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Structure of dinitrogen fixing nodules in *Erythrina poeppigiana*

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Abstract: The development and anatomy of root nodules induced in seedlings of *Erythrina poeppigiana* by a strain of *Bradyrhizobium* spp (C.R 751) is described. Infection apparently occurred via injuries where lateral roots (devoid of root hairs) emerged. The first sign of infection was a group of epidermal cells (fluffy) on the sec ondary (emerging) root that covered the nodule primordium. Here, thin-walled cells with plastids and in active division were observed but not infection threads. Infection appears to have occurred via protoxylem which was preceded by a dissolution of the vessels' cell wall. A peribacteroid, membrane-generating organelle in infected cells is described. The adjacent non-infected cells showed electron dense depositions (phenolics ?). Developed nodules were spherical 0.5 to 2 cm in diameter. The surface was corky, with lenticels. The peridermis showed several layers of suberized dead cells. The cortex consisted of external, medium and internal layers. Vascular bundles ran in the medium cortex in a centripetal way to join the root vascular system at the base of the nodule. In these vascular bundles, bacillar bacteria were observed which were confirmed to be *Bradyrhizobium* sp (results of a separate study using immunocitochemistry). Bacteroids densely filled infected cells of the nodule medulla within peribacteroid membranes. Among infected cells non-infected auxiliary cells were seen loaded with starch granules. During later stages of root development few root hairs appeared on tertiary and secondary roots. Infection also occurred via root hairs, resulting in oblong nodules caused by a tumefaction of the root cortex.

Key words: Poró, *Erythrina poeppigiana*, *Bradyrhizobium* sp, root nodules, nodule structure.

Trees of the genus *Erythrina* form a symbiosis with tropical rhizobacteria of the old cowpea group (now *Bradyrhizobium* spp.) whereby N_2 fixation is possible in special organs, the root nodules (Allen & Allen 1936). Thus it is of scientific interest to understand the physiology of the nodule as the site for this important process. Additionally, the nodulation process, as well as the structure of mature nodules, can be helpful in elucidating important biological features of the microbial-plant interaction in this tropical legume (Vance et al 1988). For general concepts of the infection process and nodulation the reader is referred to the classical works of Libbenga (1974) and Dart (1977).

Studies on the structure of *Erythrina poeppigiana* nodules are non existent to the authors' knowledge. The objectives of this work were to determine the sites for nodule initiation, the infection process and the nodule structure in *E. poeppigiana*. The results shown are preliminary, and some key interpretations await further investigation to draw definitive conclusions in view of the absence of similar studies with the same species.

METHODOLOGY

Seeds of *E. poeppigiana* were obtained from CATIE's seed bank (lot 2431) which are from a single stand of trees, to eliminate possible site effects (Nygren, *et al* 1993). Seeds were sterilized and germinated according to Somasegaran & Hoben (1985). Seedlings were transplanted to growth pouches and inoculated with strain CR 751 (strain 05, Gross, Ramírez

& Kass 1993). They were kept in the greenhouse for a month; then they were transplanted to 8 kg plastic bags with a sterile mixture 1:1 (v/v) of sand and vermiculite. A Nitrogen free solution was used in both cases (Somasegaran & Hoben 1985). Plant material was collected at different times and fixed in FAA, dehydrated in an increasing series of ethanol and dried in a critical point drier. Samples were then gold coated (200-400 nm) in an ionic generator and observed in a scanning electron microscope.

For transmission electron microscopy, plant tissue samples were fixed in Karnovsky fixer (Karnovsky 1965) plus Sorensen's phosphate buffer (0.1 M, pH 7.4) for 20 hr at 4 ^{o}C, which was followed by three washes in the buffer. Samples were dried in an increasing ethanol series, then embedded, via propylene oxide, in Spurr's resin (Spurr 1969). Thin sections were obtained in a Sorvall ultramicrotome, stained with uranyl acetate and observed in a transmission electron microscope.

RESULTS

Most of the nodules in *E. poeppigiana* were formed at the site of lateral root emergence, where an injury occured (Fig. 1), similar to *Arachis hypogaea* (Dart 1977). No root hairs were detected in the neighboring roots. The first sign of infection was an outgrowth of epidermal cells at the base of the emerging root that gave a fluffy appearance to the tissue (Fig. 2). It appears that this was the result of a meristematic stimulus induced by the bacteria, as has been shown for alfalfa (Dudley et al 1987). The nodule primordium, as well as its cells showing meristematic activity, was emerging under the epidermal cells at this stage. Infection proceeded to give a globular nodule (Fig. 3) which was attached to the root pit. Other nodules in sand or soil show lenticels on the surface. Under hydroponic culture lenticels develop into more complicated structures possibly as an adaptation to reduced oxygen availability (See Ramírez et al 1990). Some *Erythrina* species (native) are notorious for their adaptation to flooded conditions to the extent that peasants call them with the vernacular name of "poró de pantano", swamp poró. In later stages of root growth, few root hairs appeared in tertiary roots, and infection occurred additionally through these structures as in other legumes (Dart 1977). Infection gave rise to oblong nodules (Fig. 4), resulting from a swelling of the cortex. Ultrastructural studies are now under way.

The globular nodule was attached to the main root through a vasculature (Fig. 5) resulting from the convergence of vascular bundles of the nodule that ran in the middle cortex surrounding the infected nodule medulla (Fig. 12). A closer look at this vascular tissue showed typical xylem vessels and probably phloem cells (Fig. 6). At an even closer range, structures resembling bacteroids were seen in the cells (Fig. 7 and 8). Phloem cells in Fig. 7 and 8 resemble bacteroids in infected cells of the nodule medulla. If this is true, they may function as fixation threads, but more studies are needed to further substantiate this assumption. At any rate, bacteria were observed in vascular bundles of the nodule (Fig. 11, 14, 15, 16). In a separate study (results not shown) using a specific rabbit antiserum against *Bradyrhizobium* C R 751 and a pig anti-antibody (antirabbit) complexed with peroxidase plus oPD as a substrate, the bacteria were stained with a brown color of the oxidized substrate. No other tissue was stained, as were also the controls (no indigenous peroxidases nor positive reaction with pre-immune serum), thus suggesting that the bacteria were *Bradyrhizobium*. As bacteria were also observed in the root the implications call for the possibility of endogenous infection when new nodules are formed under natural conditions or under the stress of pruning, when there is almost complete senescence of existing nodules (Nygren & Ramírez 1993). This would be advantageous because it may rule out *ex planta* competition between the inoculated strains *(in planta)* and soil indigenous bradyrhizobia. Work is underway to test this hypothesis.

A nodule tangential section (fig. 12), somewhat to the periphery of the nodule, showed the cortex (external, middle and internal), as well the existence of several vascular bundles. The medulla in this section did not show many infected cells, which would be located in a deeper section plane. However, in Figure 9 infected cells of the medulla were clearly loaded with bacteroids which were surrounded by peribacteroid membranes (Fig. 10). Also in Figure 9 non-infected accessory

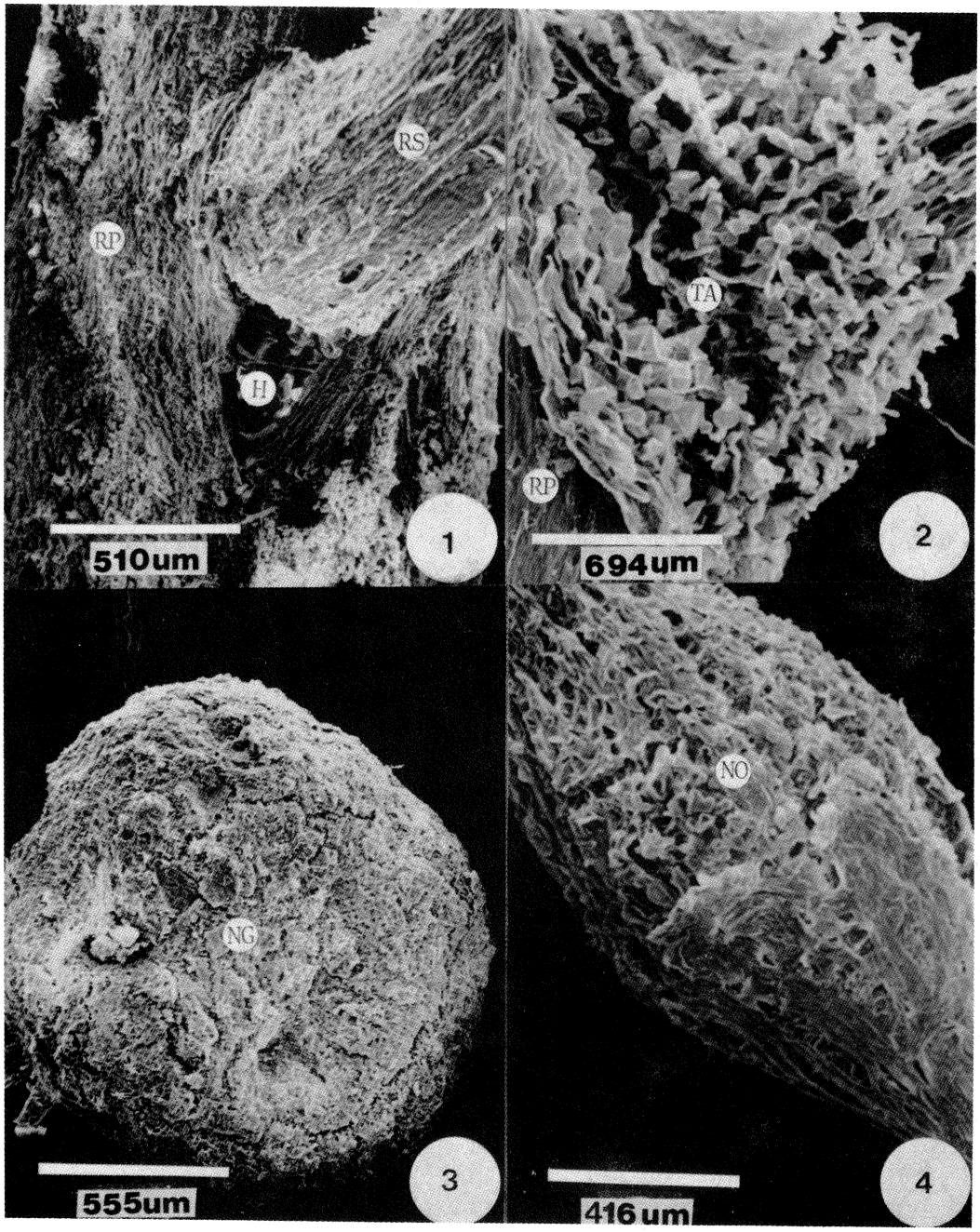

Fig. 1. Site of emergence of a lateral root in *Erythrina poeppigiana* seedlings. RP: primary root; RS: escondary root ; H: injury.
Fig. 2. SEM. Outgrowth of epidermal cells (Aerenquima?) surrounding the nodule primordium in *E. poeppigiana* seedlings, growing from the lateral. RP: principal root; TA: aerenquima.
Fig. 3. Mature nodule of *E. poeppigiana* showing a corky surface (aerenquima ?).
Fig. 4. Root nodule of *E. poeppigiana* seedlings that emerged from infection of root hairs *in the same seedlings* that also showed infection through lateral injuries. They are less common. NO: oblong nodule.

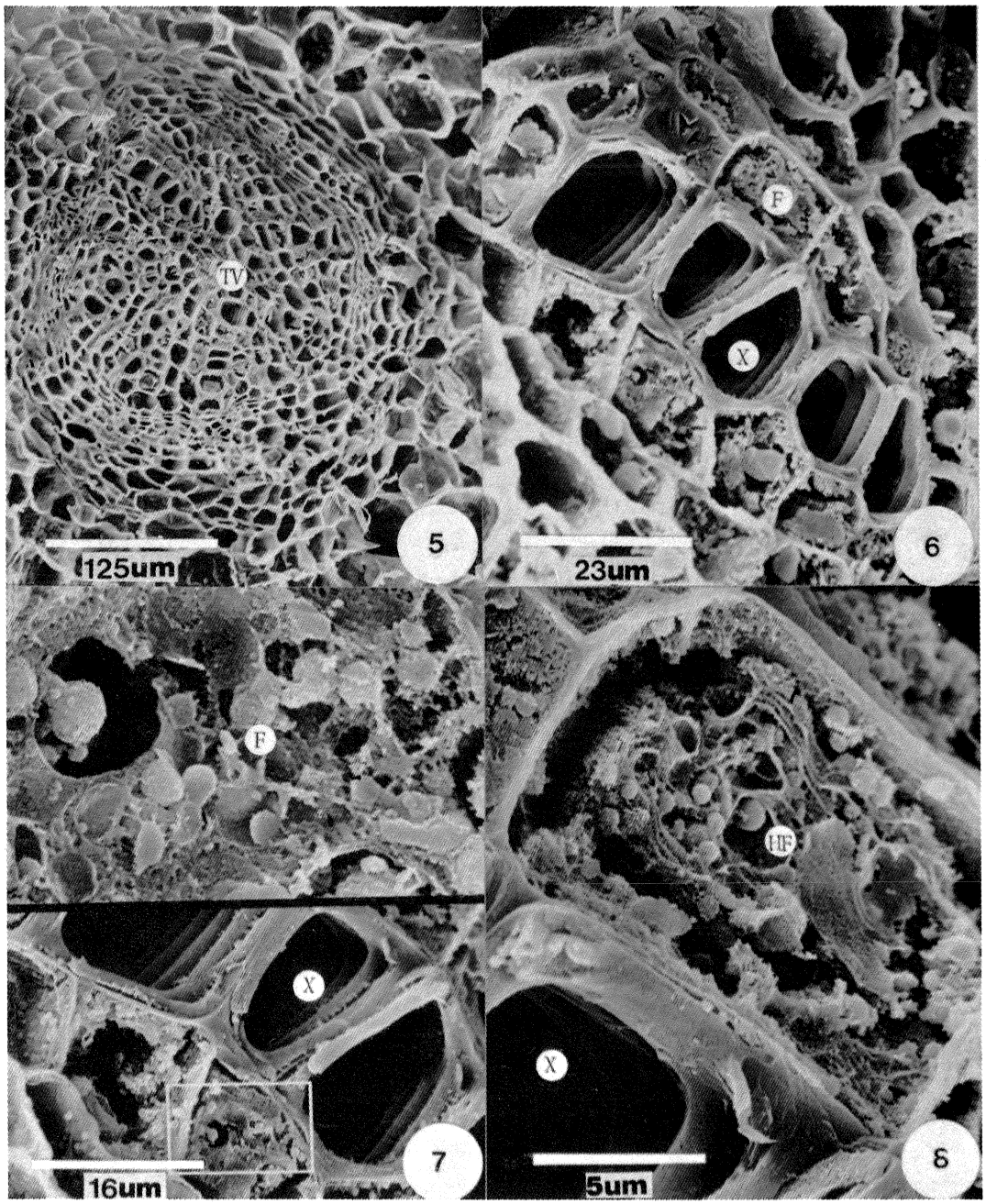

Fig. 5. Vasculature, where nodule vascular bundles converge. Vascular tissue joins the nodule to the main root that bears it.
Fig. 6. A close up of the vasculature as seen in Fig. 5.
X: xylem vessel; F: phloem.
Fig. 7. A close up of the vascular tissue that joins the nodule to the root. X: xylem vessel; F: phloem cell.
Fig. 8. Close up of F in Fig. 6. X: xylem vessel; HF: fixation thread?.

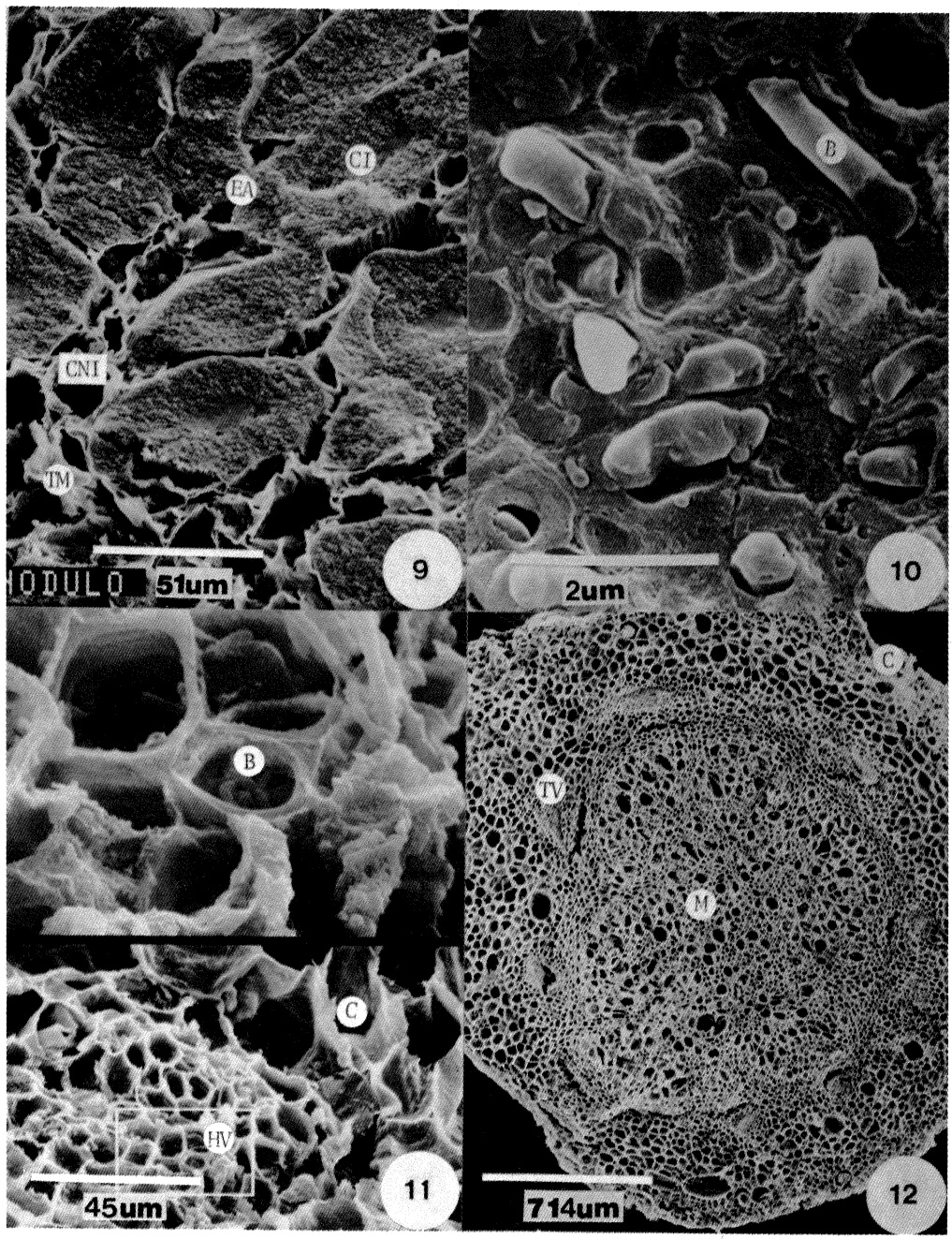

Fig. 9. Cracking phase of the medulla of a globular nodule of *E. poeppigiana*. EA: air spaces; CI: infected cell, CNI: non-infected cell; TM: medullar tissue.

Fig. 10. Cryofracture of an infected cell of the nodule medulla. Note bacteroids; bacteroides (B) and the fingerprinting of peribacteroid membranes.

Fig. 11. Tangential section (cryofracture) of a vascular vessel in the middle cortex of a globular nodule of *E. poeppigiana*. B: bacteria; C: cortex; HV: vacular bundle. Note bacteria in the vessels. Using immunocitochemistry has confirmed that bacteria are *Bradyrhizobium* spp.

Fig. 12. Tangential section (cryofracture) of a nodule of *E. poeppigiana*, somewhat polar, thus infected cells in the medulla (M) are not apparent. TV: vascular bundle. Note also the cortex in the periphery of the nodule. In this section several vascular bundles are seen surrounding the medulla.

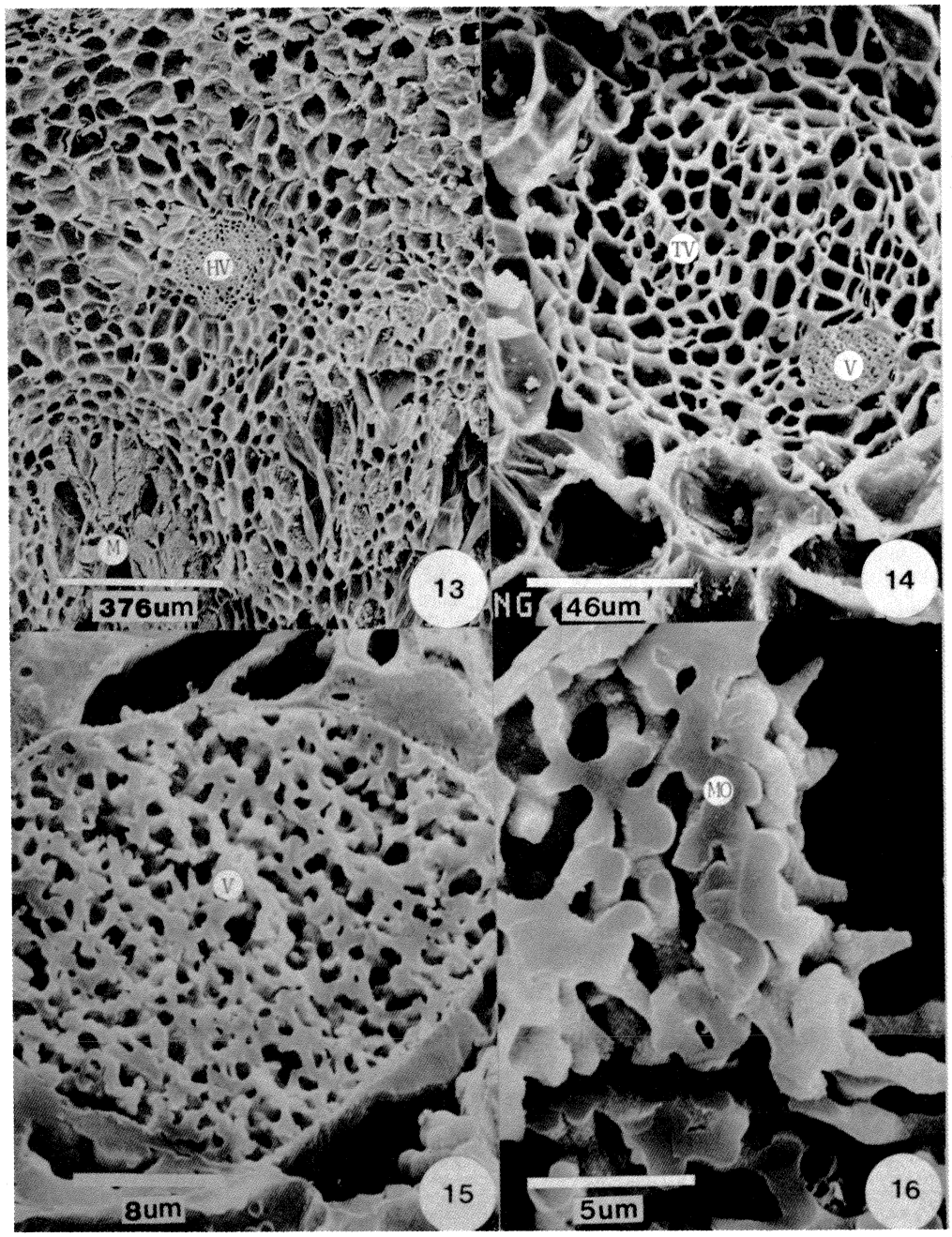

Fig. 13. Tangential section (cryofracture) of a nodule. HV: vascular bundle; M: infected cells in the medulla.
Fig. 14. TV: vascular tissue of the nodule (cryofracture) showing a vessel packed with bacteria (V).
Figs. 15 and 16. A close up of a vessel (V) in the vascular bundle of the nodule. The microorganisms (MO) appear to be bacteria. No clear explanation for this observation is available.

cells were seen next to the infected cells, as well as air spaces that may have to do with the diffusion of gases from the nodule exterior to the medulla (Dakora & Atkins 1991). The relative position of the infected tissue and the vascular bundles are seen in figure 13. The proximity of the medulla and the vascular tissue assures the constant flow of photosynthates to the infected tissue, and the outflow of fixation product from the infected cells to the N sinks in other parts of the plant.

In the cortex, larger cells were also observed with bacteria (Fig. 17, 18, 19, 10, 21) which confirms the report by Ramírez et al 1990. These bacteria also were identified as *Bradyrhizobium* using immunocytochemistry as described above. Their role is not yet clear. Additionally, other cells were seen with amorphous deposits (Fig. 25).

Transmission electron micrographs of the primordium tissue that presents meristematic activity showed cells in different stages of cell activity. Figure 26 shows the cytoplasm; Figures 27, 28 and 29 different stages of nucleus division and organelles (plastids), which in some cases showed accumulation of starch (Fig. 30, 31). In other cells (not shown), there was accumulation of a large number of starch granules. The larger differentiation in cell types was seen around what seemed young precursors of xylem vessels (Fig. 32, 33, 34, 35), characterized as having thick walls. In many instances, an outgrowth of the cytoplasm was seen dissolving the thick cell wall (Fig. 36, 37), where infection was occurring. Cells located radially, outwards from the meristematic zone were larger, with a membranous cytoplasm. Perhaps these cells are the candidates to be infected from the vessels. The cells showed great metabolic activity as indicated by the presence of plamadesmata (Fig. 38) and vesicles possibly involved in cell wall synthesis (Fig. 39, 40, 41). In other cells, another vesicles with amorphous bodies were also observed (Fig. 42, 43, 44). To what extent these structures are related to cell differentiation, i.e what cells will be infected with the bacteria and what cells will be accessory cells remains to be determined.

In several occasions, membranous structures, highly developed and apparently associated with bacteroids, were observed in cells undergoing infection (Fig. 46). These could well be structures in charge of the synthesis of new peribacteroid membranes by which the infected cells increase in size and in number of bacteroids. Other cells showed what appears to be incipient peribacteroid membranes with bacteroids inside (Fig. 47). Mature nodules showed typical bacteroids (Fig. 47). Typically each peribacteroid membrane contained 2-6 bacteroids which showed granules of polyhidroxibutyrate and polymetaphosphate (Dart 1977).

A clear picture on the infection and cell differentiation that conducts to nodule formation and development in *E. poeppigiana* have not emerged yet. Ongoing and future studies will shed light on some of the interpretation given in the present study.

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RESUMEN

Se describe el desarrollo y anatomía de nódulos radicales inducidos en plántulas de *Erythrina poeppigiana* por una cepa efectiva de *Bradyrhizobium* sp (C.R 751). La infección ocurrió aparentemente a través de las heridas en el punto de emergencia de las raíces laterales que carecían pelos radicales. El primer signo de la infección se manifestó por un grupo de células epidermales, de apariencia algodonosa, que cubría el primordio de nódulo. Aquí se observaron numerosas células de pared delgada y con plastidios en activa división celular pero no hilos de infección. La infección parece ocurrir en el protoxilema. Se describe una organella generadora de membranas peribacteroidales. Las células del primordio no infectadas y adyacentes a las anteriormente descritas, mostraron deposiciones electrodensas (¿sustancias fenólicas?). Los nódulos bien desarrollados fueron esféricos de 0,5 a 2 cm de

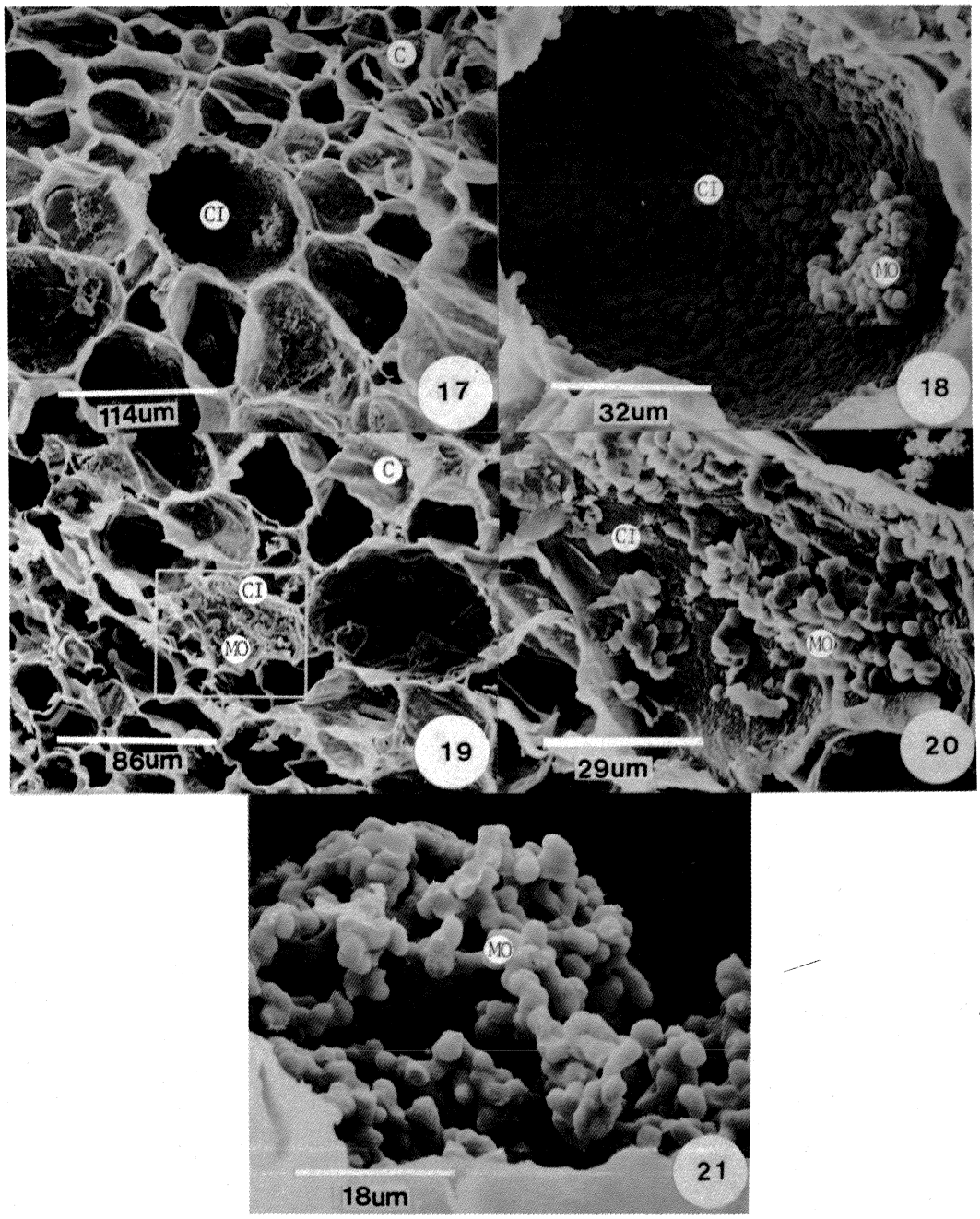

Fig. 17. Section of the nodule cortex showing an infected cell . C: nodule cortex; CI: infected cell.
Fig. 18. A close up of Fig 17, showing CI: infected cell; MO: microorganisms, they appear to be bacteria.
Figs. 19, 20 and 21. SEM. Other sections in the nodule cortex show the presence of bacteria (MO) in infected cells CI.

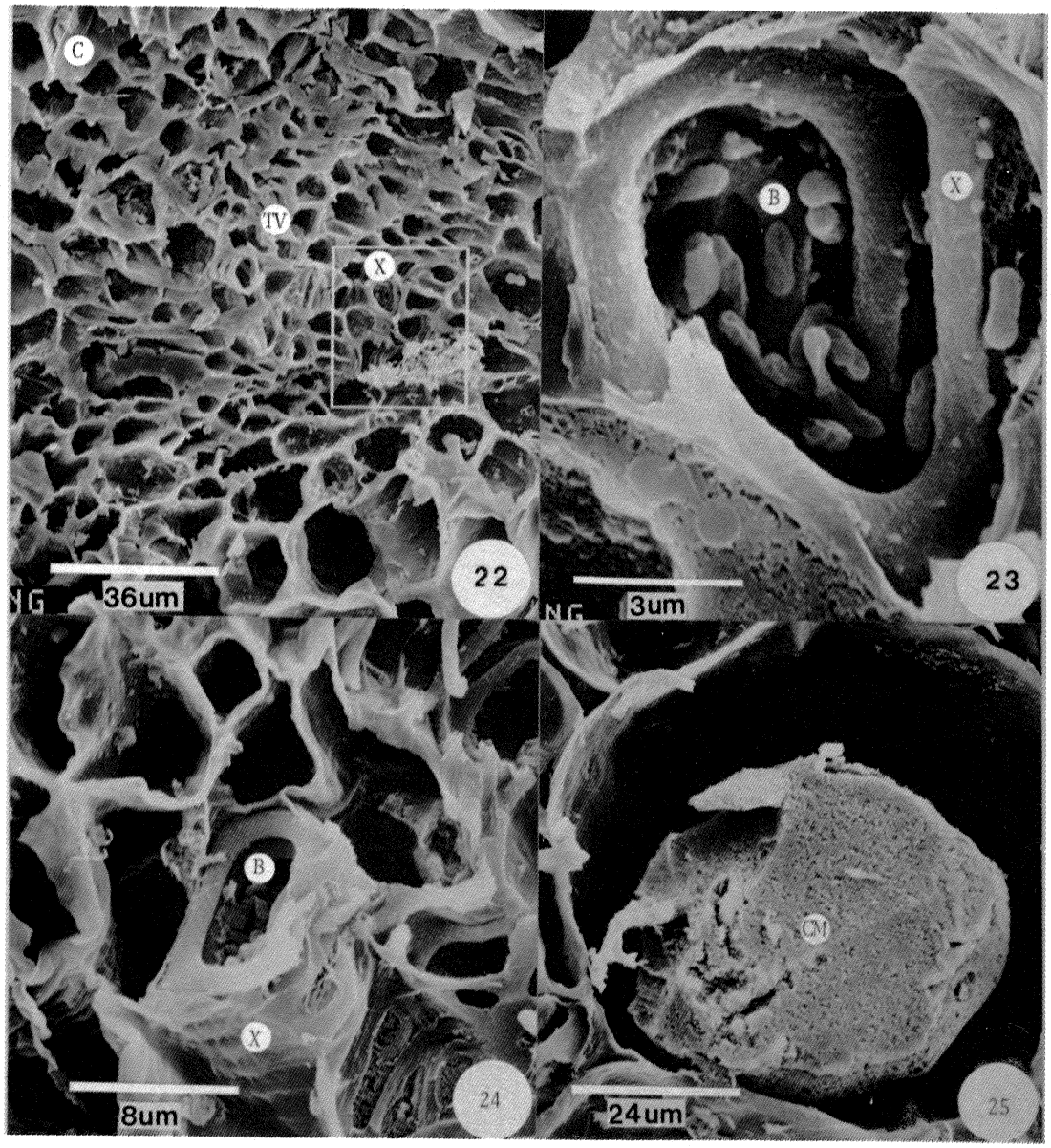

Fig. 22. TV: vascular tissue; X: xylem vessel; C: cortex.
Figs. 23 and 24. Close up of a xylem vessel (X), (B) bacteria.
Fig. 25. Cell of the cortex with CM: amorphous content.

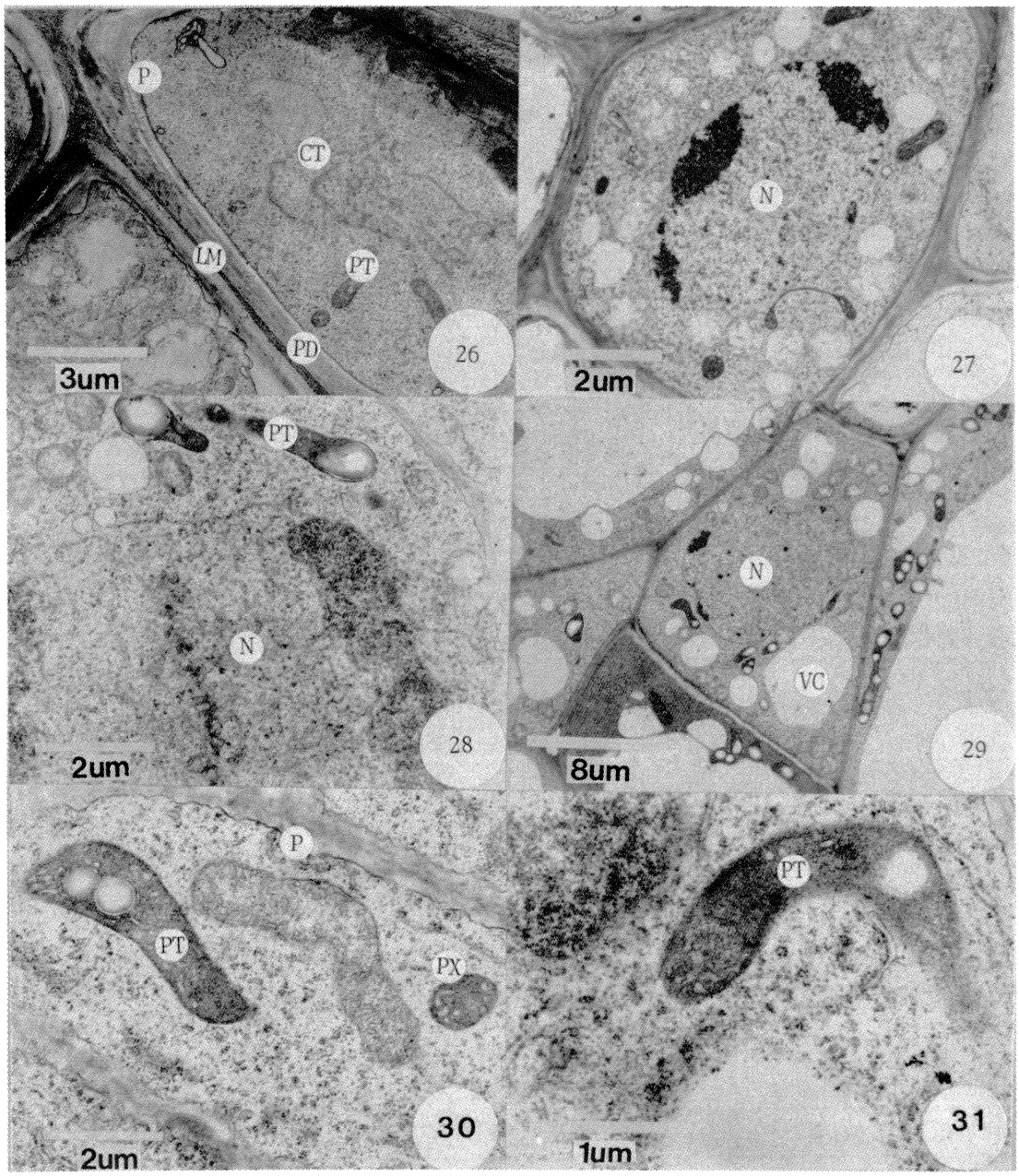

Fig. 26. Cell of the nodule primordium showing P: plasmalem; CT: cytoplasm; PT: plastid; PD: cellular wall; LM: medium lamella.
Fig. 27. Cell of the nodule primordium undergoing replication . N: nucleus.
Fig. 28. Cell of the center of the nodule primordium undergoing nuclear division. N: nucleus; PT: plastid.
Fig. 29. Cell of the center of the nodule primordium. N: nucleus; VC: vacuole.
Fig. 30. A close up of part of cell of the nodule primordium undergoing meristematic replication. P: plasma membrane; PT: plastid; PX: peroxisome.
Fig. 31. A detail of a plastid, showing incipient starch granules.

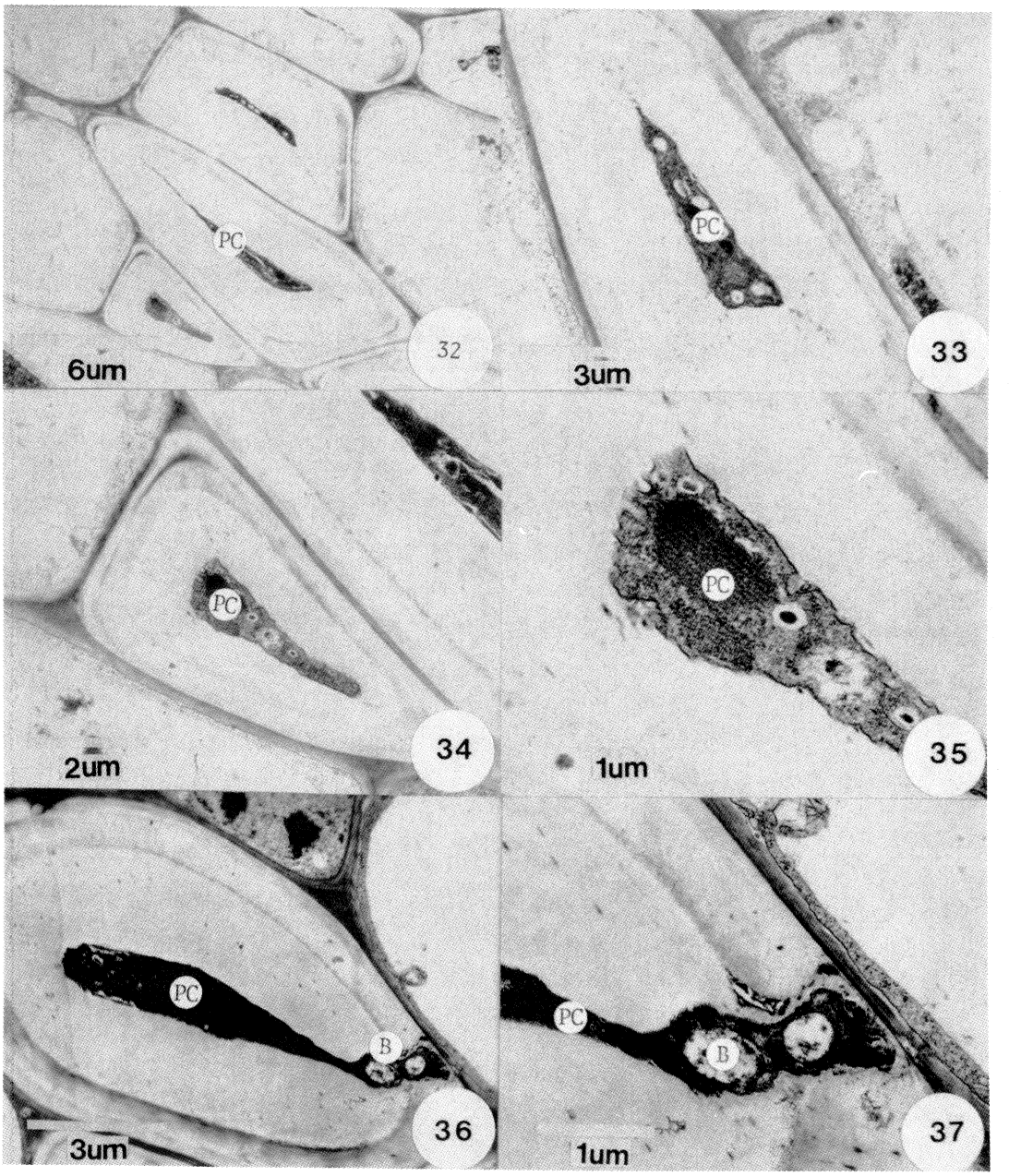

Fig. 32 and 33. Thick-walled cells appeared near the meristematic region, which resemble precursors of vascular elements (PC).
Fig. 34 and 35. A close up of one of the cells closest to the one shown in Fig. 32. Note the crystalline structure and what appears to be cross sections of bacteria. PC: precursors of xylem vessels.
Fig. 36. Another section of cells similar to the ones just shown.
Note an electron dense matrix and a thread that is "dissolving" the wall. This does not appear to be an artifact as clearly shown in Fig. 37. Similar structures have been observed repeatedly. PC: precursors of xilematic vessels. B: bacterium cell.

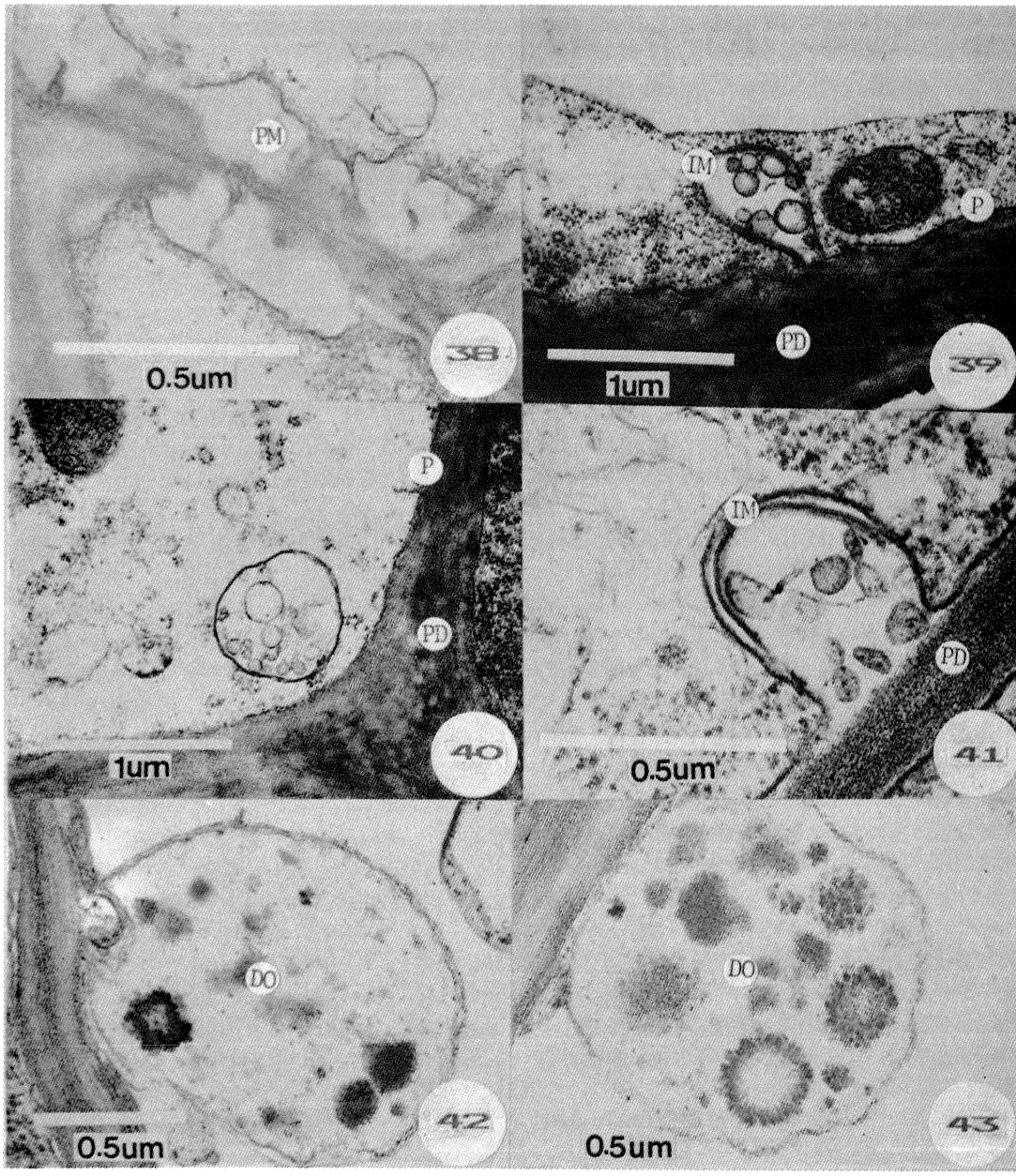

Fig. 38. Plasmodesmus (PM). Nothing special here, but in other sections it appears that associated with plasmodesmata the wall was altered.
Fig 39. PD: cellular wall; P: plasmalema; IM: membrane involution. Similar structures are shown in Figs. 40 and 41.
Figs. 42, 43 and 44. DO: osmiophilic deposits (electron dense) were seen in other cells. (Fig.44 in next page).

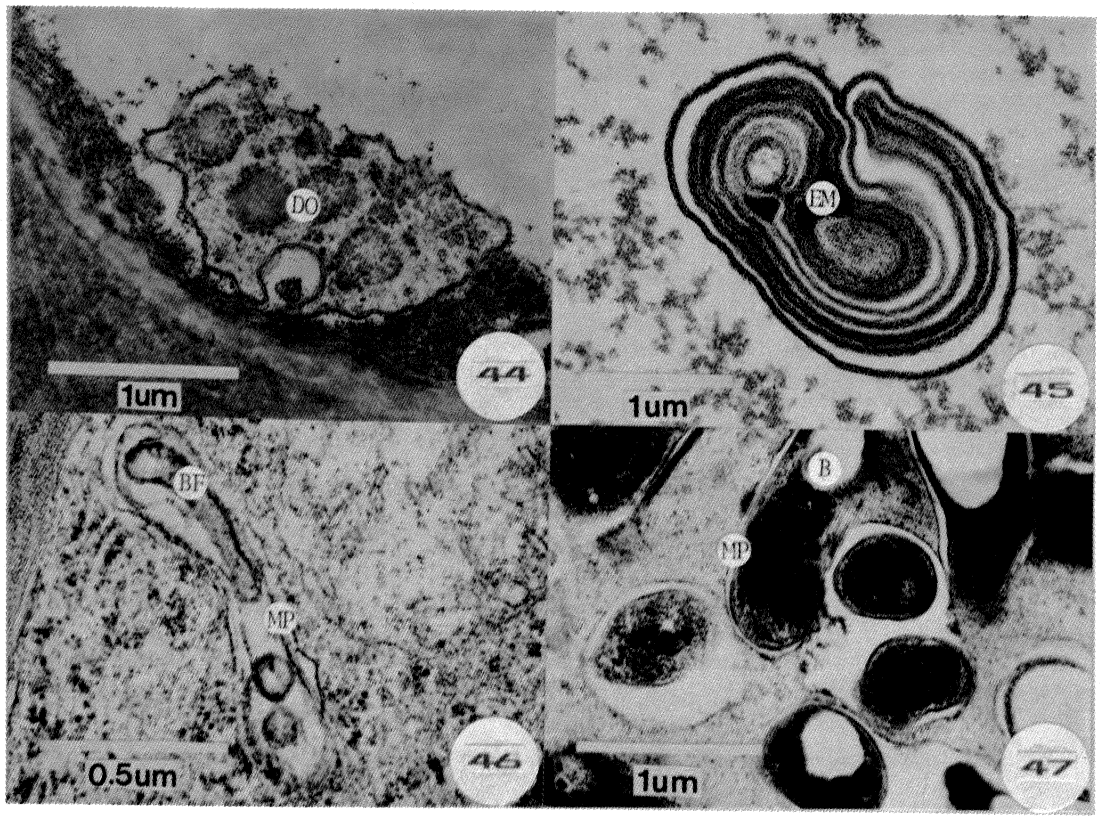

Fig. 45. EM: membranous structure. This could well be the systems in charge of assembling new peribacteroid membranes in the newly infected cell. Various similar structures were observed.
Fig. 46. Incipient peribacteroidal membranes (MP), BF bacteroid.
Fig. 47. Bacteroids in a matured infected cell. MP: peribacteroidal membrane; B: bacteroid. Note the polyhidroxibutyrate granules, white circles and the polymetaphosphate granules (dark dots).

diámetro. La superficie era corchoza, a veces con lenticelas evidentes. La peridermis mostró varias capas de células suberizadas muertas. La corteza mostró diferenciación en corteza externa, media e interna. Los haces vasculares bien organizados corrían a lo largo del nódulo, en forma centrípeta para formar uno solo, continuo con el de la raíz, en la base del nódulo. En estos haces vasculares, se observó bacterias bacilares (identificadas como *Bradyrhizobium* sp mediante immunocitoquímica en estudio paralelo). Las células de la médula mostraron numerosas membranas peribacteroidales infectadas con bacteroides. Dentro de la zona de células infectadas se visualizaron células accesorias no infectadas llenas de gránulos de almidón. En etapas ulteriores del del desarrollo radical, aparecieron pelos radicales en raíces terciarias y secundarias. Ocurrió infección para esta vía que dió orígen a nódulos oblondos resultado del crecimiento tumefacto de la corteza de la raíz en la base de los pelos radicales.

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