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Comparative Study of two Cryotechniques to Elucidate Real Functional Aspects of Legume Nodules Development*

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Summary

Legume nodules from *Lupinus albus* L. cv. Multolupa and *Phaseolus vulgaris* L. cv. Tormesina plants were subjected to two different cryotechniques (High Pressure Freezing, HPF, and Fast Freeze Fixation, FFF) in order to compare the structure obtained with respect to previous features and to deepen the relationships between the microsymbiont and the host cell in the native state. Comparing both cryotechniques, HPF yielded better general nodule ultrastructure than FFF. HPF allowed the observation of the native nodule structure since it permitted an excellent preservation. The result obtained showed that the peribacteroidal membrane was in close contact with the bacteroids, which is in contrast with the observations after chemical fixation. The narrow peribacteroid space observed between bacteroid and peribacteroid membranes appears as a dense matrix which is in agreement with the former knowledge on the existence of some enzymes and proteins in this space. O_2-released by leghemoglobin will occur in close proximity to the bacteroid membranes. The spatial relationship between Golgi apparatus and ER with the peribacteroidal membrane observed along the development of the nodule indicates that these structures are very much related with the formation of the symbiosome membrane. HPF could be the right technique to continue studying real functional aspects in developing nodules.

Key words: Cryofixation, legume, nitrogen fixation, nodule, Lupinus, Phaseolus.

Abbreviations: FFF = fast-freeze fixation; HPF = high-pressure freezing; LHb = leghemoglobin; PBM = peribacteroidal membranes; PS = peribacteroidal space.

Introduction

The symbiosis between *Rhizobium* and *Bradyrhizobium* bacteria and legume plants initiates the nodule formation on the roots of these plants (Brewin, 1993, Mylona et al., 1995). The nodule is constituted by an external part, the cortex, and a central infected zone. The infected cells contain, in addition to plant subcellular organelles, differentiated bacteria called bacteroids, capable of fixing atmospheric nitrogen. Bacteroids are surrounded by a membrane of plant origin (Mellor and Werner, 1987), the peribacteroid membrane (PBM), creating a compartment termed the symbiosome. The presence of PBM in legume nodule is one of the most obvious structural characteristics in this symbiotic organ. Depending on the fact

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whether nodule is of indeterminate or determinate type, a symbiosome can have several bacteroids residing in it.

The bacteroids function under paradoxical conditions. They require oxygen to support their respiratory energy metabolism, with the respiratory chain located in the bacteroidal membrane. But at the same time, O_2 can limit nitrogenase activity even at low concentrations. The diffusion of oxygen to the bacteroids is facilitated by leghemoglobin (LHb), a myoglobin-like-protein located in the nodule cytosol (Robertson et al., 1984; Vivo et al., 1989). Optimization of oxygen transport makes it essential that the distance between the peribacteroidal and bacteroidal membrane be as short as possible. However, generally, the common view of nodule structure, as studied by conventional methods for many years, has shown an empty peribacteroidal space (PBS) between the bacteroid membrane (BM) and the PBM, that has sometimes lead to the speculation either to the existence of an O_2-carrier between the two membranes or to the non-existence of the PBS. Since LHb was never located in this peribacteroid space by immunocytochemistry (Robertson et al., 1984; Vivo et al., 1989), the former view could not be supported.

To observe the cell in its native state it is necessary to physically cryoimmobilize the morphological and chemical components at very high cooling rates to prevent the formation of large ice crystals which cause serious damage to the tissues (Studer et al., 1992). This can be achieved by freezing cellular water in the amorphous state (vitrification). Advanced cryofixation: Fast Freeze Fixation (FFF) and High Pressure Freezing (HPF), fulfills these requirements, but HPF is the only method, till today, capable to cryoimmobilize native tissue around 500 µm thick (Moor, 1987).

Our main objective was to compare cryotechniques with conventional methods on the better insight into structure-function relationships of the root nodules, especially focusing our attention on the symbiosomes. The immunocytochemical localization of LHb in the frozen structures was also carried out to compare with previous one and to obtain a better understanding of O_2-transport from the plant cytosol to the bacteroids.

Materials and Methods

Plant growth

Surface-sterilized lupin (*Lupinus albus* L. cv. Multolupa) and bean (*Phaseolus vulgaris* L. cv. Tormesina) seeds were germinated in sterile pots filled with vermiculite and inoculated with 1 mL of bacterial suspension of *Bradyrhizobium* sp. (*Lupinus*) strain L-750 and *Rhizobium leguminosarum* bv. *phaseoli* strain JS-1 isolated in our laboratory from Sepulveda (Segovia, Spain) soils respectively.

The plants were grown in a controlled environment chamber 16/8 h light/dark cycle at 25 °C. 70 % RH and irradiance level of 190 µmol $^{-2}$ s^{-1} supplied by fluorescent tubes (Silvana Grow-Lux). Plants were supplied with N-free nutrient solution and harvested four weeks after inoculation. Sampling was done 5 h after onset of the light period.

Conventional fixation

Nodules of different age were detached from the roots and small pieces (2–3 mm wide) were taken for chemical fixation in 3 % (v/v) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, under vacuum, for 3 h at 4 °C. Some of the samples were postfixed for 2 h in 1 % (w/v) osmium tetroxide in the same buffer at 4 °C. Dehydration in acetone at room temperature was followed by infiltration and embedding in Araldite (Durcupan ACM, Fluka). Sectioning was performed with a diamond knife fitted to a Reichert-Jung Ultracut E and mounted on copper or nickel grids depending on the purpose of investigation (De Felipe et al., 1987).

High-pressure freezing fixation (HPF)

About 200 µm thick nodules slices from lupin and bean nodules were obtained with the help of sambucus pith using a hand-microtome. The slices were immersed in 1-hexadecene (Fluka) and mounted in aluminum platelets with a cavity of 2 mm in diameter and 0.2 or 0.3 mm high. They were sandwiched with a second platelet with a cavity of 0.1 or 0.2 mm. The total thickness was comprised between 0.3 and 0.5 mm. The small sandwich was introduced for cryoimmobilization in a high-pressure freezer apparatus (HPM 010, Bal-Tec, Balzers, Liechtenstein). After freezing, the small platelets were immediately transferred to liquid nitrogen in which they were stored until further freeze substitution procedure.

Fast-freeze fixation (FFF)

Samples of nodules about 0.5–1 mm thick were mounted on moist filter paper disks for fast-freeze fixation. The disks were mounted on a foam rubber block covered with a layer of mica on the stage of a Reichert-Jung cryoblock (Escaig, 1982). Excess of water was removed, and the samples were rapidly «slammed» onto a polished copper block cooled under vacuum with liquid helium to –160 °C. The frozen samples were then quickly transferred to and stored in liquid nitrogen until dehydration.

Freeze substitution (FS)

The frozen samples arising from the two freezing methods were later on processed by freeze substitution. Samples were transferred to both anhydrous acetone and anhydrous acetone containing 2 % osmium tetroxide, at –90 °C in the presence of molecular sieves (Type 4A 1/16, Union Carbide, France) to absorb water and then allowed to stay under these conditions for 3 days, followed by 2 h at –30 °C and afterwards at room temperature (Nicolas, 1991). The samples were then infiltrated in Araldite (Durcupan ACM) at different concentrations (30, 70, 100 % resin) and finally embedded in this resin. Thin sections were stained for 15 min with uranyl acetate and 5 min with lead citrate (Reynolds, 1963) and examined in an electron microscope Philips 300 at 80 kV.

Immunocytochemistry

Thin sections from nodules processed by conventional as well as by the two freezing techniques, but untreated with OsO_4 were mounted directly on uncoated grids (300 mesh) and subjected to the immunocytochemical localization of leghemoglobin as described by Vivo et al. (1989). Dilution of primary antibody was 1 : 1,000. The goat anti-rabbit IgG Au used was 15 nm diameter colloidal gold (BioCell Research Laboratories, Cardiff, U.K.).

Results

High-pressure frozen nodules

Figure 1 shows the ultrastructure of the nodule infected cells of *Phaseolus vulgaris* nodules treated by HPF and by

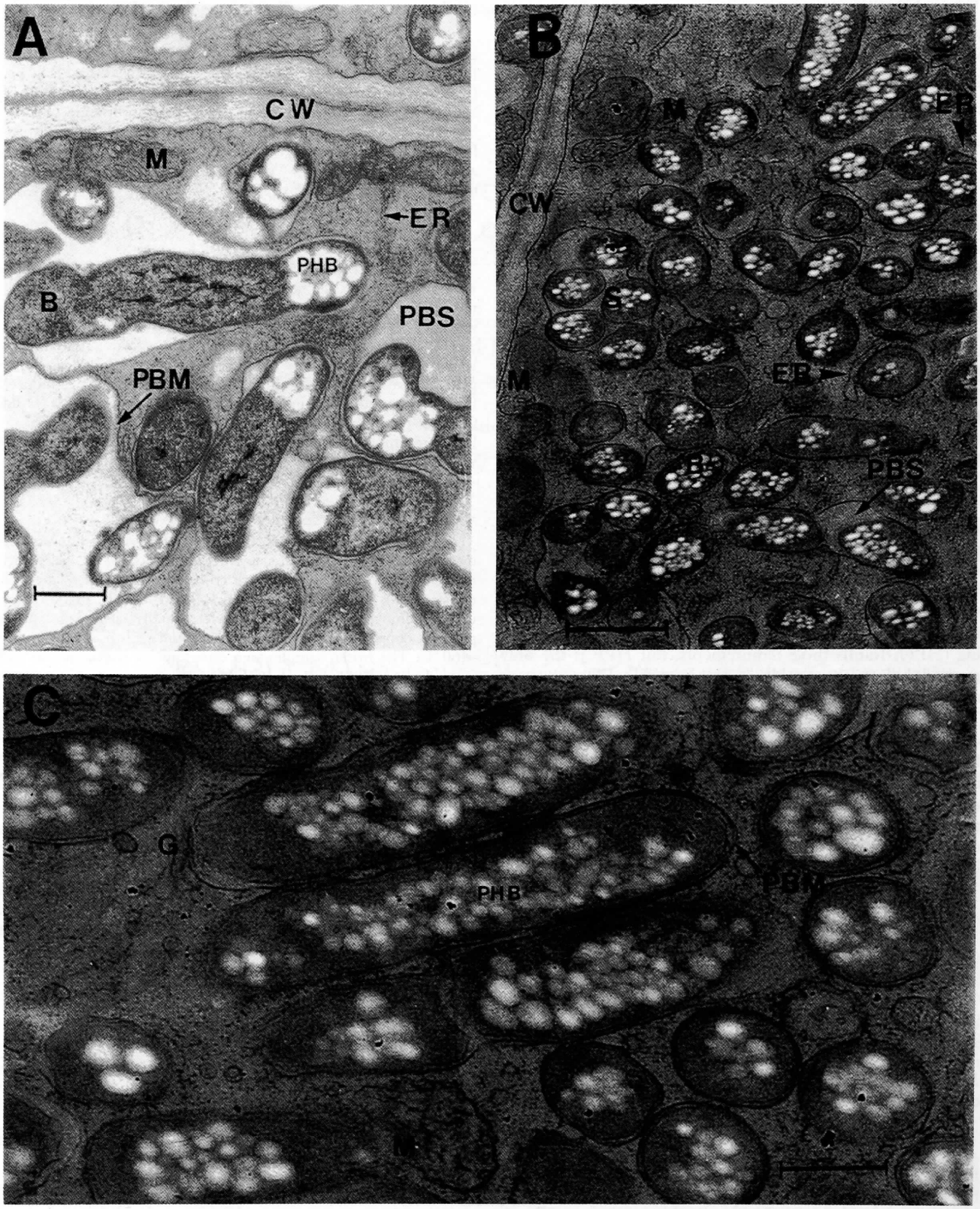
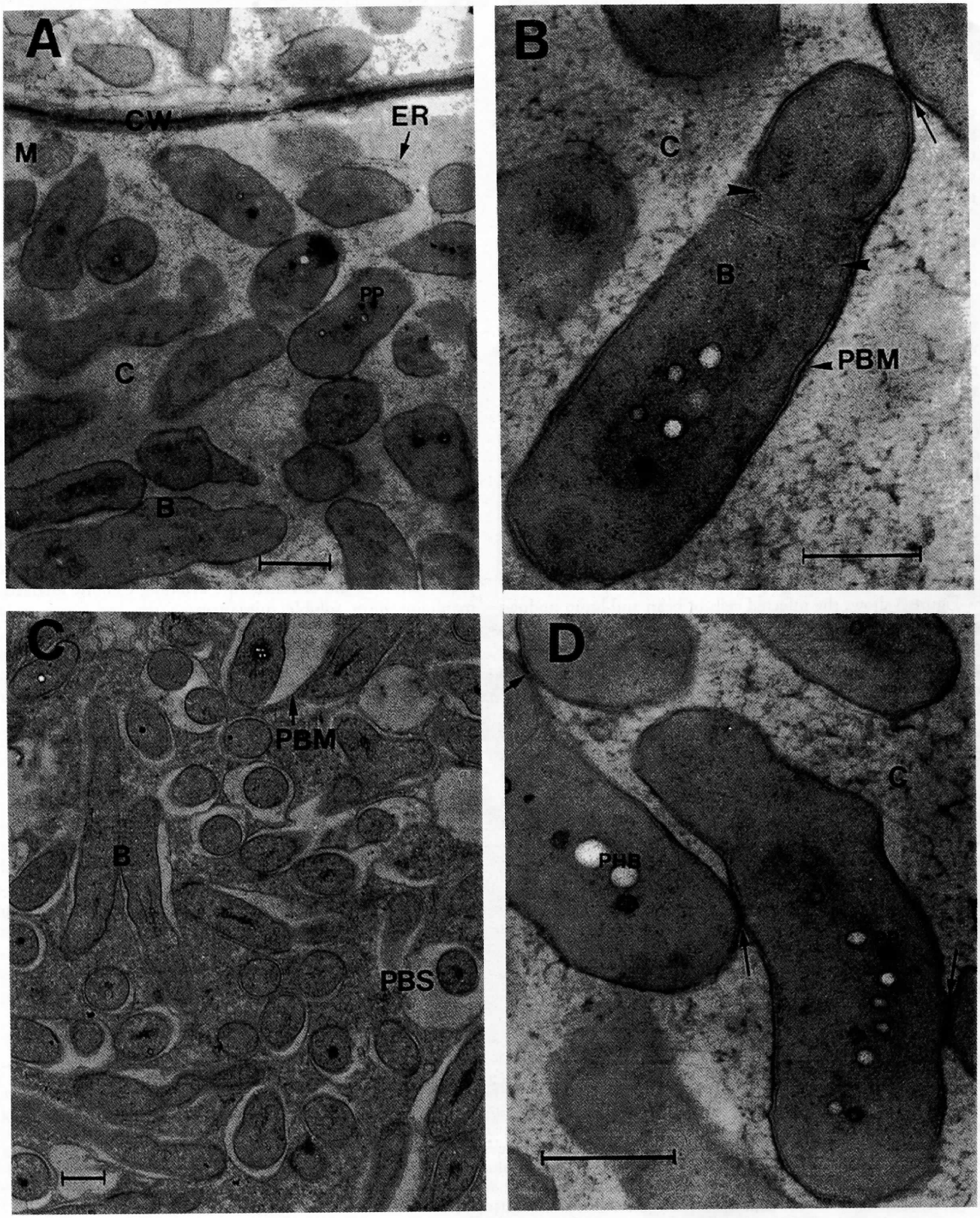


Fig. 1: Electron micrographs of bean nodules. A) Chemically fixed nodules showing several symbiosomes in infected cells. Marker bar: 0.5 µm. B) High-pressure frozen nodules showing endoplasmic reticulum cisternae closely attached to the symbiosomes. Marker bar: 1 µm. C) Bacteroids are tightly surrounded by the peribacteroidal membrane. Golgi apparatus also is in close contact with the peribacteroidal membrane. Marker bar: 0.5 µm. B, bacteroid; CW, cell wall; ER, endoplasmic reticulum; G, Golgi apparatus; M, mitochondria; PBM, peribacteroidal membrane; PBS, peribacteroidal space; PHB, polyhydroxybutyrate; S, symbiosome.


A
CW
M
ER
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B
B
C
PBM
PBM
B
PBS
D
C
PHB

conventional fixation. Figure 1 B provides a general view of the high-pressure-frozen bacteroids containing a great amount of poly-β-hydroxybutyrate granules. The peribacteroid membrane is in close contact with the bacteroids (Fig. 1 C). The narrow space between bacteroid and peribacteroid membranes looks as a dense matrix, which is greatly in contrast with the empty space found in the chemically fixed nodules (Fig. 1 A). Figure 1 B also shows some endoplasmic reticulum cisternae touching the peribacteroid membrane at some points. Golgi apparatus, sometimes closely appressed to the peribacteroid membranes, is also abundant and well preserved (Fig. 1 C). Mitochondria are located anywhere in the nodule cytosol showing very well delimited cristae.

Figure 2 presents the ultrastructure of the nodule infected cells of *Lupinus albus*. Figure 2 A shows a general view of the infected cells containing bacteroids and part of the cell walls exhibiting cell connection pores. The bacteroids contain some polyphosphate granules and very few poly-β-hydroxybutyrate. The peribacteroid membrane remains tightly wrapped around the bacteroids (Fig. 2 B) and some ER cisternae are observed in close relation with symbiosomes, as observed in bean nodules.

A notable observation, which is not commonly seen in chemical fixed nodules (Fig. 2 C), is related with the points of contact between peribacteroid membranes of adjacent symbiosomes (Figs. 2 B and 2 D). Bacteroid division (Fig. 2 B, arrowheads) is also commonly observed in the frozen nodules.

Fast-freeze fixed nodules

Figure 3 shows the infected cells of bean and lupin nodules after treating by FFF. In general, nodule cytosol of both plants has been worse preserved than by HPF technique, especially in bean nodules (Fig. 3 B). The cytosol of infected cells appeared more damaged, and was apparently less smooth and homogenous than in cells fixed by HPF techniques. The PBM in bean nodule (Fig. 3 A) is seen closely surrounding the bacteroids, and there are no noticeable differences, between the two methods, concerning the bacteroid cytoplasm. The bacteroids, inside the symbiosome, are also immersed in a dense dark matrix, as already observed by the HPF technique. In bean nodules (Fig. 3 B) several ER cisterna are visible some of them joining to the peribacteroid membranes.

Figure 3 C shows the lupin bacteroids after treating the nodules by FFF. The symbiosome was not as well preserved as in HPF, with the exception of the bacteroid cytoplasm (Fig. 2 B). Although the components of the cell wall look normal, the plasmalemma was not totally visible.

Immunocytochemistry

The localization of LHb in lupin nodules processed by HPF (Fig. 4 A) confirmed the cytosol localization. In the frozen nodules LHb is, however, in close contact with the bacteroid membrane, which is in contrast to the results obtained by chemical fixation (Fig. 4 C). So, it appears that O_2 will be transported directly from the peribacteroidal to the bacteroidal membrane, without the necessity of an O_2-carrier crossing the peribacteroidal space. Gold localization also appears on the somewhat deteriorated nodule cytosol of nodules processed by FFF (Fig. 4 B).

Discussion

Chemically fixed nodules have always shown peribacteroid spaces as empty spaces between the bacteroids and the peribacteroidal membrane. It has also been questioned how oxygen is transported to the bacteroids from the PBM to the PBS. Even the existence of an O_2-carrier to fulfill this role has been speculated.

The use of HPF and other cryotechniques based on fast-freeze fixation followed by freeze-substitution (FS) concedes substantial advantages due to the extreme rapidity of this fixation as compared to the conventional chemical fixation. The initial step physically cryoimmobilizes most molecules and thus arrests the biological reactions in a matter of milliseconds. The second step, FS, slowly removes the water still being in solid state. This procedure results in an excellent preservation of the ultrastructure avoiding osmotic artefacts, maintains most soluble substances *in situ* and keeps up a number of cell activities and protein antigenicity (Gaill et al., 1994; Hobot et al., 1985; Nicolas, 1991). Another point of interest is that the rapidity of the initial cryoimmobilization enables the capturing of unstable structures during further processes.

In addition to the observation of the extent of the peribacteroid space, the study of native nodule structure under dynamic processes was also the main objective of our research. In general, high-pressure-freezing better preserved nodule cytosol and bacteroids than fast freeze fixation. With both cryotechniques the PBM remains tightly appressed arround the bacteroids, surrounding a bacteroid in the case of lupin, and several bacteroids in bean nodules. The bacteroid ultrastructure was better preserved than the nodule cytosol, specially in the FFF technique, where the cytosol appears sometimes rather streaken or cracked. In both types of nodules, the commonly seen division bacteroids indicates that these tissues correspond to young infected cells. A common feature ob-

Fig. 2: Electron micrographs of lupin nodules. A) General view of infected cells from HPF nodules, showing bacteroids with polyphosphate granules. Some endoplasmic reticulum cysternae are found close related to the peribacteroidal membrane. B) Detail of bacteroids showing the peribacteroidal membrane tightly surrounded the bacteroid. Apparently, nodule cytosol is not as well preserved as the bacteroid cytosol. C) General view of infected cells from nodules treated by the conventional technique. The differences in the peribacteroidal spaces between A and C are clearly visible. D) Bacteroids from HPF frozen nodules showing several points of contact among each other. B, bacteroid; C, cytosol; CW, cell wall; ER, endoplasmic reticulum; M, mitochondria; PBM, peribacteroidal membrane; PBS, peribacteroidal space; PHB, polyhydroxybutyrate; PP, polyphosphate granules. Markers bars: 0.5 µm.

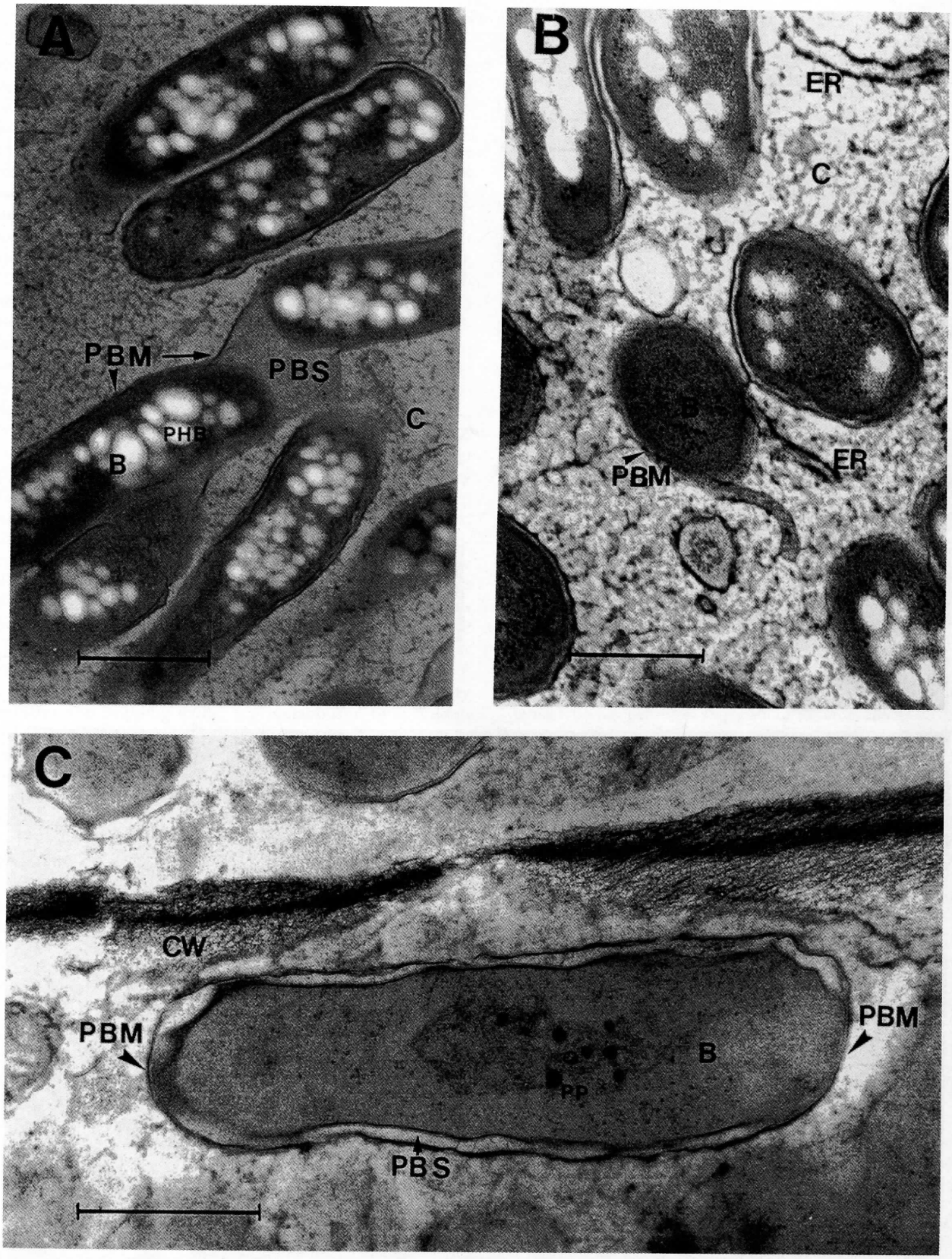

Fig. 3: Electron micrographs of bean and lupin nodules processed by FFF. A) Several bacteroids of *R. leguminosarum* are immersed in the dense matrix of the peribacteroidal space. B) Endoplasmic reticulum cisternae are attached to the symbiosome or are found in a near distance to it. The nodule cytosol apparently was more damaged than HPF nodules. C) Detail of *Bradyrhizobium* sp. (*Lupinus*) bacteroid from FFF nodule. Plasmalemma was worst preserved than by HPF. Notice that peribacteroidal membrane is attached only at some zones. B, bacteroid; CW, cell wall; ER, endoplasmic reticulum; PBM, peribacteroidal membrane; PBS, peribacteroidal space; PHB, polyhydroxybutyrate. Marker bars: 0.5 µm.

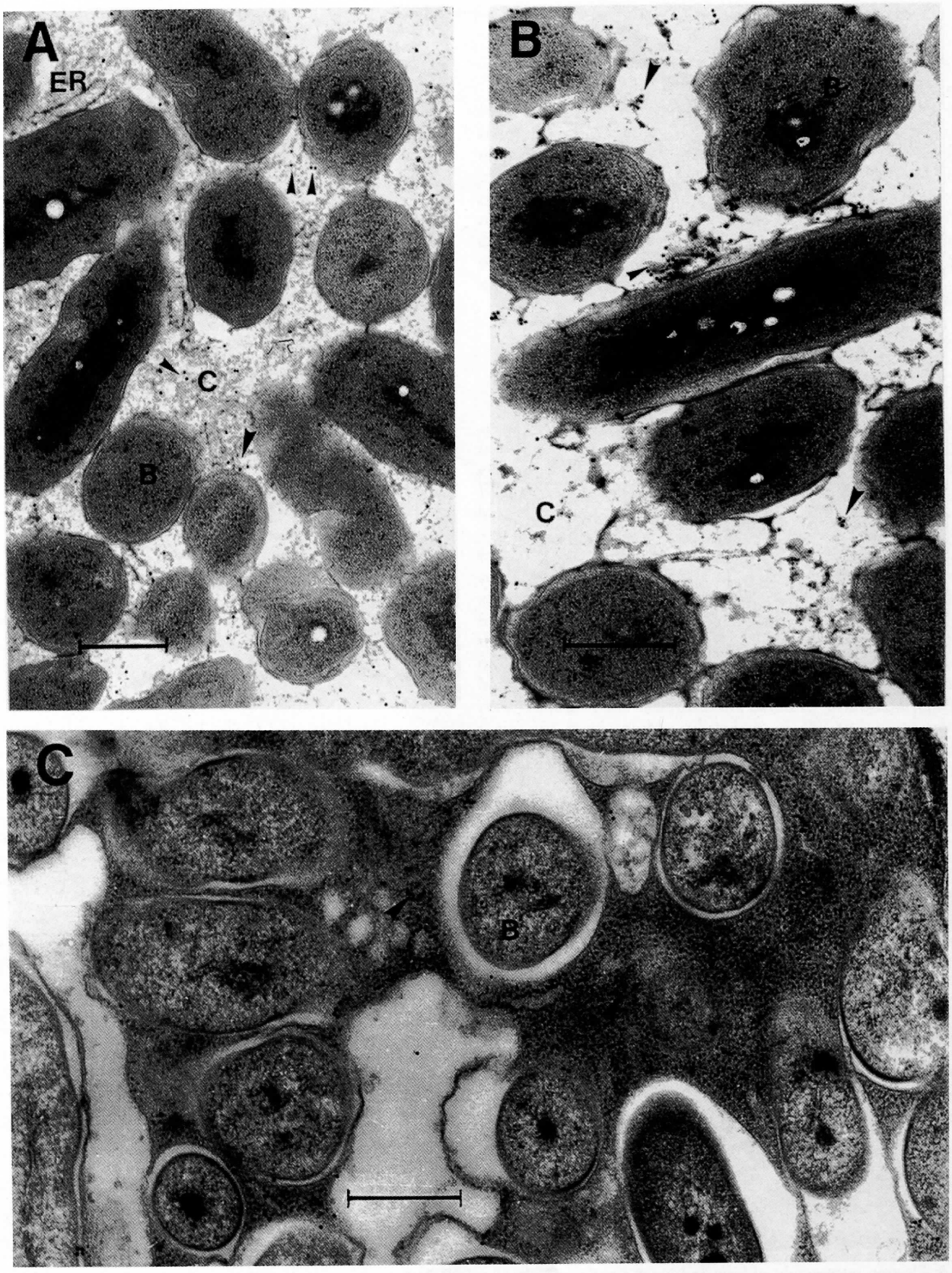

Fig. 4: Electron micrographs of lupin nodules showing immunolocalization of leghemoglobin. A) Immunolocalization in the cytosol of infected cells from HPF nodules. B) Immunolocalization in the cytosol of infected cells from FFF nodules. C) Immunolocalization in the cytosol of infected cells from chemical fixed nodules. B, bacteroid; C, cytosol; ER, endoplasmic reticulum. Marker bars: 0.5 µm.

served in the frozen nodule structures is the connection between two or more symbiosomes that could be due to either as a consequence of organelles division taking place at fixation time or as an interchange of signals among bacteroids. Another interesting observation in the morphology of the frozen structures was the close relationship of Golgi and ER cisternae with the peribacteroidal membrane; these cisternae surround symbiosomes very often. There are some speculations about the origin of the peribacteroidal membrane. Most researchers assume a pluralistic model (Roth and Stacey, 1989 a). Mellor and Werner (1987) have stated that the symbiosome membranes are primarily derived from the Golgi and not from the plasma membrane. Mellor (1989) in a review article, stated that phospholipids and fatty acid composition of the PBM resembles that of the ER. Concomitant with the release of rhizobia from infection threads, rapid proliferation of endomembrane system occurs (Verma et al., 1993). Bassarab et al. (1988) have reported that the PBM has a significantly stronger similarity to the ER than to the Golgi apparatus. In the same direction discuss Roth and Stacey (1989 b) while comparing the formation of symbiosomes with *Rhizobium japonicum* wild type and mutant strains, when they observed that insufficient ER can impair symbiosome formation. The mutant nodule cells appear to lack the ER synthesis signal required to produce the copious amounts of membrane needed for the release process. This hypothesis could be confirmed by the systematic application of these cryotechniques along the development of the nodule, and our future work will be directed to this objective.

In conclusion, cryoimmobilisation, by avoiding osmolality problems and stopping living, cell metabolism, by a sudden cold shock, lead to a good preservation of nodule ultrastructure of both plants studied. The application of these techniques to the symbiotic organs has been especially appropriate, in order to investigate the symbiosome native ultrastructure and providing information about mode of O_2 transport from the host cell cytoplasm to the bacteroids. A close interchange of nutrients and metabolites between both symbionts can easily take place (Udvardi et al., 1988). Both cryotechniques showed, at high EM symbiosome magnification a very reduced peribacteroidal space with an electron dense content. These findings are coherent with the investigations of Mellor (1988, 1989) showing some enzymes in soybean peribacteroid space and also with those of Katinakis et al. (1988) who found proteins in the pea peribacteroid space. Moreover, the oxygen transported by the LHb can be efficiently released directly to the respiratory chain placed in the bacteroidal membrane. So that, the speculation about the necessity of an O_2-carrier through the peribacteroidal space could be given up at present.

Acknowledgements

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