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Essentiality of Boron for Symbiotic Dinitrogen Fixation in Pea (*Pisum sativum*) Rhizobium Nodules[1]

Luis Bolaños, Elvira Esteban, Cristina de Lorenzo, Mercedes Fernández-Pascual, María R. de Felipe, Agustín Gárate, and Ildefonso Bonilla

Departamento de Biología (L.B., I.B.), and Departamento de Química Agrícola (E.E., A.G.), Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain; and Departamento de Fisiología y Bioquímica Vegetal, Centro de Ciencias Medioambientales, Consejo Superior de Investigaciones Científicas, Serrano 115 Duplicado, 28006 Madrid, Spain (C.d.L., M.F.-P., M.R.d.F.)

The effect of boron deficiency on symbiotic nitrogen fixation in pea (*Pisum sativum*) was examined. The absence of boron in the culture medium resulted in a decrease of the number of nodules and an alteration of nodule development leading to an inhibition of nitrogenase activity. Examination of boron-deficient nodules showed dramatic changes in cell walls and in both peribacteroid and infection thread membranes, suggesting a role for boron in the stability of these structures. These results indicate that boron is a requirement for normal nodule development and functionality.

Boron (B) is an essential micronutrient required for the normal growth of plants (Sommer and Lipman, 1928), diatoms (Smyth and Dugger, 1981), and heterocystous cyanobacteria (Bonilla et al., 1990), although it does not seem to be required for green algae (Gerloff, 1968), fungi (Shkol'nik and Maevskaya, 1977), or nonheterocystous cyanobacteria (Martinez et al., 1986). However, its primary mechanism of action still remains unknown. The unifying factor seems to be that all of the life forms that require B have cell walls, cell wall matrices, or cell envelopes, which are rich in carbohydrates (Lewis, 1980).

Thus, a role for B has been implicated in the synthesis and stability of the cell wall (Augsten and Eichorn, 1976), by forming esters with *cis*-diol groups present in the cell wall (Loomis and Durst, 1991). We have also demonstrated the essentiality of B for heterocystous N_2-fixing cyanobacteria (Mateo et al., 1986). In B-deprived cultures there was an early decrease in nitrogenase activity correlated with the disappearance of the glucolipidic layer of the heterocyst envelope, the main barrier against the oxygen inactivation of the nitrogenase (García-González et al., 1991).

In addition, many researchers have suggested a role for B in the membrane structure and function (Parr and Loughman, 1983), and studies of the direct effect of B on ion uptake have been reported (Blaser-Grill et al., 1989; Schon et al., 1990).

The goal of this study was to investigate the role of B in the symbiotic N_2 fixation process because of the importance of the relationship established across the membranes between the legume and *Rhizobium*. Brenchley and Thornton (1925) reported that B deficiency affected vascular development in *Vicia faba*, creating ineffective nodules. Here evidence is presented that shows that B is a requirement for nodule development and N_2 fixation.

MATERIALS AND METHODS

Plant Growth and Inoculation

Pea (*Pisum sativum* cv Argona) seeds were surface sterilized with 70% (v/v) ethanol and 10% (v/v) sodium hypochlorite, soaked for 4 h in sterile, distilled water, and then germinated on wet filter paper in covered stainless steel pans at 25°C. After 7 d seedlings were transferred to plastic growth pots and cultivated on B-free perlite with Fahraeus plant medium for legumes (Fahraeus, 1957). Pea plants were inoculated with 1 mL per seedling of about 10^8 cells mL^{-1} of *Rhizobium leguminosarum* bv *viciae* 3841 from an exponential culture in triptone yeast extract medium (Beringer, 1974). For growth of plants with NO_3^- as the N source, 8 mM KNO_3 was added.

Plants were maintained in a growth cabinet at 25°C day/22°C night temperatures with a 16-h photoperiod and an irradiance of 190 μmol m^{-2} s^{-1}. RH was kept between 60 and 70%.

For B-free cultures, B was removed from the micronutrient solution. For B-normal cultures, B (as H_3BO_3) was added to a final concentration of 0.1 mg L^{-1} B. All solutions were prepared and stored in polyethylene containers previously tested to prevent release of B even under sterilizing conditions (Mateo et al., 1986). The presence of B in the solutions and media was determined prior to using them, and no B was detected (detection limit was 0.02 μg mL^{-1}). B concentration was determined using azomethine H at pH 5.1 (Wolf, 1974) and a Technicon automatic analytical system (Martinez et al., 1986).

Analytical Methods

Ten plants for each treatment were harvested weekly. Nitrogenase activity was determined by acetylene reduction.

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* Corresponding author; fax 34-1-397-83-44.

Abbreviation: pbm, peribacteroid membrane.

The ethylene produced was measured with a Shimadzu GC-8A gas chromatograph, and the acetylene reduction activity was computed according to the method of Stewart et al. (1967) after acetylene reduction assay nodules were picked out and weighed.

Microscopy

Nodules were selected at a comparable stage of development. Samples were processed for EM according to the method of De Felipe et al. (1987). Sections (1 μm) were cut with a Reichert OM-U2 ultramicrotome with a diamond knife and stained with toluidine blue for light microscopy observations. For histochemical purposes the periodic acid-Schiff reaction was applied (Jensen, 1962). Semithin sections were mounted and photographed in a Zeiss Axiophot photomicroscope. Ultrathin sections were examined after they had been stained with lead citrate under a Phillips 300 electron microscope at an accelerating voltage of 80 kV.

Reproducibility

Data in figures are mean values from four independent experiments in which 10 samples were used for each individual experiment.

RESULTS

Nodules developed in the absence of B were smaller in size (Fig. 1B) and in weight (Fig. 2A) than the control. Most of the nodules from low-B plants appeared pale in contrast with the bigger pink control (+B) nodules after 3 weeks of culture (Fig. 1). Figure 1B (arrows) shows necrotic stages in root tips, which are typical in B-deficient plants. B starvation resulted in a 50% inhibition of acetylene reduction activity after 2 weeks of treatment and about a 70% inhibition after 3 weeks, indicating that B-deficient nodules were mostly nonfunctional after 3 and 4 weeks of treatment (Fig. 2B).

Light microscopic examination (Fig. 3) of nodules (3 and 4 weeks old) showed dramatic changes and alterations in the structure of B-deficient nodules (Fig. 3, B and D) compared with control nodules (Fig. 3, A and C). Most of the cells appeared enlarged and irregularly shaped. There is no evident differentiation between nodular tissues (infected zone and inner and outer cortex). With respect to cell walls, some regions were thicker than normal, and others were thinner or even without wall deposition in B-deficient nodules. Cell breakage also takes place in B-deficient nodules.

Control nodules accumulated starch grains in the infected cells close to the inner cortex (Fig. 3E). The accumulation of starch grains in the central infected tissue (mainly in the interstitial and not the infected cells of B-deficient nodules, Fig. 3F) suggests that nodule metabolism was impaired under B-deficiency conditions, which is in complete accordance with the observed disorganized structure.

Symbiosomes showed an advanced stage of senescence, with a degeneration of the pbm and a complete alteration of bacteroid structure in B-deficient nodules. Symbiosomes were found with either an altered pbm or without membranes, and ghosts of degraded pbm were also found in the same cell (Fig. 4B). Comparison of infection threads in control and B-deficient nodules (Fig. 4, C and D) showed clear alterations in the latter. First, the membrane of B-deficient infection threads appeared degraded and broken at certain sites; second, bacteria inside the thread appeared osmiophilic, probably due to the general advanced stage of senescence.

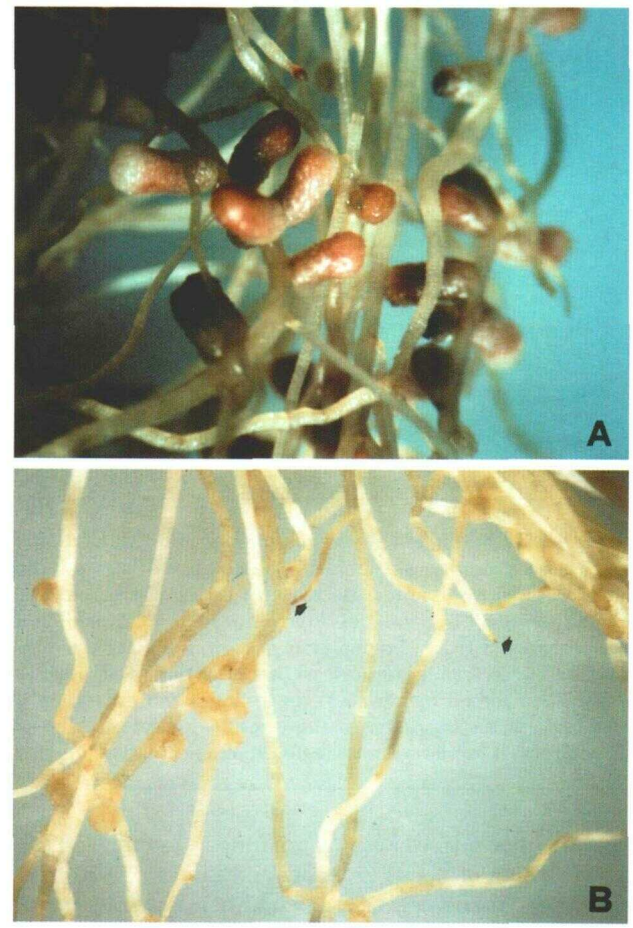

Figure 1. Pea root nodules developed with 0.1 mg L^{-1} B (A) or without added B (B) in the culture media. Arrows show necrotic root tips from B-deficient plants.

The structure of cell walls in B-deficient nodules, when observed under the electron microscope, corroborated the observations made by light microscopy. The infected cells were sinuous in shape, and their cell walls were irregularly developed (Fig. 4F, arrowheads).

DISCUSSION

As a nutrient for plants, B is specially required in meristematic cells (Raven, 1980) more than in mature tissues. Thus, the first effects of B deficiency appear in meristems, as described long ago by Sommer and Sorokin (1928). A high meristem B requirement occurs because of the low phloem mobility of B from shoots to other parts of the plant, leading to a higher accumulation of B in leaves (Raven, 1980). Because very little B is redistributed from shoots, a continuous external supply of B during the development of the plant is

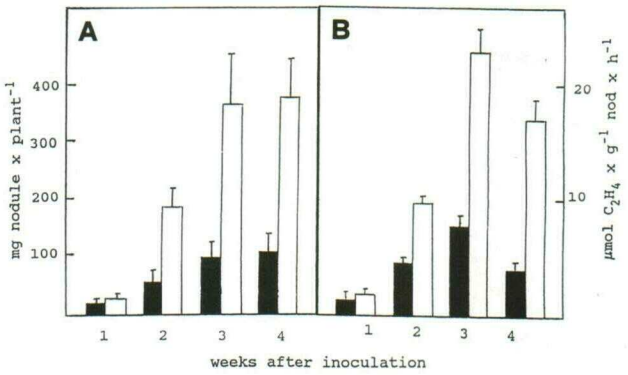

Figure 2. Effect of B deprivation on the weight (A) and nitrogenase (acetylene reduction) activity (B) of pea root nodules. □, Control; ■, B deficient.

imperative (Gupta, 1979); otherwise, a B deficiency occurs in roots, as we also found in B-deficient pea root (data not shown). Approximately 24 h after B was removed from the nutrient solution, root elongation stopped (Cohen and Lepper, 1977), whereas this effect appeared later on the shoots due to the higher concentration of nutrients in leaves (Loomis and Durst, 1991). We also found this effect in pea roots grown in low B, which produced necrotic root tips (Fig. 1B, arrows).

B concentration in nodules from low-B peas was also very low compared with the control (+B) nodules (data not shown). Nodule development starts with the induction of a new meristem (Rolfe and Gresshoff, 1988) where, after invasion by *Rhizobium* and maturation, N_2 fixation begins. With the B deficiency in this new meristem, the pea nodule structure and function were damaged, leading to a lack of nitrogen for the pea. Nodulated peas were necrotic after growing for 30 d in the absence of B, probably because of a lack of nitrogen, whereas peas growing with NO_3^- as the nitrogen source did not necrose (data not shown).

During nodule development an extensive synthesis of membrane of about 30- to 50-fold of that in other tissues occurs in infected cells to build the pbm of each symbiosome (Robertson et al., 1984; Bradley et al., 1986). Because most of the B in plants is bound in cell walls (Thellier et al., 1979) and membranes (Torchia and Hirsch, 1982; Parr and Loughman, 1983), it is logical to find high levels of B in nodules (data not shown) and to observe the dramatic alterations produced by B deficiency in this structure.

The structural alteration of B-deprived nodules showing senescence correlates with the progressive inhibition of nitrogenase activity (Fig. 2B). Most of the nodules from B-deficient plants were not functional (Fig. 2B). Brenchley and Thornton (1925) reported a low number and ineffective nodules in B-deficient *V. faba*. Because B was not essential for *Rhizobium*, these authors attributed the alterations to an effect of B on the vascular tissue, which would not allow normal transport of nutrients from root to nodule. Although this effect cannot be ruled out, our results show that the vascular tissue was less damaged than the other nodule tissues (Fig. 3). The accumulation of starch grains found in the interstitial cells of B-deficient nodules suggested that nodule metabolism is impaired by B deficiency.

A similar starch accumulation has been suggested to be a feature of stress in indeterminate nodules (Arrese-Igor et al., 1993). It may indicate that the vascular tissue was transporting sugars that were stored as starch from the plant to the nodule, as has been reported for spontaneous nonfunctional nodules (Caetano-Anolles et al., 1993). In these spontaneous nodules, sugars were not metabolized in nodules from B-deficient plants because of alterations caused by the B deficiency. Therefore, alterations in nodule metabolism produced by B deficiency should be due to a direct effect on nodule structure development and functionality rather than to the effect directly produced on the plant.

The electron micrographs of B-deprived nodules show abnormal infection structures. The growth of an infection

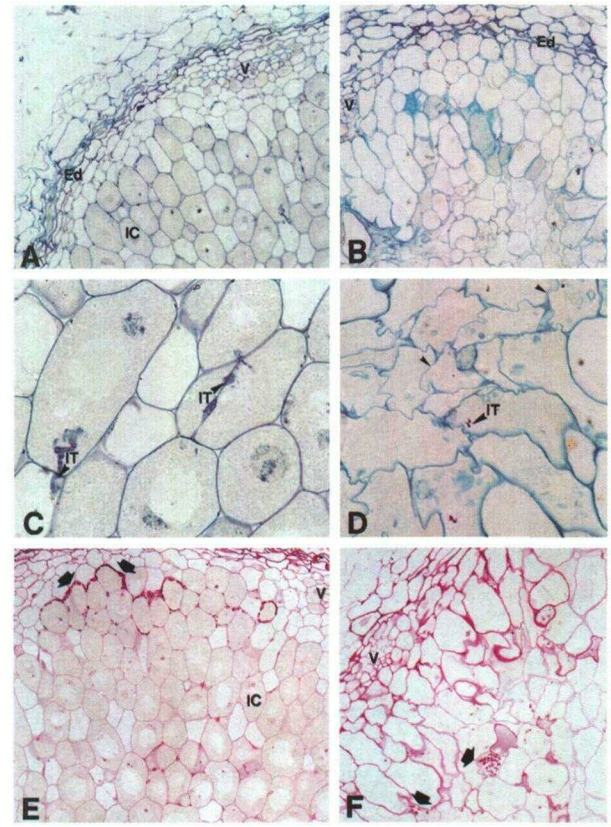

Figure 3. Light micrographs of pea nodules grown in the presence (A, C, and E) and in absence (B, D, and F) of B. A, Control nodule stained with toluidine blue. Cortex and infected zone are well differentiated; ×225. B, B-deficient nodule. Nodule structure is highly disorganized, and nodule tissues are not easily distinguishable; ×225. C, Control nodule showing well-developed infection threads; ×710. D, Infected zone in a B-deficient nodule. Cells are irregularly shaped (arrowheads), and infection threads are not completely developed; ×710. E, Periodic acid-Schiff reaction in a control nodule. Starch grains (arrows) are located in the periphery of the infected cells close to the inner cortex; ×225. F, Periodic acid-Schiff reaction in a B-deficient nodule. Accumulation of starch grains (arrows) occurs in the interstitial cells of the infected zone; ×225. Ed, Nodule endodermis; IC, infected cells; IT, infection thread; V, vascular bundle.

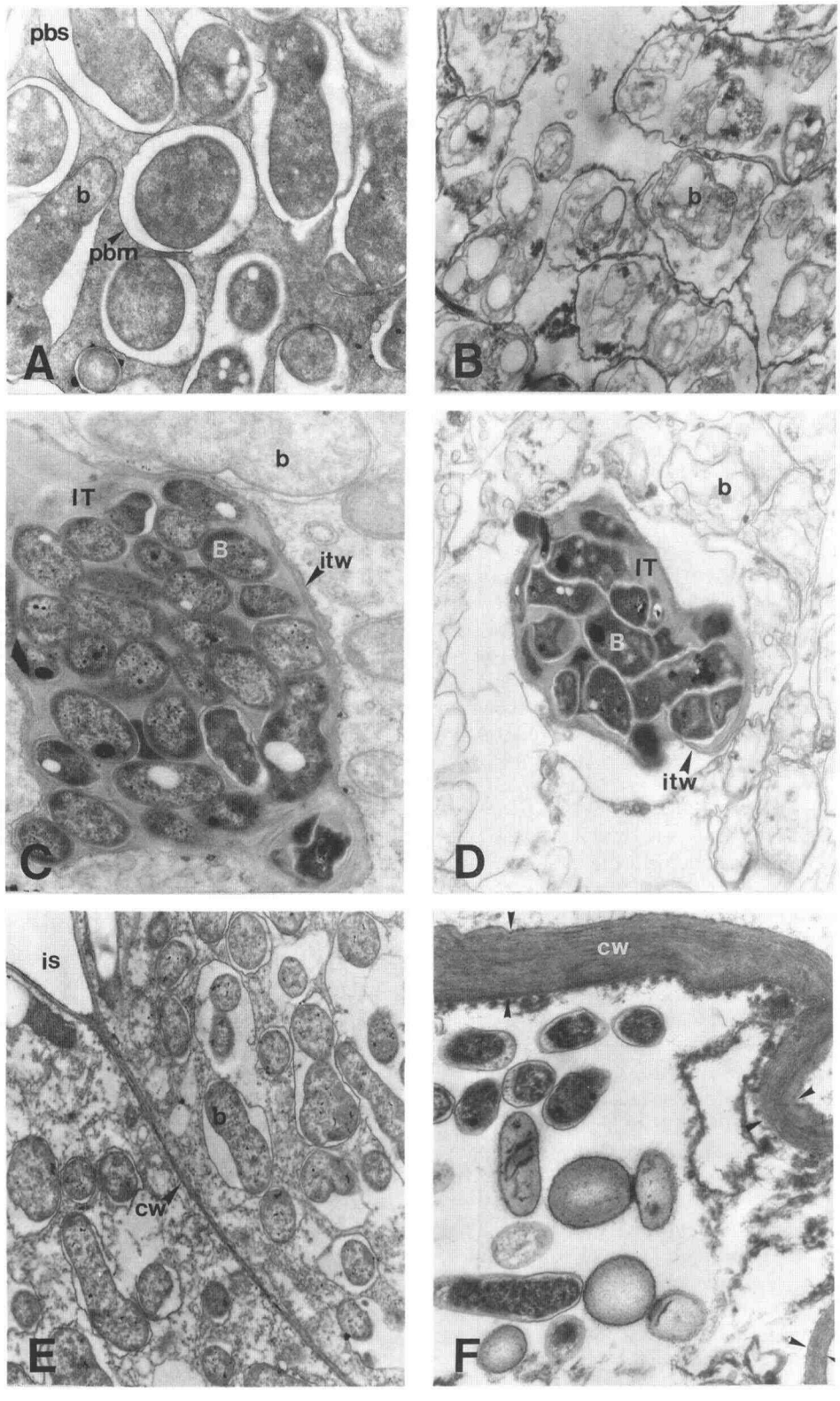

Figure 4. Electron micrographs of pea nodules grown in the presence (A, C, and E) and absence (B, D, and F) of B. A, Symbiosomes in a control nodule. pbm's are easily observable; ×11,300. B, Ghosts of pbm and bacteroid membranes in a B-deficient nodule; ×13,000. C, A transverse section of an infection thread in a control nodule. Integrity of thread wall can be observed; ×20,700. D, Transverse section of an infection thread in a B-deficient nodule. Infection thread wall and membrane are lost at certain sites. Bacteria look highly osmiophilic and irregularly shaped, and lysis of bacteria inside the thread has occurred; ×16,900. E, Cell wall of uniform thickness between two infected cells in a control nodule; ×16,900. F, Infected cell of a B-deficient nodule. Cell wall thickness is irregular (arrowheads), and cell and bacteroid structure is altered; ×24,400. B, Bacteria, b, bacteroid; cw, cell wall; is, intercellular space; IT, infection thread; itw, infection thread wall; pbs, peribacteroid space.

thread is driven by the deposition of primary cell wall components (Rae et al., 1992) to build a tunnel-like structure that grows downward in the cell (Brewin, 1991). Because this wall material is stabilized by B (Augsten and Eichorn, 1976), the correct structure and growth of the infection threads and, consequently, the correct progress of infective bacteria to the invasion of the cell is altered by the absence of B. As shown in Figure 4D, bacteria are able to invade the cells by cracks in the infection threads. B-deficient walls appeared typically enlarged (Rajaratman and Lowry, 1974), as shown in Figure 4F, which correlates with the enlarged and irregularly shaped cells observed under the light microscope (Fig. 3). Despite their enlargement, B-deficient walls are very fragile and easily broken (Loomis and Durst, 1991) and appeared to be broken in micrographs (Fig. 3).

Degradation of pbm and infection thread membranes was apparent by EM (Fig. 4, B and F), indicating a role for B in the stability of these membranes as suggested for plant membranes (Parr and Loughman, 1983; Goldbach et al., 1991). The integrity of the pbm is particularly important for a correct establishment of the nutritional relation between plant and bacteria (Perotto et al., 1991; Verma, 1992). Thus, when affected by B deficiency, symbiosomes have a damaged pbm and will be nonfunctional, which would elicit responses of pathogenesis (Félix et al., 1991) and a process of premature senescence by the failure of nitrogenase activity (Brewin, 1991), leading to the lysis of the internal structure of the nodule.

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