

Mineral Nutrition for Legume-Rhizobia Symbiosis: B, Ca, N, P, S, K, Fe, Mo, Co, and Ni: A Review

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Abstract The intensification and expansion of modern agriculture starting in the middle of the 20th century accounted for a substantial increase in crop yield. However, productivity growth has led to an extraordinary simplification of farming systems and greater reliance on external inputs. The extensive use of pesticides and fertilizers are the cause of frequent health problems and pollution of natural ecosystems. Such evidence has led to debate about the sustainability of current intensive agricultural practices. Organic farming, which aims to produce healthy food and to respect the environment, emerges as an alternative to the negative consequences of conventional farming. In the context of sustainable organic agriculture, the successful use of biological nitrogen fixation without a decrease in productivity will reduce chemical fertilization. For that, it is important to have previous knowledge of mineral nutrient requirements to optimize symbiotic nitrogen fixation and legume crop production. Here, we first review the basic concepts of mineral nutrition, as well as the importance of mineral nutrients specifically for biological nitrogen fixation in the legume-rhizobia symbiosis. Second, a broad summary of the roles of boron and calcium in plants, with special attention to their key functions in nitrogen fixation and legume-rhizobia symbiosis, will be the central topic of this review. Symbiotic nitrogen fixation is an optimal alternative to reducing the application of chemical N-fertilizer, but demand for some nutrients is higher for legume nodule development and function than for non-nodulated legumes, and corrections of nutrient deficiencies are sometimes needed to ensure crop success. Phosphorus is a common limiting nutrient of nodulated legume growth, because of phosphate requirements for nodulation and for the very energy-expensive nitrogen fixation reaction. The enhancement of the association of nodulated-legumes with vesicular-arbuscular mycorrhizas, improving phosphorus uptake, is an ecological and cheap way to correct P limitation. Sulphur and potassium are not usually limiting nutrients for nodulated legumes, although a K^+ supplement for osmoadaptation has to

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be considered for growth in saline soils. Similarly, although demand for cobalt or nickel is higher with nodulated than with non-nodulated legumes, the soil limitations of these micronutrients are unclear. Conversely, iron and molybdenum limitations for nodulated legumes are common, even in soil with sufficient Fe and Mo, because of the anaerobic and acidic environment inside the nodule that limits the availability of these micronutrients. Therefore, Fe and Mo fertilization cannot be ruled out in sustainable agriculture based on nodulated legumes. Among mineral nutrients, B and Ca are undoubtedly the nutrients with a major effect on legume symbiosis. Both nodulation and nitrogen fixation depend on B and Ca^{2+} , with calcium more necessary for early symbiotic events and B for nodule maturation. Boron deficiency is very common, and there is a risk of toxicity following B fertilization because it appears at concentrations close to sufficiency; and, in boron-deficient soils, an early small supplement of calcium prevents the effects of B limitation during nodulation. Therefore, a proper B–Ca feeding will greatly correct boron deficiency and improve crop production. Overall, improvement of symbiotic nitrogen fixation in legumes, combined with mycorrhizal associations, is a natural fertilizing alternative to conventional chemical fertilizers. Nevertheless, small and controlled application of conventional farming practices has to be considered to correct nutrient limitations, increase crop production, and satisfy the high demand of agriculturally derived food.

Keywords Boron · Calcium · Cobalt · Iron · Legume-symbiosis · Mineral nutrition · Molybdenum · Nickel · Nitrogen fixation · Nodule development · Phosphorus · Potassium · Sustainable agriculture

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1 Introduction

The need to satisfy nutritional demands on behalf of the world's current population is leading to the exponential increase of agricultural production. Such a high demand has been translated into a massive and sometimes indiscriminate application of

pesticides and nitrogen fertilizers as the unique human response to the lack of nitrogen available for plants in cultivable soils (Peoples et al., 1995). Since the middle of the 20th century, world chemical fertilizer consumption has increased dramatically. Global fertilizer consumption in 2000 was 136 million (Lal, 2004). Fertilizer use has leveled off in highly populated countries such as the United States (about 19 million tons per year since 1984) and India (16 million tons per year since 1998). China is now the top consumer of chemical fertilizers, with more than 40 million tons in 2004. European Union statistics point to excessive nitrogen fertilization (55 kg ha^{-1} per year) and application of active pesticides (2.0 kg ha^{-1} in 2001) (Eurostat, 2006). Massive application of fertilizers and pesticides affects both farmers and residents of rural areas and creates pollution of natural ecosystems associated with agriculture (Liebman, 2001; Matson et al., 1997). Serious problems derived from these practices have led to a call for sustainable agriculture based on organic ecological farming, which produces healthy food. The high costs of increasing the production of fertilizers for this practice are undoubtedly a problem at the economic level; and increased production has also been translated into another serious constraint, not only for humans, but for the survival of the biosphere: the contamination of continental waters by increasing nitrate contents until toxic levels cause the consequent eutrophication of lakes and rivers. For example, in the United States, the estimated environmental and health care costs of the recommended use of pesticides are about \$10 billion per year—the excessive fertilizer use costs \$2.5 billion from wasted fertilizer inputs, and the costs of public and environmental health issues related to soil erosion by conventional modern agriculture exceed \$45 billion yearly (Pimentel, 2005; Pimentel et al., 1995).

The use of N as a fertilizer has degraded huge land extensions around the world and biological nitrogen fixation as an alternative to chemical fertilization is required to replace tons of fertilizers (Burriss, 1994). This natural way of supplying nitrogen for plants is due to the capacity of certain soil microorganisms to fix atmospheric N_2 and to transform it into ammonium, which can be used by the plant when the fixing microorganism establishes a symbiotic relationship with it. Biological nitrogen fixation has attracted great agronomic interest. It is estimated that rhizobial symbiosis, with over one hundred agriculturally important legumes, accounts for at least half of the annual amount of nitrogen fixation in soil ecosystems (Peoples and Craswell, 1992). This plant–microorganism symbiosis offers a series of advantages over N fertilizer, among which are the high efficiency in the utilization of N by the plant, sometimes near 100%; the minimization of leaching of nitrogenous fertilizers to the soil; and the reduction of soil and water contamination.

All living N_2 -fixers are prokaryotes that do not share a homogenous taxonomic group; the only characteristic they share is the presence of the nitrogenase enzyme complex (for a review, see Sprent and Sprent, 1990). They include phototrophic organisms like bacteria of the families Rhodospirillaceae, Chlorobiaceae, and Cyanobacteriae; chemoautotrophs like *Thiobacillus*, *Xanthobacter*, and *Desulfovibrio*; heterotrophs like *Azotobacter*, *Enterobacter*, *Klebsiella*, and *Clostridium*; and bacteria of the Frankiaceae and Rhizobiaceae families. These organisms can fix nitrogen as free-living forms (with the exception of the Rhizobiaceae), or by

establishing symbiotic relationships with other organisms. The most extended N₂-fixing symbioses involving higher plants are those established between rhizobia and legumes and by *Frankia* with actinorhizal plants. Actinorhiza pioneers plants in devastated soils; *Frankia* symbioses acquire great environmental importance for the recovery of eroded soils (Tate, 1995). Meanwhile, rhizobia-legume symbioses have an enormous potential to produce food for humans (grain legumes) (Wani et al., 1995) or animals (pasture) (Thomas, 1995); to renew cultivable soils by the practice of culture rotation; and definitely to reduce the use of chemical fertilizers.

Since the classic studies of Hellriegel and Wilfarth (1888), clearly establishing that microbes inside the root nodules allowed legumes to obtain N from the air, the interaction between rhizobia and legumes has been widely studied, but some aspects are still unknown. One of them is the influence of various nutrients required by the system both during the establishment or/and development of the symbiosis and during nodule organogenesis.

2 Mineral Nutrition in the Legume-Rhizobia Nitrogen Fixing Symbiosis

After more than one century of research, great knowledge about molecular aspects of the legume-rhizobia interaction has been acquired. Nevertheless, several shadowy aspects of physiological, environmental, and nutritional subjects affecting one or both symbiotic partners, or specifically their interaction, still remain to be elucidated before we fully understand the symbiotic process.

In general, plant development depends on several genetic and environmental factors. Considering a plant in a concrete environment, the more important factors for growth are light, water, CO₂, and nutrients. Atmospheric CO₂ and soil water contribute C, O, and H that makes up about 90–95% of the plant's dry weight. The remaining 5–10% (N, P, K, S, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, Cl, Ni, plus Na or Si in some plant species) is called the mineral fraction. Despite their low, or even very low, quantitative presence, all of these mineral nutrients are absolutely essential for processes related to plant growth and development, including plant-microbe interactions like those resulting in legume-rhizobia symbiosis. For example, Mo is required at a concentration of only 0.1 ppm (part per million, mg kg⁻¹ dry weight), but it is absolutely essential. Several plants can tolerate and accumulate heavy metals at concentrations unusually high (Memon et al., 2001), but heavy metals are not essential nutrients for those plants. Consequently, it has to be clearly stated that neither the presence nor the concentration of a mineral element are valid criteria of essentiality. In the absence of a given mineral, only the fact that a plant is unable to complete its vital cycle can grant the category of essential nutrient to that mineral. To learn about plant mineral nutrition, Marschner (1995) and Epstein and Bloom (2005) are excellent monographic books.

It is important to emphasize that nutrient deficiencies can affect not only plants but also rhizobia soil populations (reviewed by O'Hara, 2001). A considerable variability of response among genus, species, and strains makes nutritional effects on bacteria highly unknown. Furthermore, competition between plants and soil microorganisms for nutrients can induce deficiencies in rhizobia, especially of those nutrients with low availability in many soils, like phosphorus or iron, affecting the nodulation capacity. Besides the effects on both symbiotic partners, some nutrients can directly play a specific role in some stages of the symbiosis development. Finally, nutrient balance can modify the absorption and accumulation of other mineral nutrients, affecting the growth of both symbionts, and the regulation of gene expression that governs the interaction. Therefore, integrated approaches involving plant physiology, microbiology, and molecular biology studies are required to fully understand nutritional stresses in the legume-rhizobia symbiosis. Meanwhile, this review will be focused on mineral nutrients with a strong effect on the symbiotic process, rather than on the growth of free-living bacteria and plants.

Although any one of the above listed 17 nutrients considered essential for all plants is also essential for legume-rhizobia symbiosis, some of them play particular roles during the symbiotic interaction. Of course, N has to be highlighted as the element that has to be fixed from atmospheric N_2 by bacterial nitrogenase. In addition, other mineral nutrients with a more specific effect on the interaction, including elements like Co or Ni, either required for the microsymbiont or exclusively for the N_2 -fixing event, are briefly reviewed herein. Furthermore, B and Ca are two nutrients described as highly in demand for nodulated plants; both of them have strong effects on nodulation and nitrogen fixation (Redondo-Nieto et al., 2003), and a relationship between B and Ca in many physiological plant processes was early stated (Reeve and Shive, 1944). Therefore the roles of both mineral nutrients in symbiotic nitrogen fixation will be more profusely described.

As stated above, deficiencies of nutrients herein reviewed, especially B and Ca, affect the symbiotic process, and hence crop production. The legume-rhizobia symbiosis is a highly regulated process of organogenesis, and different mineral nutrients have strong effects on different developmental stages of the symbiotic interaction, and/or on the nitrogen fixation process itself. Knowledge of the roles of mineral nutrients is useful in order to establish the right nutrient supplements to improve crop legume production without affecting ecological farming practices.

2.1 Macronutrients

2.1.1 Nitrogen

The symbiosis between soil rhizobia and legumes is not obligatory. In soils with enough available N, both bacteria and plants may remain unassociated during their full life cycle. However, N deficiency triggers the interaction, and only while low

N conditions are maintained does symbiosis develop successfully into a nodule where atmospheric molecular N_2 is reduced. Depending on the moment of application, the supply of combined nitrogen reduces nodulation by reduction of bacterial adsorption to the roots (Munns, 1968) or by diminution of infection (Abdel-Wahab and Abd-Alla, 1995); inhibits leghemoglobin synthesis (Bisseling et al., 1978) and nitrogenase activity; and accelerates nodule senescence (Becana and Sprent, 1987). Maximum N_2 -fixation to satisfy N needs of a plant requires that the legume be adequately nodulated, therefore optimization of rhizobial infection in a context of sustainable agriculture demands a reduction of fertilizer-N application.

2.1.2 Phosphorus, Sulfur, and Potassium

Among macronutrients, along with calcium (to be treated later on), P is a common limiting nutrient of N_2 -fixing legume crop production in many areas (Pereira and Bliss, 1989). Phosphate acts on nodulation and nitrogen fixation (Ssali and Keya, 1983). In energy terms, nitrogen fixation is a very expensive process, exceeding 16–18 mol ATP per mol N_2 fixed (Bergersen, 1991); consequently, N_2 -fixing legumes will require more P than those supplied with combined nitrogen (Cassman et al., 1981a, b). In a context of sustainable agriculture, infection with vesicular-arbuscular mycorrhizas can greatly improve phosphorus uptake (Bucher, 2007) to satisfy the high demand of nodulating legumes, especially in soils with low P availability.

The effects of sulphur and potassium are usually less dramatic, although symbiotic systems have been described as more sensitive to low K than the legumes themselves (Sangakkara et al., 1996). However, in saline soils K^+ acquires great importance as an osmolyte for adaptation (Zahran, 1999). Taking into account that nearly 50% of the world's irrigated land is categorized as having potential salinity problems (Rhoades and Loveday, 1990), a supplement of K^+ has to be considered in order to successfully cultivate symbiotic legumes in saline soils.

2.2 Micronutrients

2.2.1 Iron, Molybdenum

These two nutrients are especially required for nodules. The nitrogenase enzyme system consists of two components: component I is a MoFe protein and component II is Fe-protein. Moreover, other proteins requiring Fe as a cofactor important for symbiotic N_2 fixation are abundant inside the nodules; among them, heme-containing proteins like the oxygen carrier leghemoglobin and the cytochromes or Fe-S proteins such as ferredoxin. Therefore, a particularly high requirement of Fe exists in legume nodules (O'Hara et al., 1988), and low iron availability in soils will affect more nodulated than combined N-fed legumes. Moreover, the anaerobiosis required for N_2 -fixation could impair the process of reduction of Fe^{3+} to Fe^{2+} , aggravating a possible Fe limitation (Romera et al., 2004). Similarly, the supply of Mo to soils with low availability of this micronutrient has to be increased for the

development of N₂-fixing legumes. Molybdenum limitation occurs in some naturally acidic, poorly buffered soils. The acidifying effect of nitrogenase activity can also reduce Mo availability, affecting crop legume production (Doerge et al., 1985). Therefore, despite the advantages of improved biological nitrogen fixation for sustainable agriculture, a risk of iron or molybdenum deficiencies has to be considered in symbiotic legumes once the nodule is developed and the nitrogen fixation process takes place.

2.2.2 Cobalt

Cobalt deficiency affects nodule development and function at different levels (Dilworth et al., 1979). The requirement of Co for N₂-fixing nodules was reported by Ahmed and Evans (1960). The dependence of Co, the cobalamin coenzyme B₁₂ content, leghemoglobin, and N₂-fixation was later demonstrated (Kliwer and Evans, 1963). Cobalamin (vitamin B₁₂), which has Co(III) as the metal component, is required for enzymes such as methionine syntase; ribonucleotide reductase, involved in bacteroid differentiation (Dilworth and Bisseling, 1984); methylmalonyl-coenzyme A mutase, involved in the synthesis of heme groups; and leghemoglobin or bacterial cytochromes (Riley and Dilworth, 1985). Therefore, Co is one of the micronutrients with a strong effect not only during nodule development but also during nodule function.

2.2.3 Nickel

About 200 µg Ni are enough to fully satisfy plant demands, forming complexes with several enzymes (Dalton et al., 1988). Therefore, there is no clear evidence of Ni deficiency in soils, although beneficial effects of Ni supply to plants fed with urea in calcareous soil have been reported (Singh et al., 1990). Both plant and rhizobial ureases are Ni-requiring enzymes. In legumes like bean or soybean, developing determinate nodules, ureides are the dominant form of transport from nodules to shoots of fixed nitrogen (Atkins, 1987). Urea is an intermediate of nitrogen and ureide metabolism, and has to be degraded by urease. Otherwise, accumulation of urea will lead to leaf necrosis (Krogmeier et al., 1991). Besides urease, rhizobial hydrogenase also requires Ni. These enzymes recycle hydrogen generated by nitrogenase enzymes, increasing the efficiency of the nitrogen fixation process (Maier and Triplett, 1996). A low level of Ni in agricultural soils limits hydrogenase activity (Ureta et al., 2005).

3 Major Importance of Boron and Calcium in Legume Symbiosis

The study of the interaction between B and Ca is an important topic of research on mineral nutrition of plants. The content of either nutrient influences the tissue distribution (Ramón et al., 1990) and the requirements of the other for optimal plant growth (Teasdale and Richards, 1990). Boron is required for plants

at micromolar concentrations, but it has been implicated in several physiological processes (Blevins and Lukaszewski, 1998; Brown et al., 2002): cell wall structure and synthesis; membrane structure and membrane associated reactions; reproduction; nitrogen fixation; and phenolics metabolism. The diversity of plant processes affected by B leads to pleiotropic effects on plant development due to a deficiency of this micronutrient. Although the primary role of B in plants is unknown, its special chemistry, with the capacity of borate anions to form stable covalent links with cis-diols of carbohydrate moieties of molecules, turning them functional, has been established as the basis of any function of B (Bolaños et al., 2004b).

Calcium is also implicated in a large number of physiological processes in plants (Leonard and Hepler, 1990). Although the traditional functions of calcium in plants are also related to cell wall structure and membrane structure and function, recent reviews have focused on cytosolic free Ca^{2+} as one of the most important messengers involved in signal-response coupling (Rudd and Franklin-Tong, 2001; Sanders et al., 2002). Although most of plant Ca is linked to the cell wall and membrane, maintaining a small amount of cytosolic free Ca^{2+} is important because several physiological processes are accompanied by changes in cytoplasmic calcium concentration (Trewavas and Malhó, 1998). Moreover, a number of external stimuli lead to changes in cytosolic Ca^{2+} , which acts as a second messenger in the signaling between environmental factors and plant responses (Bush, 1995). Our previous studies in cyanobacteria demonstrated that calcium is implicated in the stability of heterocyst envelopes, and consequently in the protection of nitrogenase activity under stress conditions (Fernández-Piñas et al., 1995). Moreover, calcium may be involved in early signalling in response to temperature shocks, salinity, and osmotic stress in cyanobacteria (Torrecilla et al., 2000, 2001); and in heterocyst differentiation (Torrecilla et al., 2004). Therefore, a major challenge for future research will be to identify cellular targets of Ca^{2+} signals for cell differentiation and the primary sensors that perceive stresses and trigger Ca^{2+} signalling.

Evidence of a physiological B–Ca interaction was described for transport processes across the cell membrane (Tang and De la Fuente, 1986), although most of the investigations regarding the B–Ca relationship have been focused on the structure and function of the cell wall. In that sense, Kobayashi et al. (1999) demonstrated that Ca^{2+} promoted *in vitro* formation of dimers of borate-rhamnogalacturan II, and proposed that Ca^{2+} stabilizes pectin polysaccharides of the cell wall through ionic and coordinate bonding in the polygalacturonic acid region. At the signal transduction level, B could be implicated in the liberation of cytosolic Ca^{2+} by the cyclic-ADP ribose pathway (as proposed by Eckhert et al., 2007, following studies on prostate cancer) that regulates, among other things, ABA signaling (Wu et al., 1997).

Concerning nitrogen fixing organisms, a nutritional relationship between B and Ca in cyanobacteria was shown by our group. These microorganisms require B for maintaining the envelope of specialized N_2 -fixing heterocysts (Bonilla et al., 1990). A supplement of extra Ca to cultures growing in the absence of B (Bolaños et al., 1993), or the addition of B to Ca-deficient treatments (Bonilla et al., 1995) resulted in a recovery of heterocyst structure and nitrogenase activity. In rhizobia-legume

symbiosis the studies of Carpena et al. (2000) suggest a specific B–Ca relationship in nodulated pea plants; they described the effect of low B on Ca concentration and a Ca-mediated mobilization of B from old to new growing tissues in B-deficient plants.

3.1 Boron and Nitrogen Fixing Rhizobia-Legume Symbioses

Legume (and also actinorhizal) N₂-fixing symbioses involve the development of a new plant organ, generally in the root, the nodule. New synthesis and deposition of wall and membrane material occurs to build a nodule; and several rhizobia and plant macromolecules decorated with cis-diol rich glycosil-moieties are implicated in plant-bacterial cell surface interactions. Therefore, B is a clue element in the establishment and maintenance of these symbioses.

The requirement of B for symbiotic N₂ fixation in legumes was very early suggested by Brenchley and Thornton (1925) in *Vicia faba* and confirmed at the end of the past century in nodulated *Pisum sativum* (Bolaños et al., 1994) (Fig. 1) and *Phaseolus vulgaris* (Bonilla et al., 1997a) plants (Fig. 2). In nodulated legumes, B deficiency led to a high reduction of nodules and nitrogenase activity (Table 1). Besides the extensive synthesis of new membranes and walls, rearrangements and changes in cell wall structure during nodule development explain a high requirement for B and typical symptoms of B-deficiency in the structure of nodules developed without B (Brewin, 2004). Studies at a molecular level have revealed that hydroxyproline-/proline-rich glycoproteins (Bonilla et al., 1997a) and pectin polysaccharides (Bonilla et al., 1997b; Redondo-Nieto et al., 2003) are abnormally assembled, leading to aberrant nodule cell walls.

Not only cell wall components, but also bacteria and plant-derived glycoconjugates containing cis-diol groups able to interact with borate anions, play essential roles in the correct establishment of the symbiosis between legumes and rhizobia

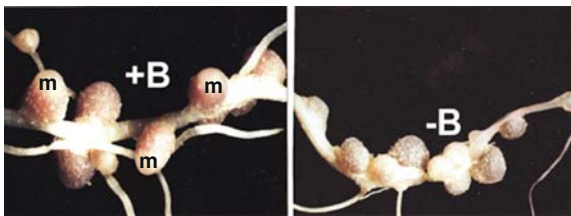
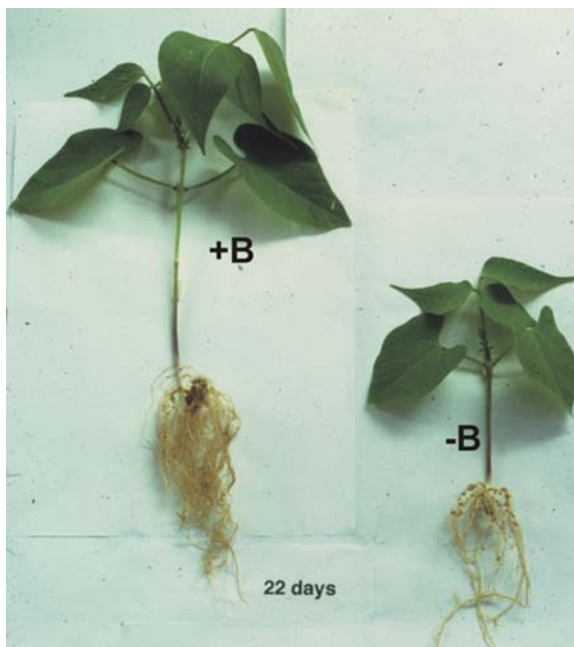


Fig. 1 Effects of B deficiency on pea (*Pisum sativum* L.) nodule development. Pea plants were grown with (+B) or without (–B) boron and inoculated with *Rhizobium leguminosarum* bv. *Viciae*. Note that 3 weeks post inoculation, nodules from B-sufficient plants have a colourless apical meristem (m) and a central zone of a red color due to the presence of the oxygen carrier leghemoglobin. By contrast, nodules from B-deficient plants are of a small size and of a pale color, because they develop abnormally in the absence of B and do not have leghemoglobin, being not functional. +B plants were fed with a growth solution containing 9.3 μ M B; –B plants were grown with no added B

Fig. 2 Effects of B deficiency on the development of bean (*Phaseolus vulgaris* L.) nodulated plants 22 days after inoculation with *Rhizobium etli*. +B with boron, -B: without boron. B-deficiency led to poor nodulation and root development. +B: plants were fed with a growth solution containing $9.3 \mu\text{M}$ B; -B: plants were grown with no added B



(Kannenberg and Brewin, 1994). Therefore, a B function is expected not only to stabilize nodule structure but also to modulate bacteria-plant interactions for the maintenance of a correct symbiotic relationship.

A legume-rhizobia symbiosis starts with the exchange of diffusible signal molecules between both partners, resulting in the activation of rhizobial *nod* (nodulation) genes in response to flavonoids exuded by the legume root (Spaink, 2000). The products of *nod* gene activity are the Nod (nodulation) factors, lipochitin-oligosaccharides that induce root hair deformation, cortical cell division (Dénarié and Cullimore, 1993), and preinfection structures in curled root hairs (van Brussel

Table 1 Effects of B deficiency (-B) on nodulation and nitrogenase activity of several legumes 4 weeks post inoculation with the appropriate host rhizobial strain. Nitrogenase was measured as acetylene reduction activity (ARA) (100% corresponds to 183 ± 36 , 621 ± 137 , and $8.1 \pm 3.6 \text{ nmol C}_2\text{H}_2 \text{ root}^{-1}\text{h}^{-1}$ for *Pisum sativum*, *Phaseolus vulgaris*, and *Medicago sativa*, respectively). The normal B treatment (+B) was $9.3 \mu\text{M}$ added as boric acid (H_3BO_3)

	Nodules per root		% Nitrogenase (ARA activity)	
	+B	-B	+B	-B
<i>Pisum sativum</i> - <i>Rhizobium leguminosarum</i>	170 ± 37	53 ± 26	100%	15%
<i>Phaseolus vulgaris</i> - <i>Rhizobium etli</i>	328 ± 65	143 ± 43	100%	28%
<i>Medicago sativa</i> - <i>Sinorhizobium meliloti</i>	17 ± 6	5 ± 3	100%	0%

et al., 1992) in the appropriate host legume. Boron deficiency leads to a very much reduced nodulation because of the very low *nod* gene induction activity of root exudates from B-deficient legumes (Redondo-Nieto et al., 2001). This effect might be a reflection of the phenolic, and hence flavonoid, metabolism, which is affected by boron nutrition (Ruiz et al., 1998), so that B deficiency can modify the presence or release of flavonoid compounds that in turn induce the expression of *nod* genes. Besides diffusible signals, colonization of the root surface by rhizobial cells is also diminished in B-deficient plants (Redondo-Nieto et al., 2001).

Following early preinfection plant-rhizobia signalling, induction of cortical cell division by Nod factors leads to a nodule primordium. Meanwhile, rhizobia colonizing curled root hairs make contact with the plant cell surface and invade the plant through a transcellular tunnel (the infection thread) sheathed with cell wall material (Rae et al., 1992), followed by an endocytosis-like process from unwalled infection droplets (Brewin, 1991, 2004). Infection threads in B-deficient legumes are extremely enlarged and aborted prior to bacterial release (Bolaños et al., 1996), even in the root hair that has previously reached the cortical cell (Redondo-Nieto et al., 2001). Furthermore, both indeterminate (pea) and determinate (bean) nodules appear almost uninvaded when they are induced in the absence of B (Bolaños et al., 1994; Bonilla et al., 1997a). During the growth of infection threads, rhizobia are embedded by a matrix containing plant-derived glycoproteins, including a root nodule extensin-like glycoprotein apparently important for intra- or intermolecular cross-linking (Rathbun et al., 2002). Boron can modulate interactions between the infection thread matrix glycoproteins and the bacteria cell surface, to promote nodule invasion (Bolaños et al., 1996). In the absence of B, root nodule extensin can attach to the cell surface of rhizobia. Therefore, the bacterium can be trapped, avoiding the endocytosis process leading to poorly invaded nodules.

After gaining the cytosol compartment, rhizobia are now called bacteroids, and are surrounded by a plant-derived membrane (peribacteroid membrane). They grow, divide, and develop into differentiated symbiosomes when biological nitrogen fixation takes place. The peribacteroid membrane harbors a differentiated glycocalyx composed of glycoproteins and glycolipids which codifferentiate with bacteroids (Perotto et al., 1991), and several thousand symbiosomes occupy each infected cell; consequently, an extensive synthesis and differentiation of membrane takes place at rates about 30- to 50-fold higher than in other tissues (Robertson and Lyttleton, 1984). During symbiosome maturation, new proteins are targeted to the symbiosome compartment to constitute a peribacteroid fluid. Two isoforms of a nodule-specific lectin-like glycoprotein (*Pisum sativum* nodule lectin, PsNLEC-1) seem to be implicated in bacteroid maturation (Dahiya et al., 1997; Sherrier et al., 1997). It has been shown that the glycosyl-moiety of these glycoproteins interacts with both the surface of the bacteroid and the symbiosomal membrane (Bolaños et al., 2004b). Therefore, this interaction seems to play a direct role for symbiosome development, since pea mutants lacking the symbiosomal form of PsNLEC 1 (Dahiya et al., 1998) or cell surface defective rhizobia (Perotto et al., 1994), that do not interact physically with PsNLEC 1, do not develop N₂-fixing bacteroids. The detection by specific antibodies of sugar groups of PsNLEC-1 demonstrates that the carbohydrate-moiety

of this protein was modified in the absence of B. Localization in ultra-thin pea nodule sections of PsNLEC-1 glycoproteins showed that they were accumulated in Golgi-derived or cytoplasmic vesicles, instead of symbiosomes in B-deficient nodules. This indicates a failure of the targeting of Ps-NLEC glycoproteins to the peribacteroid fluid of symbiosomes in B-deficient nodules (Bolaños et al., 2001). Aberrant bacteroid differentiation in the absence of B has been recently related to some glycoproteins that are possible borate ligands and that appear associated to the glycocalyx of the peribacteroid membrane of dividing symbiosomes. They are called RGII-glycoproteins, because they share antigenicity with rhamnogalacturan II pectin polysaccharide (Redondo-Nieto et al., 2007). These glycoproteins were never detected in B-deficient cells, suggesting that they are stabilized on the glycocalyx through borate bridges and that association of the carbohydrate moiety of PsNLEC-1 with the peribacteroid membrane is mediated by RGII-glycoproteins.

Overall studies justify that boron is undoubtedly the micronutrient with the highest demand increase for symbiotic legumes. Although B is widely distributed both in the lithosphere and the hydrosphere, usually only soluble B (about 10% of total B in soil) is available to plants, making boron deficiency more common than deficiency in any other plant micronutrient worldwide (Shorrocks, 1997). Therefore, boron deficiency is a constraint for sustainable agriculture based on legume-rhizobia symbiosis; nevertheless, both the sufficiency and the toxicity of boron for nodulated legumes are in a narrow range of concentrations (Redondo-Nieto et al., 2003), and boron application following diagnosis of boron deficiency has to be extremely accurate.

3.2 Calcium and Nitrogen Fixing Rhizobia-Legume Symbioses

A role of Ca^{2+} for N_2 -fixation in legumes was first reported by Greenwood and Hallsworth (1960). Later, Lowter and Loneragan (1968) described that a high Ca^{2+} supply was required to induce a high number of nodules in the plants, and Munns (1970) described a higher Ca^{2+} requirement for early infection events. These studies indicate an important role for Ca^{2+} in plant-bacteria signalling and recognition. The activity of *nod* genes is higher when the amount of Ca^{2+} for plant growth increases. Richardson et al. (1988) demonstrated that high Ca^{2+} increased the amount of *nod*-gene-inducing compounds in root exudates. This effect can be due to the role of Ca^{2+} on the synthesis of flavonoids. Application of external Ca^{2+} to plants enhances the phenylalanine ammonia-lyase activity (Castañeda and Pérez, 1996), the key enzyme in the flavonoid synthesis pathway.

Calcium is also required for an optimal root hair colonization (Lodeiro et al., 1995). Attachment of rhizobia is mediated by plant and bacterial components able to use Ca^{2+} as a ligand to reinforce the adhesion. Calcium ions can therefore strengthen the activity of plant lectins or rhizobial Ca^{2+} -dependent ricadhesines (Smit et al., 1989). Moreover, bacterial exopolysaccharide (EPS) can form a gel in the presence of cations as Ca^{2+} , being a non-specific mechanism for rhizobial attachment (Morris et al., 1989).

Besides early preinfection interactions, Ca^{2+} plays an important role in signal transduction during Nod factors perception and nodule organogenesis (Charron et al., 2004). Calcium has been demonstrated to act as a second messenger in Nod-factor signal transduction (Cárdenas et al., 2000; Lhuissier et al., 2001). Following Nod factor application, an influx of Ca^{2+} at the root hair tip (Felle et al., 1998) was the first detectable effect. This could lead to an efflux of Cl^- and membrane depolarization (Downie and Walker, 1999), causing an increase of cytosolic Ca^{2+} within a few minutes at the root hair tip (Cárdenas et al., 1999; Felle et al., 1999). First infection events, including root hair tip swelling, vacuolation, endoplasmic reticulum alignment with the plasma membrane, nuclear movement to the swelling, and inward growth of the cell wall to initiate the infection thread, can be related to these Ca^{2+} dynamics. About 3 min after Nod factor application, a reorganization of the actin cytoskeleton starts (Cárdenas et al., 1998). The cascade involved in the transduction of Nod factor signalling is mediated by a G-protein and phospholipases C (Pingret et al., 1998) that are also fully activated by Ca^{2+} . Hydrolysis of phosphatidylinositol biphosphate (PIP_2) by phospholipases C produces water soluble IP_3 that can regulate actin binding proteins (ABP)-mediated rearrangements of actin filaments and bundles. Furthermore, there are other later Ca^{2+} spikes originating from the perinuclear region of the root tip approximately 9 min after Nod factor application, which extend for at least 60 min–3 h (Ehrhardt et al., 1996). Although the role of these spikes is still unclear, there is some information concerning gene expression (Schultze and Kondorosi, 1998; Felle et al., 1999) that is important for cell cycle regulation during nodule organogenesis.

3.3 B–Ca Relationship in Biological Nitrogen Fixation

As stated above, investigating the roles of boron in nitrogen fixation, we demonstrated a relationship between the micronutrient and calcium in cyanobacteria, but also in nodulated legumes. Rhizobia-legume symbiosis is highly influenced by B and Ca^{2+} at the different steps of nodule development and organogenesis. Particularly important is the recovery effect of B deficiency by addition of Ca^{2+} , which is translated to a plant, mainly root, development (Fig. 3). Pea, bean, and alfalfa plants grown in media containing different concentrations of B and Ca^{2+} , and inoculated with their host rhizobia, develop different amounts of nodules and nitrogen-fixing activity, depending of the level of either nutrient in the growth media (Table 2), indicating that the relationship between B and Ca can be clearly stated (Redondo-Nieto et al., 2003).

Determination of *nod* gene activity, which is very low after exposure of *Rhizobium* to root exudates derived from B-deficient plants, demonstrates a higher induction capacity in plants treated with high concentrations of Ca^{2+} . Besides the exchange of diffusible signals, Ca^{2+} can also increase root colonization by rhizobia, which is diminished by B deficiency. Moreover, the phenomena of cell invasion and spreading inhibited by B deficiency were also recovered by addition of Ca^{2+} . However, Ca^{2+} could not prevent alterations of B-deficient nodule cell wall structure,



Fig. 3 Effects of different B and Ca treatments on the development of pea (*Pisum sativum* L.) (upper side) and bean (*Phaseolus vulgaris* L.) (bottom side) nodulated plants. Note that deficiency of B (-B+Ca treatment) led to poor nodulation and root development, and that supplementation with Ca (-B+2Ca treatment) partially prevented the effects of B deficiency and resulted in increased nodulation and root development. +B: 9.3 μ M B; +Ca: 0.68 mM Ca^{2+}

confirming that both nutrients are essential for wall architecture Ca^{2+} (Redondo-Nieto et al., 2003). Furthermore, previous results indicate that Ca^{2+} cannot prevent abnormal PsNLEC-1 targeting to the symbiosomal compartment under B deficiency (Redondo-Nieto, 2002), indicating a specific role for B in Golgi-derived vesicle targeting. Fig. 4 summarizes all of these results.

Table 2 Effects of different B and Ca treatments on nodulation and nitrogenase activity of nodulated *Pisum sativum* L. plants. Nitrogenase was measured as acetylene reduction activity (ARA)(100% corresponds to $183 \pm 36 \text{ nmol C}_2\text{H}_2 \text{ root}^{-1}\text{h}^{-1}$). The normal B treatment (+B) was $9.3 \mu\text{M}$ and the toxic treatment ++B was $46.5 \mu\text{M}$, added as boric acid (H_3BO_3). The normal Ca treatment (+Ca) was 0.68 mM added as calcium chloride (CaCl_2)

	Nodules per root			% Nitrogenase activity (ARA)		
	+B	-B	++B	+B	-B	++B
+Ca	170 ± 37	53 ± 26	77 ± 31	100%	15%	26%
-Ca	22 ± 11	26 ± 10	18 ± 12	20%	10%	43%
+2Ca	267 ± 51	245 ± 64	231 ± 56	80%	50%	21%

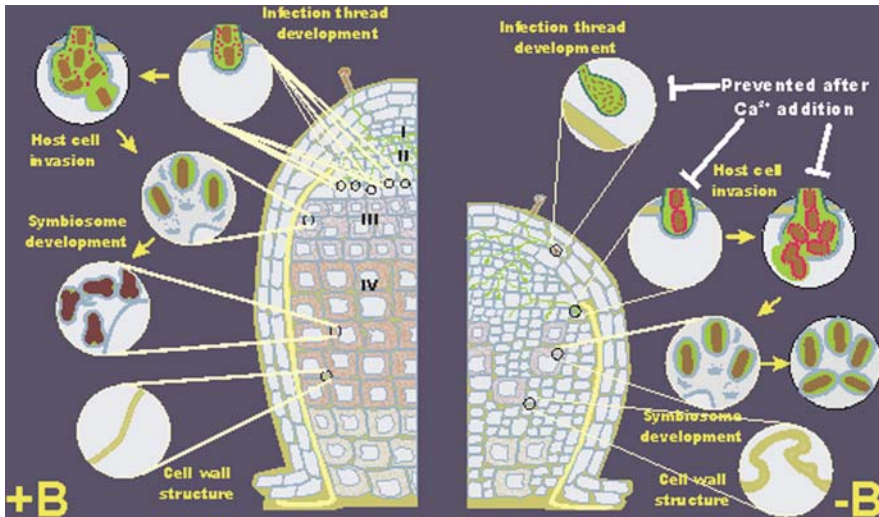


Fig. 4 Effects of B deficiency on the different processes of nodule development in the legume-rhizobia symbiosis (*left panel*). Prevention of B deficiency by Ca^{2+} addition (*right panel*). Boron deficiency inhibits infection threads development, reducing nodule invasion. Both effects are prevented by increasing Ca^{2+} supply. Therefore, Ca^{2+} appears more important than B during early events of nodule development. The absence of B also alters symbiosome differentiation and nodule organogenesis, leading to non- N_2 -fixing nodules. Different zones of nodule development (I: nodule meristem, II: infection thread development, III: bacteroid proliferation, and IV: nitrogen fixation) are not clearly differentiated in B deficient nodules. Therefore, nodule organogenesis and maturation are absolutely B-dependent because deficiency effects on these processes cannot be prevented by Ca^{2+} . Finally, both nutrients are essential for maintaining nodule cell wall structure

Another insight into a B–Ca relationship comes from the study of gene expression during nodule development. Genetic studies of nodulation of *Medicago truncatula* showed that expression of more than 60% of the analysed genes (including genes involved in the cell cycle, cell wall assembly, and ribosome biogenesis) was affected by boron deficiency; and that, in some cases, a supplement of Ca^{2+} could reverse gene expression to a normal level (Redondo-Nieto et al., 2002). The

cyclic-ADP ribose pathway involved in release of internal Ca^{2+} is presumably modulated by B (Eckhert et al., 2007). Investigating whether those genes regulated by a B–Ca ratio are influenced by the cyclic-ADP ribose pathway will shed new light on the role of B in signal transduction.

Cyclic-ADP ribose is the central mediator of plant signaling involving abscisic acid (ABA), the primary phytohormone that mediates plant responses to stresses such as cold, drought, and salinity signaling (Wu et al., 1997). Interestingly, the interaction between calcium and boron seems to be more evident and important in nodule development and nitrogen fixation under salt stress. While pea plants cultivated under saline conditions did not develop nodules in a normal nutrient solution, modifying levels of B and Ca could increase nodulation and nitrogen fixation and recover plant development to 70% of that of plants grown without salt stress (El-Hamdaoui et al., 2003).

4 Conclusion

The knowledge of nutritional requirements and the role of different mineral nutrients during each step of the development of a legume-rhizobia symbiosis, as well as the impact of mineral nutrients on the process of nitrogen fixation, are imperative in a context of sustainable agriculture. Depending on the soil features, a particularly important factor for optimization of symbiotic N_2 -fixation is the availability of phosphorus, potassium, iron, and molybdenum. Infection with vesicular-arbuscular mycorrhizas improves phosphorus uptake; therefore, the “triple symbiosis” legume-rhizobia-mycorrhiza will reduce N- and P-chemical fertilizer. Nevertheless, sustainable agriculture has to include a few of the conventional farming practices in a controlled way to optimize crop production without ecological risk. That is the case with potassium fertilization in saline soils; or the application of micronutrients with reduced availability due to the nodule environment or to the nitrogen fixation process, like iron or molybdenum; or those with a higher requirement for the development of symbiosis or the nitrogenase function, like cobalt or nickel. Our studies have demonstrated during the last two decades that boron is certainly the micronutrient whose deficiency has the most impact in nodule development and nitrogen fixation in legume symbioses. Because boron deficiency is very common worldwide, the diagnosis of B availability is very important prior cultivation of nodulated legumes. Our studies also show a relationship between boron and calcium during legume nodulation and symbiotic nitrogen fixation, both under physiological and under stress conditions. Nodulation and nitrogen fixation in legume-*Rhizobium* symbioses is dependent on B and Ca^{2+} . During the early events of nodulation, B was essential for *nod* gene induction, root hair curling, and adsorption of bacteria to the root surface, though Ca^{2+} addition could prevent the inhibitory effects of B deficiency and increased nodule number. High concentrations of Ca^{2+} also enhanced cell and tissue invasion by *Rhizobium*, which were highly impaired by B deficiency, although Ca^{2+} could not restore nodule structure. Taking into account that boron concentrations leading to either sufficiency or toxicity are quite precise, small calcium supplements

can be used for the correction of boron deficiency without a high application of B-fertilizer, mainly at the early stages of nodule development when Ca^{2+} can partially prevent B-deficiency.

Furthermore, the study of symbiosis under salt stress indicates that proper B and Ca nutrition can facilitate salt tolerance. Therefore, such studies should accompany genetic approaches searching for tolerant cultivars, in order to establish the best nutritional conditions for each type of legume, which will ensure the success of symbiosis, plant development, and crop production in saline soils.

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