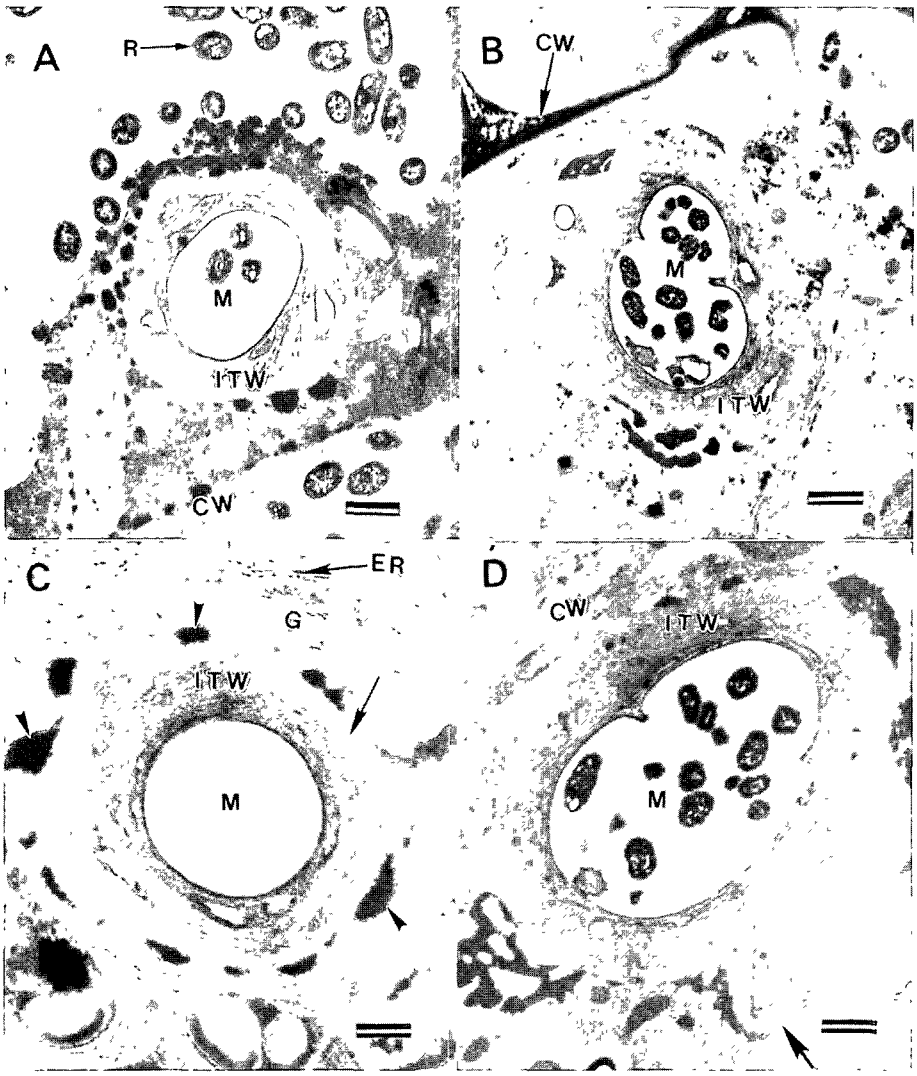

Fig. 5. Transmission electron micrographs of thin sections of 4 day-old stem nodules (A) Intercellular rhizobia (R) near the apex of the nodule An adjacent cell shows a cross-section of an infection thread Bar = 0 8 μm (B) The host cell shows signs of disintegration Osmiophilic inclusions can also be seen (arrows). Bar = 1 8 μm Higher magnification views of the infection threads (C and D). (C) The infection thread is filled with what appears to be a homogeneous, amorphous matrix (M). The cell also contains prominent Golgi apparatus (G) and amyloplasts The infection thread wall (ITW) is made up of fibrillar material and contains several small vacuoles At the periphery of the infection thread wall, pockets of dark staining materials are seen (arrowheads) Bar = 0.6 μm (D) Enlargement of the infection thread wall and formation of finger-like projections (arrow) The infection thread contain several free living rhizobia dispersed in the homogeneous matrix Bar = 0.7 μm. CW, cell wall, ER, endoplasmic reticulum.

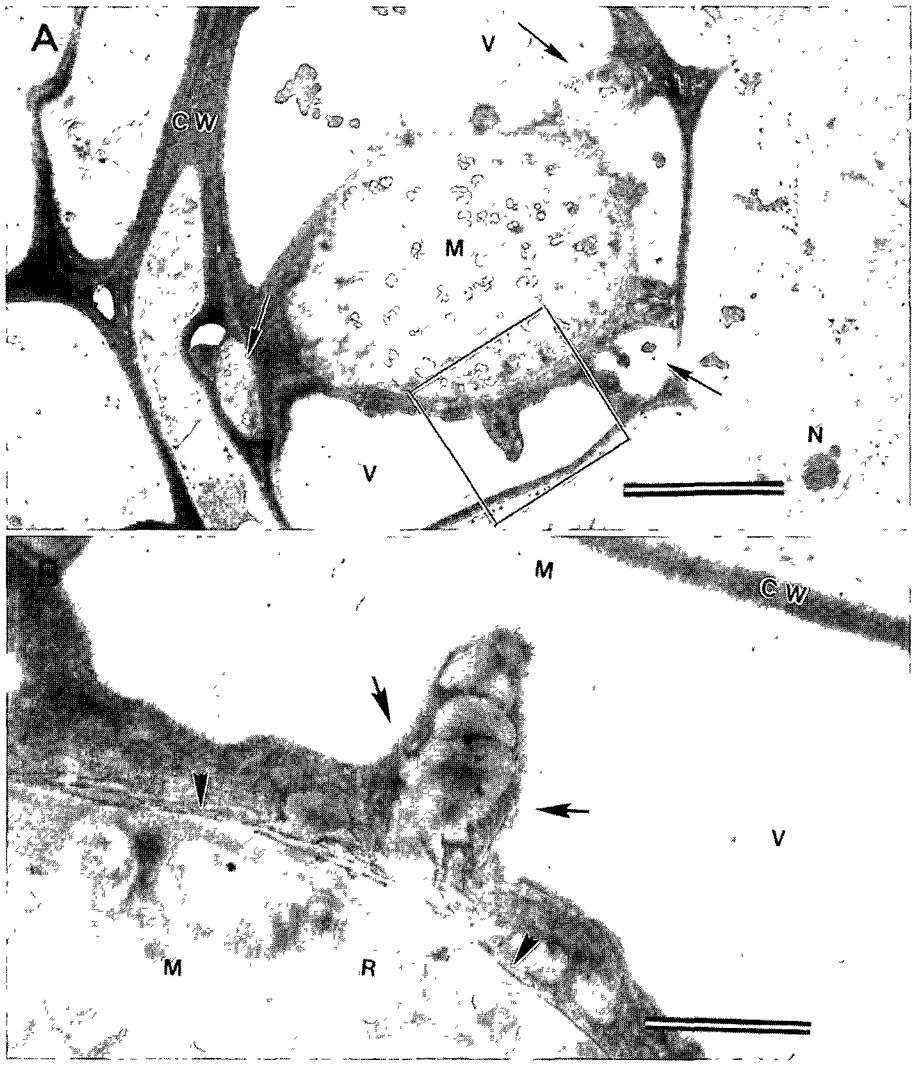

Fig. 6. High magnification electron micrographs of enlarged infection pocket revealing the presence of several free-living bacteria The infection pocket is surrounded by a thick wall Note the finger-like projections from the enlarged infection pockets Some of these projections are in contact with the host cell wall Bar = 6 μm (B) High-magnification view of the boxed region in Fig 6A. The cell wall surrounding the infection pocket is delineated from the host cytosol by plasma membrane Note the presence of a granular region between the plasma membrane and the projections from the infection pocket The bacteria within the infection pockets contain poly-b-hydroxybutyrate granules The host cell also contains numerous mitochondria. Bar = 1 μm CW, cell wall, M, mitochondria; N, nucleus, R, rhizobia; V, vacuole

material also contains small vacuoles, which accumulate dark-staining inclusions (Fig. 5D).

Enlargement of the infection threads is principally due to the proliferation of the contained rhizobia (Fig 6A). The enlarged infection thread now occupies much of the volume of the cell (Fig. 6A). Additionally, the large vacuoles of host cells are diminished in size as the infection thread enlarges. The cytoplasm is delimited from the infection thread by means of a membrane, which clearly differentiates the host cell from the infection thread (Fig. 6B). At this developmental stage, the release of the rhizobia into the cytosol of the host cells was not observed. When a 10-day-old nodule was viewed with the scanning electron microscope, large pockets of infected cells could be easily identified (Fig. 7A, stars). At a higher magnification, the infected cells within the nodules revealed numerous rhizobia (Fig. 7B). Transmission electron microscopic examination of 15-day-old nodules confirms that the entire cell becomes occupied by rhizobia, which have now differentiated into bacteriods. Unlike the cells in which infection threads were prominent, walls of cells occupied by rhizobia showed no accumulation of dark-staining materials (compare Figs. 6A and 7C). The bacteriods were surrounded by symbiosomes, which often enclosed more than one rhizobium (Fig. 7C).

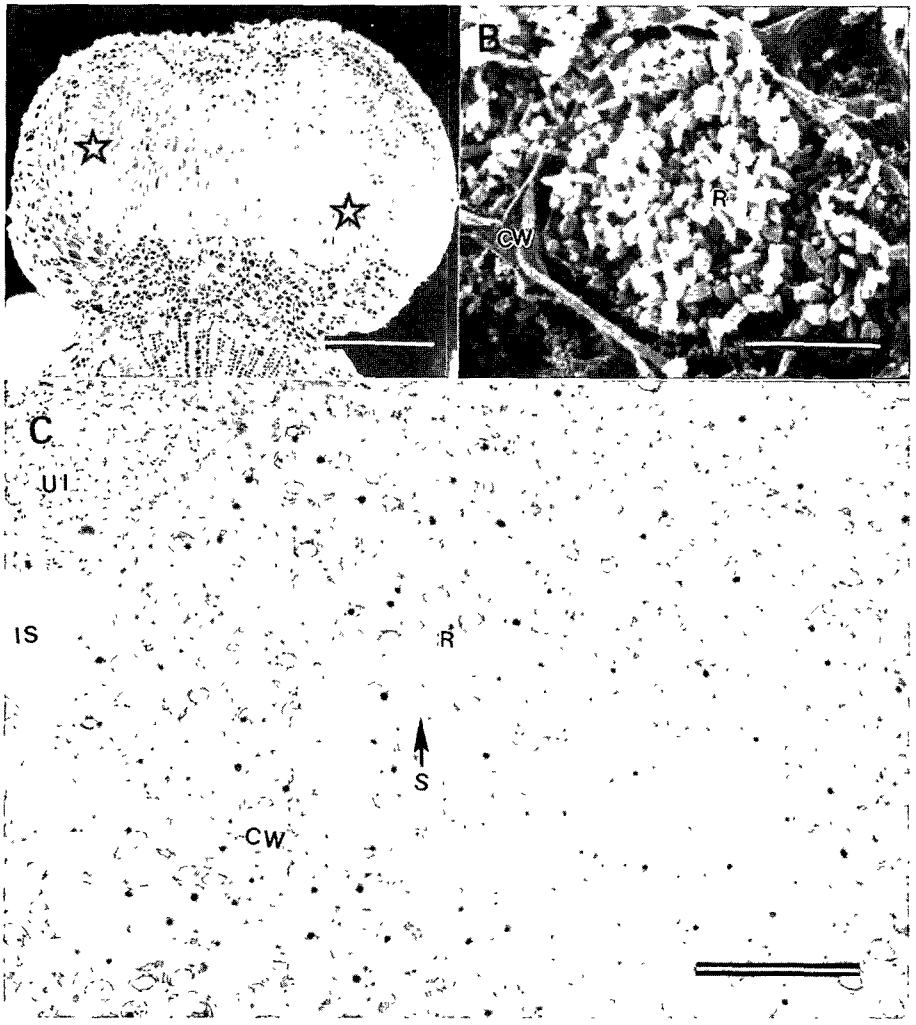

Fig. 7. (A and B) Scanning electron micrographs of 10-day-old *Sesbania rostrata* stem nodules Regions within the swollen nodule which are infected with rhizobia are represented by stars. Vascular bundles are seen at the base of the central rhizobial containing region Bar = 500 μm (B) A high magnification view of the bacterial filled region Bar = 10 μm (C) Electron micrograph of thin section of a mature stem nodule The bacteria are enclosed within symbiosomes Most of the symbiosomes contain more than one bacterium Bar = 4 μm. CW, cell wall; IS, intercellular space; R, rhizobia, S, symbiosome; UI, uninfected cell.

DISCUSSION

Sinorhizobium sp MUS10 enters the host through cracks located at the tip of incipient adventitious root primordia. This mode of entry is reminiscent of that reported for specific tropical legumes in which the rhizobia enter into the root cortex through a crack generated by the emerging lateral roots (Chandler, 1978, Sprent & Faria, 1988; Alazzard & Duboux, 1990; Ndoye *et al.*, 1994; Rana & Krishnan, 1995) In the case of *S. rostrata* stem nodules, there are no root hairs associated with adventitious root primordia At least three modes of entry for rhizobia into respective host plants have been documented (Sprent & Faria, 1988). The bacterium can invade through wounds, root hairs, and epidermal cells Entry through wounds, often called "crack entry" is considered a "primitive" type of infection (Ndoye *et al.*, 1994).

Establishment of infection pockets induces two distinct changes within the adventitious root primordia The first change, which resembles hypersensitive response, involves deterioration and collapse of host cells surrounding the infection pockets Fragments of plant cell wall, generated during infection process, are thought to be responsible for initiating the hypersensitive reaction (McNeil *et al.*, 1984). Occurrence of fragmented cell wall material surrounding the infection pockets is common in MUS10-induced stem nodules. A similar situation occurs during the early phase of infection in *Stylosanthes* spp (Chandler *et al*, 1982) and *A. afraspera* (Alazzard & Duboux, 1990). The second pronounced change observed is the stimulation of cell division

in the cortex. In contrast to plant cells near the vicinity of the infection pockets, which undergo marked deterioration, inner cortical cells are stimulated to divide rapidly. Diffusible chemical signals, the Nod-factors (Truchet *et al.*, 1991; Geurts & Bisseling, 2002), mediate the meristematic activity of cortical cells in legume roots. Structure of the Nod factors produced by *S. saheli*, *S. teranga* bv. *sesbaniae*, and *A. caulinodans*, three symbionts of *S. rostrata*, has been elucidated (Lorquin *et al* , 1997). These Nod factors contain two unique features in the terminal, reducing glucosamine residue, an arabinosyl group on C-3 and fucosyl substitution on C-6 (Lorquin *et al.*, 1997; Mergaert *et al.*, 1997). The Nod factor structure of MUS10 is currently being investigated to determine if it also exhibits this double glycosylation of the terminal glucosamine residue

The fissure in the adventitious root primordia provides a niche for MUS10 to rapidly multiply and form infection pockets. Rhizobia in the infection pockets were enclosed within homogeneous material. Although the homogenous material is speculated to be exo-polysaccharide (Tsien *et al.*, 1983), neither the chemical composition nor function of the material is known. Biochemical analysis has demonstrated that proline-rich proteins are a major component of the infection thread matrix (Sherrier & VandenBosch, 1994). Rhizobia from the infection pockets are conveyed to the meristematic cortical cells by means of infection threads. Infection threads are numerous in MUS10-induced stem nodules and always originate from the cell walls of the infected cells. The electron micrographs presented in this study provide in-depth information on the structure and the origin of the infection threads. Further, the scanning electron micrographs clearly show that the infection threads can harbor several rows of the bacterium Since infection threads appear to arise from cell walls, the general consensus is that the structures are of plant origin. The elegant work of Higashi *et al* (1987), however, suggests that the infection threads are structurally distinct from the cell wall. Driselase, a cell-wall degrading enzyme, had no effect on the infection thread within *Astragalus indica* nodules, while the cell walls were digested Because infection threads are numerous in *S. rostrata* stem nodules, it should be possible to biochemically purify the infection threads and examine their chemical composition.

Even though the mode of infection of MUS10 can be considered "primitive" (crack entry), the rhizobia within the infection threads are released into the actively dividing cortical cells where they differentiate into bacteroids. In this regard, they differ from all Caesalpinioid legumes, and some genera of Papilionoideae (Sprent & Faria, 1988) and *Paraspopnia* (Trinick, 1979), where the rhizobia are not released from the infection threads A comparison of the micrographs of early development of nodules in *P. rigida* presented by Lancelle & Torrey (1985) with the present study reveals striking similarities between the two systems. In both cases, the rhizobia multiply in the intercellular spaces and infection threads are initiated from host cell walls. Rhizobia within the infection threads are embedded in a homogeneous matrix and are surrounded by host cell wall. The host cells near the vicinity of the infection pockets degenerate indicating that the plant recognizes the invading rhizobium as a pathogen rather than a symbiont. However, these two systems diverge at the latter stages of nodule development. In *Parasponia*, the bacteria are retained in the infection threads through out the nodule development, while in *S. rostrata*, stem nodules are released into the cortical cells. The retention of bacteria within the infection threads has been regarded as evolutionarily "primitive" (Sprent & Faria, 1988). The ultrastructure of *S. rostrata* stem nodules exhibits both primitive and advanced anatomical features. This state, perhaps, represents an intermediate condition between nodule development in non-legumes such as *Parasponia* and the well advanced soybean-*Rhizobium* interactions (Newcomb *et al.*, 1979)

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