Journal of Experimental Botany, Vol. 41, No. 230, pp. 1199–1206, September 1990

Development of Stem Nodules in a Tropical Forage Legume, *Aeschynomene afraspera*

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Received 30 October 1989

ABSTRACT

Rhizobial infection occurred on the stem of *Aeschynomene afraspera* at the site of emergence of adventitious root primordia. Rhizobia invaded cortical cells at the base of the root primordium. The first infected cell enlarged and collapsed after rhizobia had multiplied in large numbers. At this time, a meristematic zone was initiated some distance beneath the first infected cell. Rhizobial penetration into the deeper cortex was by progressive collapse of infected cells towards the meristematic zone; rhizobia entering the cortical cells by invagination of the host cell wall. At the entry point, rhizobia were embedded in digitate cell wall material. These infection structures were restricted, always originating from the cell wall of an adjacent infected cell. Soon after infection, the cell collapsed progressively forming infection strand-like structures which developed up to the meristematic zone. When infection had reached the meristematic zone, invaded host cells ceased to collapse but divided repeatedly to form the nodule.

Key words: *Aeschynomene afraspera*, stem nodulation.

INTRODUCTION

A few tropical legumes, belonging to the genera *Aeschynomene* and *Sesbania*, are able to form nitrogen-fixing nodules both on stems and roots. Stem nodulation, restricted to date to only one species of *Sesbania, S. rostrata* (Dreyfus and Dommergues, 1981), is much more widespread within the genus *Aeschynomene* (Alazard, 1985).

Recent interest has been generated in *Aeschynomene afraspera* cv. Leonard which is characterized by a profuse stem nodulation and a high nitrogen-fixing potential (Alazard and Duhoux, 1987). Moreover, this plant can be successfully used as a green manure in rice cultivation, increasing the rice grain yield by 80% (Alazard and Becker, 1987).

There are two different ways by which the infection and nodule development process in root nodulated legumes can occur [see reviews by Dart (1977) and Bauer (1981)]. The first process, which occurs in the majority of temperate legumes, involves the formation of infection threads within root hairs followed by the release of rhizobia in the cortical cells of the host plant. The second process, which occurs in a relatively small number of tropical legumes such as *Arachis* (Allen and Allen, 1940; Chandler, 1978) and *Stylosanthes* (Ranga Rao, 1977; Chandler, Date, and Roughley, 1982), is characterized by the absence of infection threads and involves direct invasion of cortical cells at the site of emerging lateral roots. Intercellular infection is initiated at the basal junction between the root hair cells and adjacent epidermal cells. Rhizobia invade the host cell by localized cell wall degradation. Further development of the nodule occurs by repeated division of the infected host cells.

Both stem and root nodules of *Aeschynomene indica* are known to arise near emerging lateral roots (Arora, 1954). When root hairs are absent, it is presumed that the mode of entry is through epidermal cells of the cortex. No infection threads are formed in the nodules, hence rhizobia are spread by cell division.

Studies by Tsien, Dreyfus, and Schmidt (1983) and Duhoux (1984) on *Sesbania rostrata* are the first reports on the infection process and nodule morphogenesis in stem nodulated legumes. In *Sesbania rostrata*, rhizobial

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infection is intercellularly initiated between the basal cells of the protruding root primordium forming infection pockets in which rhizobia multiply in large numbers. At the same time, a meristematic zone arises in the adjacent cortical cells of the root primordium. Infection threads then originate from the intercellular pockets of infection and divide into several branches that invade the meristematic cells.

Most of the studies in *Aeschynomene* sp. have dealt with the ultrastructure of established root or stem nodules (Yatazawa, Yoshida, and Maeda, 1984; Vaughn and Elmore, 1985). To our knowledge, there are no reports on the infection process and early development of *Aeschynomene* stem nodules. We report here studies on the early stages of infection and stem nodule development in *A. afraspera.*

MATERIALS AND METHODS

Plant material and inoculation

Aeschynomene afraspera plants were grown in a greenhouse in pots containing waterlogged sterile soil as described previously (Alazard and Duhoux, 1987). Stems were inoculated by applying a 10-fold dilution of a broth culture of *Rhizobium* sp. strain ORS 322 on to the stem with a small spraying bottle or a sterile brush (Alazard, 1985).

Microscopy of nodules

Nodules were fixed for both light and transmission electron microscopy 24 h after inoculation and daily thereafter for 12 d, so that successive stages of stem nodule development could be noted.

Materials for transmission electron microscopy were fixed in 2·5% glutaraldehyde (v/v) in 0·2 M Na-cacodylate buffer, pH 7·0 for 1 h under vacuum at room temperature. The fixed material was washed four times in the same buffer, post-fixed for 1 h in buffered 1% OsO_4 (w/v), rinsed, dehydrated and embedded in Epon 812. Ultra-thin sections were stained with 2% uranyl acetate and lead citrate (Reynolds, 1963) prior to observation on a Siemens Elmiskop 101 electron microscope.

Materials for light microscopy were fixed overnight in a solution containing 32·5% ethanol, 5% acetic acid and 5% formaldehyde (v/v), and embedded in Paraplast (+) (Brunswich Co.), or were cut from Epon blocks prepared as above. Sections were stained with Regaud's iron–haematoxylin stain (Lison, 1960).

RESULTS AND DISCUSSION

Nodulation sites on the stem of A. afraspera

As with other stem nodulated legumes, stem nodulation of *A. afraspera* occurred at predetermined sites independently of bacterial infection (Alazard and Duhoux, 1987). A stem nodulation site of *Aeschynomene afraspera* consisted of a root primordium protruding beneath epidermal cells. The site became susceptible to rhizobial infection only when a circular cavity was formed between the central root primordium and the surrounding cortical tissue (Plate 1A, D). At this stage, only a thin layer of flattened epidermal cells overlayed the apex of the protruding root primordium. This epidermal layer presented surface breaks due to mechanical forces associated with the developing root primordium, which allowed rhizobial access to the inside of the stem nodulation site. Root hairs were not observed in the epidermis of the root primordium.

Infection of the stem nodulation site

The first evidence of stem infection was the presence, 2 d after inoculation, of an enlarged star-shaped cell near the epidermal cell layer (Plate 1B, C). This irregularly and loosely outlined cell had a voluminous nucleus and nucleolus, and was filled with rhizobia. At the same time, or shortly after the enlarged cell was detectable, a meristematic zone arose in the inner cortex at a distance beneath the first infected structure (Plate 1B, C), suggesting that rhizobia could trigger meristem initiation from a distance by means of a diffusible chemical signal as described in temperate legumes (Truchet, Michel, and Denarie, 1980). This infection structure was transient. Transverse sections through the inoculated stem nodulation site at a slightly later stage of development revealed that infection was initiated in the cortex of the root primordium (Plate 1D, E) and showed an advanced stage of collapse of the initially infected cell. The collapsed cell was compressed by neighbouring cells and appeared as a densely stained star-shaped patch. It was filled with rhizobia embedded within a very electron-dense material (Plate 1F).

Our observations were inadequate to provide information on rhizobial invasion of the first infected cell.

Development of infection

The mechanism for intracellular invasion of host cells and progressive collapse was evidenced in the subsequent development of infection. Rhizobia entered the host cell by invagination of the cell wall (Plate 2A). The rhizobia appeared to come from the inner wall of the host cell. At the entry point, rhizobia were embedded in new cell wall material bounded by plasma membrane. The newly-formed wall material at this point was reminiscent of that observed for infection thread initiation in root hairs (Callaham and Torrey, 1981). However, the infection structures described here were restricted and did not extend beyond the boundaries of the host cell. In addition, rhizobia were not surrounded by an electron-transparent matrix as found in true infection threads (Callaham and Torrey, 1981; Turgeon and Bauer, 1985). Cell invasion appeared to have a deleterious effect on the host cell. It created disorganization of the cytoplasm (Plate 2A) and soon after, the cell collapsed (Plate 2B). Infection advanced from cell to cell as shown by internal invaginations of the host cell wall in the adjacent cell. Then rhizobia penetrated into the deeper stem cortex by the successive collapse of invaded cells and intercellular infection strand-like structures were formed in the resulting spaces.

This infection mode leading to the death of the initially

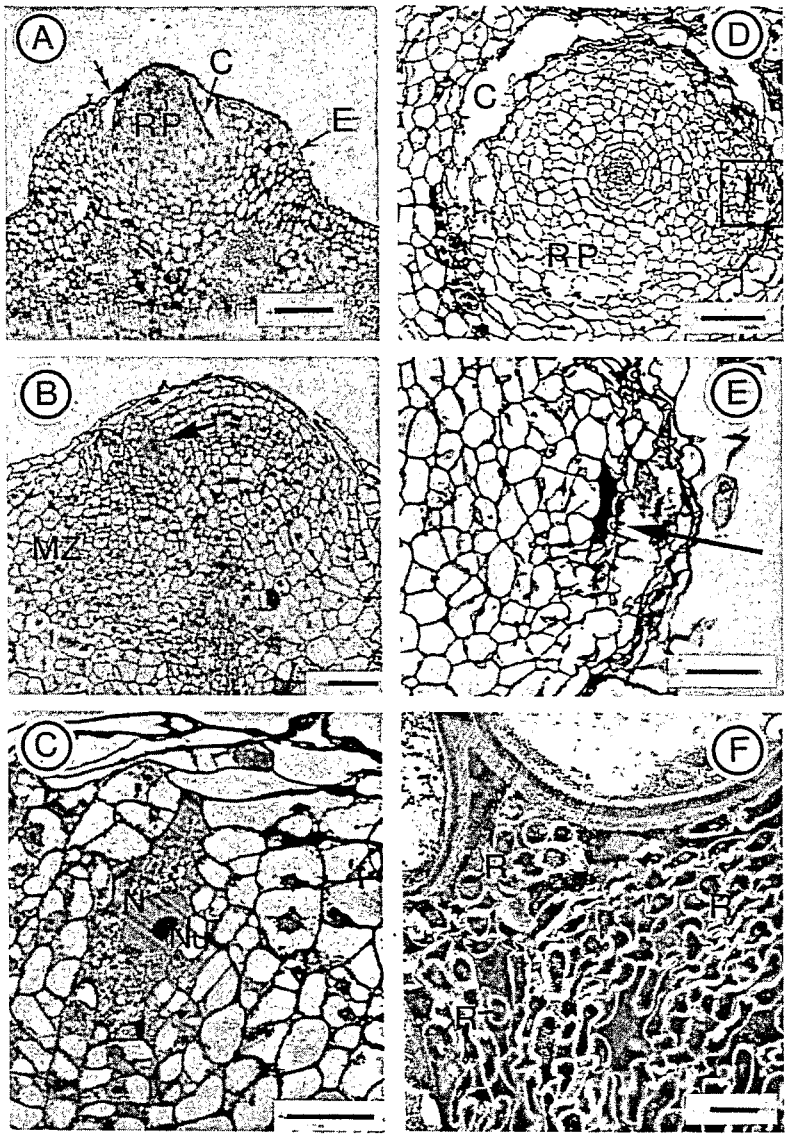

PLATE 1. (A) Light micrograph of a median longitudinal section through an uninoculated nodulation site on the stem of *A. afraspera*. The protruding root primordium (RP) is overlayed with a thin layer of flattened epidermal cells (double arrows) and leaves a circular cavity (C) around its base. Stem epidermis (E). Paraplast (+) block; iron-haematoxylin stain. ×76 (Bar=0·25 mm). (B) Light micrograph of a longitudinal section through a nodulation site, 2 d after inoculation, showing an enlarged infected cell (arrows) in the cortex near the circular cavity. Note the meristematic zone (MZ) induced beneath the infected cell. Epon block; iron–haematoxylin stain. ×80 (Bar=100μm) (C) Enlargement of the first infected host cell shown in (B). This loosely outlined cell has a prominent nucleus and nucleolus as compared with those in adjacent cells and is probably degenerating as evidenced by cytoplasm content and irregular boundaries of this cell. Nucleus (N); nucleolus (Nu). ×140 (Bar=50 μm). (D) Light micrograph of a transverse section through an inoculated nodulation site at a later stage in development showing the collapsed cell (boxed area) located in the outer cortical cells of the root primordium. Note the circular cavity (C) encircling the root primordium (RP). Epon block; iron–haematoxylin stain. ×100 (Bar=0·1 mm). (E) Enlargement of boxed-in area of (D) showing the collapsed cell (arrow) compressed by neighbouring cells. The collapsed cells resemble intercellular spaces filled with darkly stained material. ×220 (Bar=50 μm). (F) Transmission electron micrograph of the collapsed cell shown in (E), with numerous rhizobia (R) embedded in a very electron-dense material. ×7000 (Bar=1 μm).

invaded cell was similar to that described in *Stylosanthes* spp. (Chandler *et al.*, 1982) and resembled a defence mechanism to invasion by phytopathogens more than a symbiotic interaction.

As a result of the collapse of infected cells, infection strand-like structures were observed in the cortex, leading away from the fissure at the base of the root primordium up to the meristematic zone (Plate 3A, B). At this stage, a layer of tannin-containing cells partially surrounded the meristematic zone (Plate 3A).

Meristematic cells were constituted by small uninfected thin-walled cells containing a large centrally-located nucleus plus numerous vacuoles and plastids in a dense cytoplasm (Plate 3C).

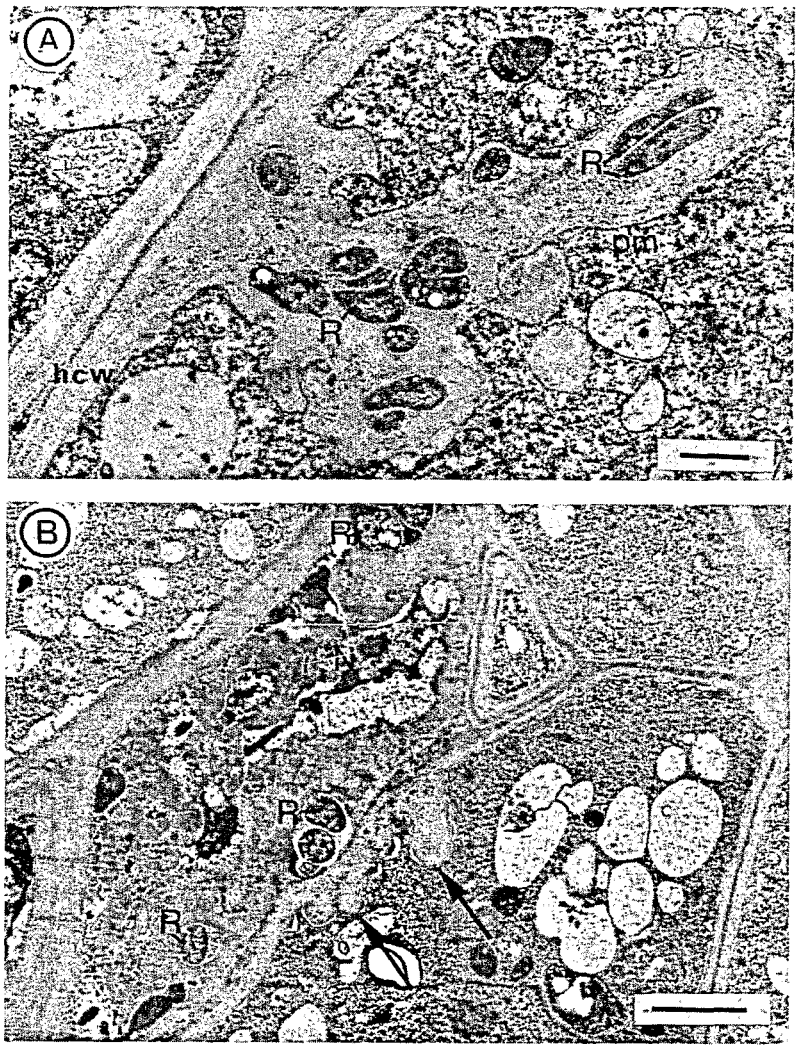

PLATE 2. (A) Transmission electron micrograph showing intracellular penetration of rhizobia. Rhizobia (R) are embedded in cell wall material surrounded by plasma membrane (pm). Host cell wall (hcw). Note the disorganization of the invaded cell cytoplasm. ×11 000 (Bar=1 μm). (B) Transmission electron micrograph of an inner collapsed cell. The cytoplasm is degenerated but the nucleus (N) is still recognizable. Note the invaginations of cell wall in the adjacent cell (arrows). Rhizobia (R). ×7500 (Bar=2 μm).

Occasionally more than one strand could be observed in the same nodulation site suggesting separate infection origins (Plate 3A, B).

Nodule development

When the infection strand reached the meristematic zone, 24 to 48 h after the first infected cell could be observed, the infected meristematic cells did not collapse. Meristematic cells appeared infected with a few rhizobia enclosed singly in a peribacteroid membrane (Plate 4A). At this stage, the presence of rhizobia did not induce the collapse of the host cell (Plate 4A). At the entry point into the meristematic cells, rhizobia were not embedded within the cell wall material (Plate 4B, C).

A phase of rapid multiplication of rhizobia occurred while the infected cells divided repeatedly, leading to an increase in the volume of the nodule (Plates 4A, 5A).

There were no uninfected cells in the central core of the infected tissue and true infection threads were never observed during the nodule development stage.

Rhizobia in the central tissue of the nodule were rod-shaped or elongated, similar to their free-living form (Plate 4C). They were singly enclosed in a peribacteroid membrane probably originating from the plasma membrane. They had a dense fibrillar nuclear material and some electron-transparent regions at the polar end, probably corresponding to poly-3-hydroxybutyric acid (PHB) inclusions (Plate 5B).

The infection process leading to stem nodule formation in *Aeschynomene afraspera* is quite different from that described for the other stem nodulated legume *Sesbania*

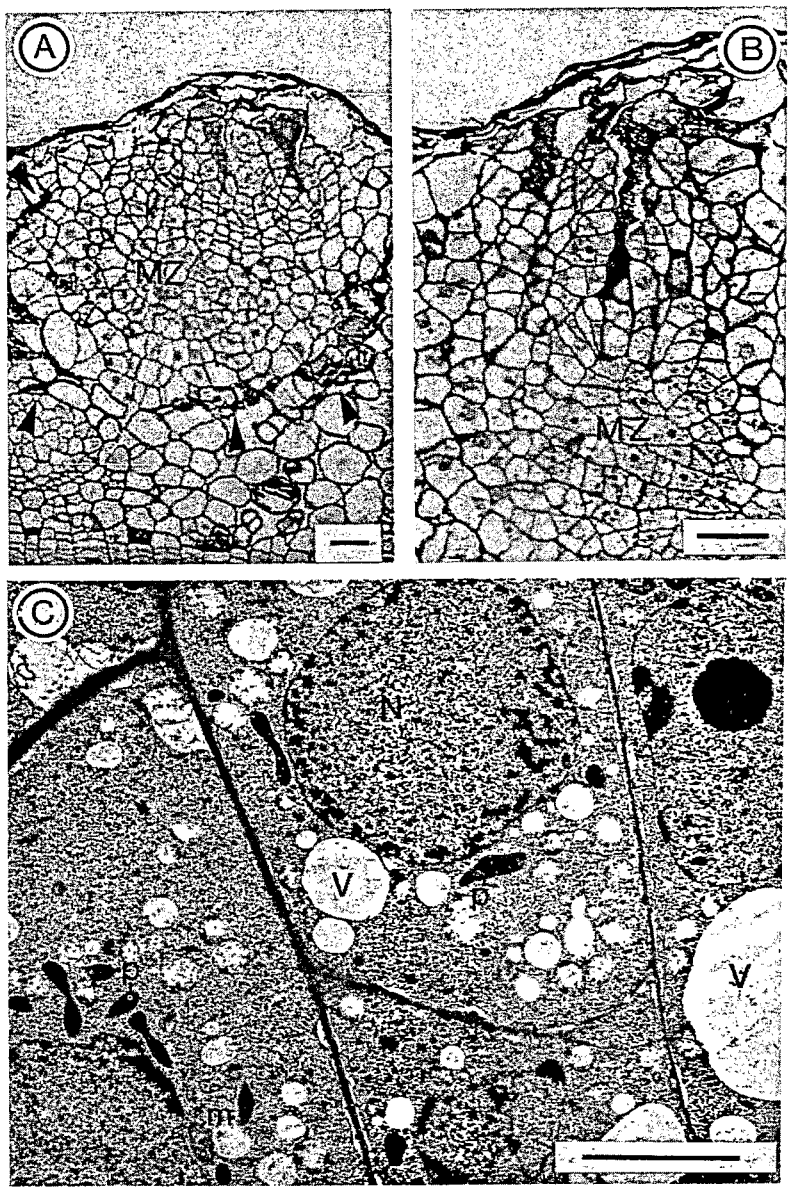

PLATE 3. (A) Light micrograph of longitudinal section through a 4-d-old nodule showing two initially infected cells which have collapsed. The meristematic zone is surrounded by a layer of cells containing darkly stained tannins (large arrowheads). Meristematic zone (MZ). Epon block; iron–haematoxylin stain. × 120 (Bar = 50 μm). (B) Development of an infection strand constituted by linear juxtaposition of collapsed cells deeper in the cortex up to the meristematic zone (MZ). × 220 (Bar = 50 μm). (C) Electron micrograph of thin section in the meristematic zone 3 d after inoculation. Uninfected cells have a large centrally located nucleus (N) in vacuolated (V) cytoplasm. Plastids (P); mitochondria (m). × 5000 (Bar = 5 μm).

rostrata. On the other hand, it appears closely related to that of alternate modes of infection described in threadless legumes, particularly in *Stylosanthes* spp. where early invaded cells collapse (Chandler *et al.*, 1982). The situation in *Arachis* (Chandler, 1978) is rather different, in that the penetration of rhizobia in the deeper cortex is via progression by separating the cells at the middle lamellae.

Aeschynomene, *Arachis*, and *Stylosanthes* which belong to the legume tribe Aeschynomeneae (Polhill, Raven, and Stirton, 1981) form nodules of the aeschynomenoid type that is located in the axils of lateral roots (Corby, 1981). It is interesting taxonomically that a similar mode of infection occurs in these genera. In addition, the aeschynomenoid nodules are also found in most genera of the tribe Dalbergieae and in the monogeneric tribe Adesmieae (Faria, Franco, Jesus, Menandro, Baitello, Mucci, Dobereiner, and Sprent, 1984). It is likely that all these plants could be sharing a common mode of entry (Sprent, 1989). Mode of entry in ways other than through root hairs may

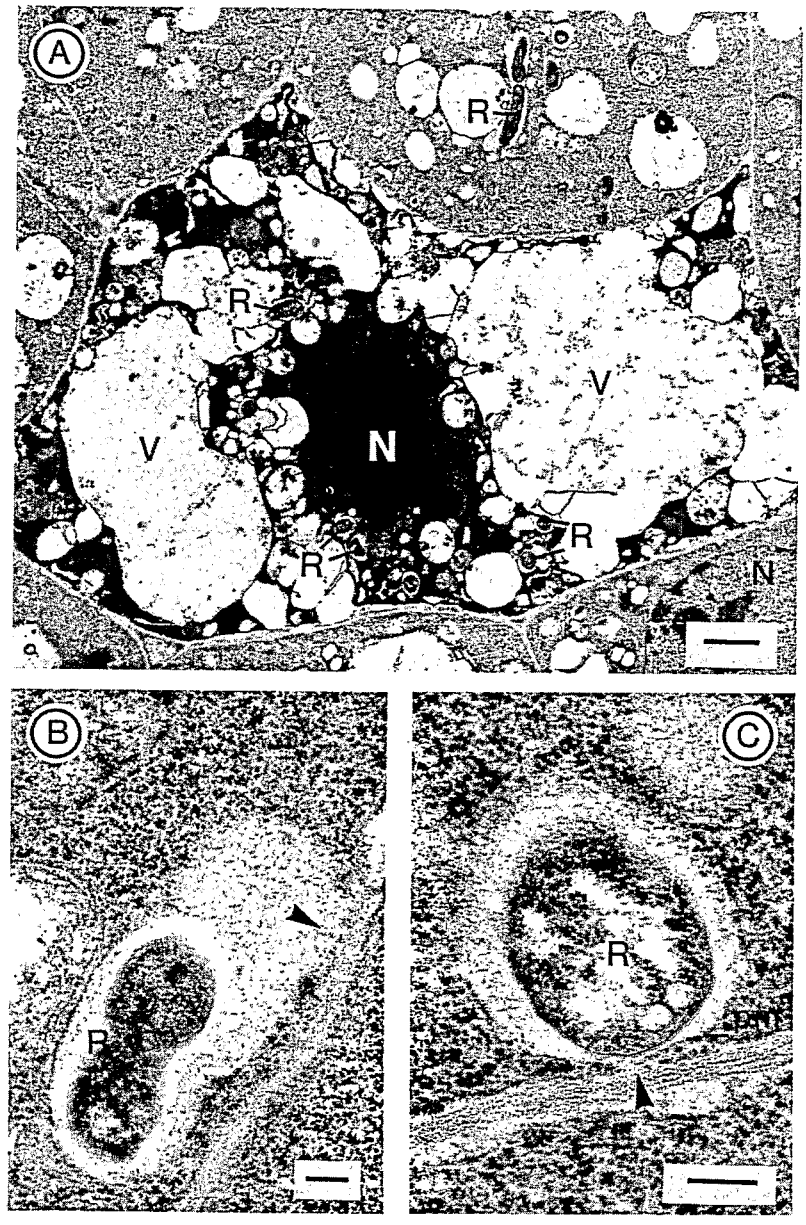

PLATE 4. (A) Transmission electron micrograph after the infection strand had reached the meristematic zone. The collapsing cell is surrounded by meristematic cells. Its darkly stained cytoplasm is highly vacuolated (V) and contains numerous rhizobia (R). A few rhizobia are present in an adjacent meristematic cell. Nucleus (N). ×2500 (Bar = 2 μm). (B and C) Rhizobium (R) can often be seen in direct contact (arrowheads) with the cell wall of the meristematic cells suggesting that they are penetrating into the meristematic cells. Peribacteroid membrane (pm). (B): ×20 000 (Bar = 0·2 μm); C: ×30 000 (Bar = 0·2 μm).

be more common in legumes, since the infection process has been described in only a small number of leguminous plants, particularly in tropical legume genera where even nodulation has not yet been examined (Faria *et al.*, 1984).

Many questions still remain regarding the rhizobial entry into the first infected cells and into meristematic cells at the end of the infection strands. Further investigations are needed to clarify these mechanisms and, more generally, to clarify the mode of infection in legumes where infection thread formation does not occur.

ACKNOWLEDGEMENTS

The authors wish to thank Dr B. K. Robertson for reading the manuscript, Dr G. Truchet for suggestions while this work was in progress, and Dr X. Mattei at the University of Dakar for providing the electron microscopy facilities.

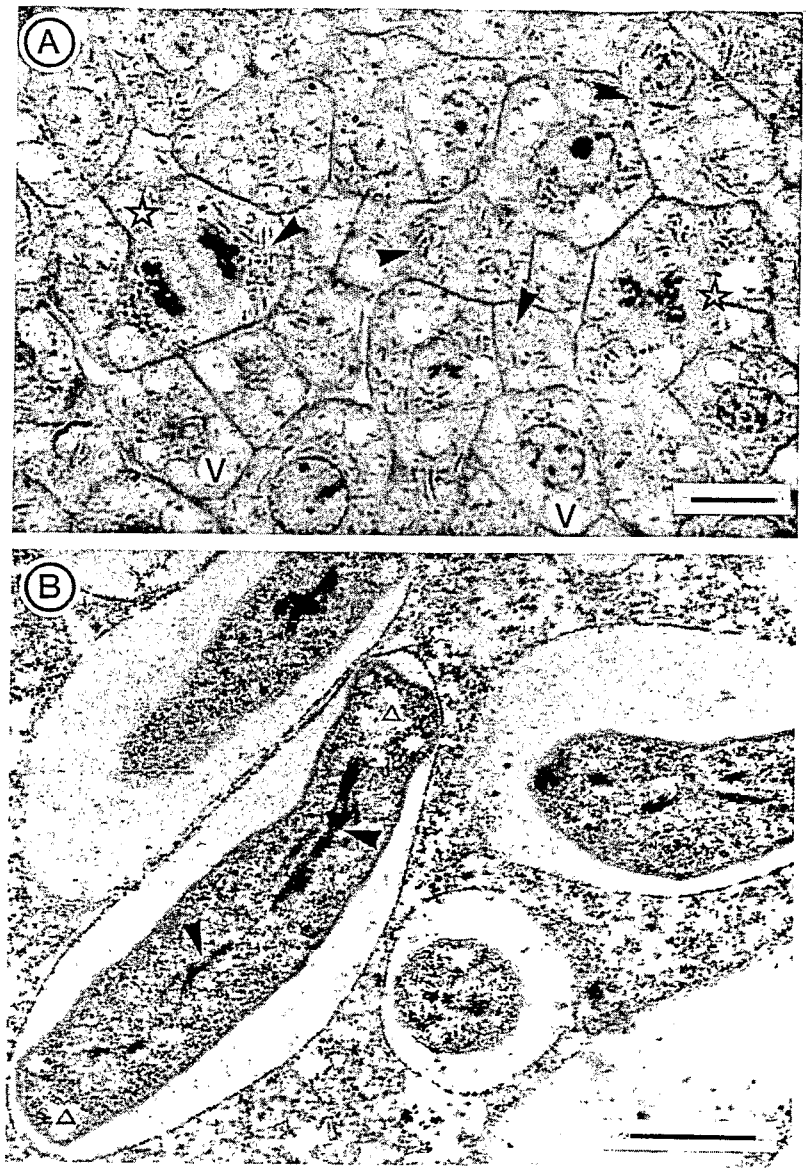

PLATE 5. (A) High magnification of part of the central tissue of a 12-d-old nodule showing rod-shaped rhizobia (arrows) in the host cells. Infected cells are vacuolated (V) and show different stages of mitosis (*). Infection threads are never observed. × 1300 (Bar = 10 μm). (B) Transmission electron micrograph showing rhizobia in the central tissue of the nodule. Rod-shaped rhizobia are enclosed singly in a peribacteroid membrane, and have a condensed central fibrillar nucleoid (arrowheads) with poly-b-hydroxybutyrate (PHBA) inclusions (Δ). × 40 000 (Bar = 0·5 μm).

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