



## Review Article

# Role of Some of Mineral Nutrients in Biological Nitrogen Fixation

Weria Weisany<sup>1\*</sup>, Yaghoub Raei<sup>2</sup>, Kaveh Haji Allahverdipoor<sup>3</sup>

<sup>1</sup> Ph.D student in Agro Ecology, Department of Plant Ecophysiology, Faculty of Agriculture, Tabriz University and

<sup>1</sup> Member of Young Researchers and Elite Club, Islamic Azad University, Sanandaj Branch, Iran.

\* Corresponding author: E-mail: Weria.Wisany@gmail.com

<sup>2</sup>Department of Plant Ecophysiology, Faculty of Agriculture, Tabriz University, Tabriz, Iran

<sup>3</sup>Department of Agronomy and Plant Breeding, Faculty of agriculture, University of Kurdistan, Sanandaj, Iran

### ABSTRACT

Atmospheric nitrogen fixation probably contributes at most about 10% of the total annual yield of fixed nitrogen. The most important source of fixed nitrogen derives from the activity of certain soil bacteria that absorb atmospheric N<sub>2</sub> gas and convert it into ammonium. The process of biological nitrogen fixation offers an economical attractive and ecological advantage by of reducing external nitrogen input and improving the quality and quantity of internal resources. Mineral nutrients may influence N<sub>2</sub> fixation in legumes and nonlegumes at various stages of the symbiotic process: infection and nodule development, nodule function, and host plant growth. Here, 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. For healthy and vigorous growth, intact plants need to take up from the soil: relatively large amounts of some inorganic elements: ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P) and sulphur (S); and, small quantities of other elements: iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). The enhancing effect of low levels of combined nitrogen on N<sub>2</sub> fixation in legumes is related to the lag phase between root infection and the onset of N<sub>2</sub> fixation.

Phosphorus (P) is second only to nitrogen as an essential mineral fertilizer for crop production. At any given time, a substantial component of soil P is in the form of poorly soluble mineral phosphates. A high phosphorus supply is needed for nodulation. When legumes dependent on symbiotic nitrogen receive an inadequate supply of phosphorus, they may therefore also suffer from nitrogen deficiency. Potassium and sulphur are not usually limiting nutrients for nodulated legumes, although a K<sup>+</sup> supplement for osmoadaptation has to be considered for growth in saline soils. 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<sup>2+</sup>, with calcium more necessary for early symbiotic events and B for nodule maturation.

Copper plays a role in proteins that are required for N<sub>2</sub> fixation in rhizobia. Cu deficiency decreased nitrogen fixation in subterranean clover. Iron is required for several key enzymes of the nitrogenase complex as well as for the electron carrier ferredoxin and for some hydrogenases. A particular high iron requirement exists in legumes for the heme component of hemoglobin. As molybdenum is a metal component of nitrogenase, all N<sub>2</sub>-fixing systems have a specific high molybdenum requirement. Molybdenum deficiency-induced nitrogen deficiency in legumes relying on N<sub>2</sub> fixation is widespread, particularly in acid mineral soils of the humid and sub humid tropics. A specific role for nickel in nitrogen-fixing bacteria is now well established with the determination that a nickel-dependent hydrogenase is active in many rhizobial bacteria. Cobalt is required for the synthesis of leghemoglobin and, thus, for the growth of legumes relying on symbiotically fixed nitrogen, is an essential mineral nutrient. It has been established that Rhizobium and other N<sub>2</sub>-fixing microorganisms have an absolute cobalt requirement whether or not they are growing within nodules and regardless of whether they are dependent on a nitrogen supply from N<sub>2</sub> fixation or from mineral nitrogen. There for, in sustainable agriculture systems, the reduction of chemical fertilizers application is one of the most important principles, and nitrogen fixation by legumes is a natural fertilizing alternative to conventional chemical fertilizers. Thus, it is necessary to review of the effects of mineral nutrients on the rate of nitrogen fixation.

**Key Words:** legumes, mineral nutrients, nitrogen fixation

Received 24/02/2013 Accepted 21/03/2013

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## INTRODUCTION

Chemical (metallic or non-metallic) natures, the form taken up by plants, biochemical functions and quantitative differences in functional requirements, the mineral nutrients have been divided into two or more categories. The distinction based on quantitative differences in functional requirements-macronutrient and micronutrient elements [51] is widely followed. These essential mineral elements include six macronutrients of N, K, P, S, Mg and Ca, which are present in relatively large amounts in plant tissues ( $\text{mg g}^{-1}$  of dry tissue). In addition, micronutrients play important roles as constituents of organic structures, constituents or activators of enzymes (Cu, Fe, Mn, Mo, Ni, and Zn), electron carriers or in osmoregulation. They also function in the regulation of metabolism (Cl, Cu, Fe, Mn, and Zn), reproduction and protection of plant against abiotic and biotic stresses [69].

The major source of fixed-N to the biosphere is biological  $\text{N}_2$ -fixation by microorganisms known collectively as diazotrophs; they provide about 60% of the total annual input. No eukaryote is known to have this capability [17]. The most commonly "fixed" forms of nitrogen are ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) ions. Nitrogen fixation is the process, which, as a component of the biogeochemical Nitrogen Cycle, drives the conversion of atmospheric  $\text{N}_2$  into ammonium ions.

Several key abiotic factors limit legume productivity and nitrogen fixation in world agriculture. These may be summarised as extremes of temperature, water availability and nutrient availability, and toxic soil factors such as sodicity and pH. Mineral nutrients may influence  $\text{N}_2$  fixation in legumes and nonlegumes at various levels of the symbiotic interactions: infection and nodule development, nodule function, and host plant growth [61]. Robson [65] summarized the nature of the interaction between nutrient supply and combined nitrogen on legume growth as a means for estimating symbiotic sensitivity to their supply or concentration. He concluded that Co and Mo are required in high amounts for symbiotic nitrogen fixation for host-plant growth than Cu, Ca and P. Although there is currently experimental evidence for specific requirements for 11 nutrients (B, Ca, Co, Cu, Fe, K, Mo, Ni, P, Se and Zn) for symbiotic development in some species of legume, only four of these elements (Ca, P, Fe and Mo) appear to cause significant limitations on the productivity of symbiotic legumes in some agricultural soils [61].

Lynd and Ansman [52] found reductions in nodule number of peanut when K was applied alone, but not when P and Ca were added. A stimulatory effect of the rate of  $60\text{mg kg}^{-1}$  of  $\text{NH}_4\text{NO}_3$  on nodulation also has been reported in presence of P [35].

We begin this review with an introduction to the mineral nutrients essential for the legume-Rhizobium symbiosis. It is important to separate effects of nutrient deficiencies on the host plant from effects on the symbiotic system. Nutrient constraints to nodule development and function will be reviewed with an emphasis on the mineral nutrition of symbiotic rhizobia present in the root and nodule.

### Essential mineral nutrients

The essential mineral nutrients for symbiotic legume nitrogen fixation are those required for the normal establishment and functioning of the symbiosis. Based on this definition, adapted from Arnon and Stout [8], the following chemical elements are known to be essential for the legume-Rhizobium symbiosis: C, H, O, N, P, S, K, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, Cl, Ni and Co. Each essential nutrient has specific physiological and biochemical roles and there are minimal nutrient concentrations required within both legumes and rhizobia to sustain metabolic function at rates which do not limit growth [38].

## EFFECT OF MACRONUTRIENTS ON NITROGEN FIXATION

### Nitrogen

Presence of mineral N in the soil inhibits both nodule formation and nitrogenase activity [74]. Anne-Sophie Voisin *et al.* [6] reported that mineral N in the soil inhibited symbiotic nitrogen fixation but it was relative to start of nodulation and  $\text{N}_2$  fixation at early vegetative growth at low concentration. The inhibitory effects of mineral N on nodulation and  $\text{N}_2$  fixation of soybean are clear at high concentrations ( $>5\text{ mM}$ ), but far less at lower concentrations. However, nitrogen fertilization affects nodulation of bean plants and therefore the usually-recommended rates of  $40\text{-}60\text{ kg N ha}^{-1}$  suppress  $\text{N}_2$  fixation [67,36]. Although there are a few reports on positive effects of low nitrate concentrations on  $\text{N}_2$  fixation in legume species such as soybean [75,39,40], Olsson *et al.* [62] showed that plants reduce carbon allocation to arbuscular mycorrhizae when grown in high compared to low nitrogen agar media.

### **Phosphorus**

Phosphorus is used in numerous molecular and biochemical plant processes, particularly in energy acquisition, storage and utilization [33]. The deficiency of phosphorous supply and availability remains a severe limitation on nitrogen fixation and symbiotic interactions. This requirement might be higher than for root or shoot growth of the host plant. There are marked differences in rhizobial and plant requirements for P [10,63] with the slow- growing more tolerant to low P than the fast-growing rhizobia [10]. Nodules themselves are strong sinks for P [41] and nodulation and N<sub>2</sub> fixation are strongly influenced by P availability [49,71,68]. When legumes-dependent on symbiotic nitrogen receive an inadequate supply of phosphorus, they may suffer nitrogen deficiency. The phosphorus content per unit dry weight is usually considerably higher in the nodules than in the roots and shoots, particularly at low external phosphorus supply [1]. Nitrogen fixing plants have an increased requirement for P over those receiving direct nitrogen fertilization, probably due to need for nodule development and signal transduction, and to P-lipids in the large number of bacterioids [37]. Also, capability of developing nodules to compete with other vegetative sinks (root and shoot meristems) for phosphorus at limited external supply may be different between legume species [44,66].

### **Potassium**

Potassium is not an integral constituent of any metabolite but serves to activate numerous enzymes, serves as a counter ion and is the major cationic inorganic cellular osmoticum [33]. The growth rate of internodes is affected [27], (and some dicotyledonous species may form rosettes [12]. With the advance of K deficiency, old leaves show the first symptoms as under such conditions K is trans located from older to younger leaves and growing tips via the phloem A qualitative requirement for K has been demonstrated for some rhizobia [70,79]. Vincent [79] suggested that *R. trifolii* and *R. meliloti* show restricted growth when K is omitted from a defined medium and a linear response in cell yield up to 0.006 mM was obtained in batch culture.

### **Calcium**

Calcium is a macronutrient for plants, yet it is actively excluded from plant cytoplasm Calcium has several distinct functions within higher plants. Inhibition of nodulation is a major limiting factor in N<sub>2</sub> fixation of many legume species grown in acid mineral soils. Increase in soil pH by Hming is therefore very effective in increasing nodule number, for example in common bean [20] or alfalfa [64]. Various factors are responsible for poor nodulation in acid mineral soils, high concentrations of protons and of monomeric aluminium [5] and in particular, low calcium concentrations. With regard to legume plants under N<sub>2</sub>-fixing symbiosis, subclover plant chlorosis under Ca deficiency due to impaired N<sub>2</sub> fixation has been described [9]. Calcium deficiency, decreased nitrogen fixation in nodules of *T. subterraneum* [9], *G. Max* [14] and *M. Sativa* [53], also affects attachment of rhizobia to root hairs [72] and nodulation and nodule development [5]. Lastly, a calcium-spiking phenomenon is initiated in root-hair cells of legumes by nodulation actors and rhizobia [81].

Since that report, there have been others on the role of calcium redistribution in the infection process [32,56], including the demonstration that Nod factors can induce such changes even in tissue culture cells [22,80]. There is no doubt that the identification of the Nod factor receptor and the unraveling of the downstream signal transduction pathway will be a major challenge for rhizobiologists in their quest to understand the developmental pathway that culminates in the formation of the root nodule.

### **Sulfur**

Sulfur is an essential element for growth and physiological functioning of plants. The sulfur-containing amino acids cysteine and methionine play a significant role in the structure, conformation, and function of proteins and enzymes in vegetative plant tissue [76].

Although synthetic media for the growth of rhizobia commonly contain S [11,70], until recently little attempt has been made to define S requirements quantitatively [55]. O'Hara et al. [58,59,60] examined the S nutrition of two strains of *B. japonicum* and two strains of *Brady rhizobium* sp. using batch and chemostat cultures. High levels of contaminating S present in media components had to be removed before S limitation occurred in batch culture. Growth of the four Brady rhizobia strains became limited in chemostat culture when the concentrations of S in the inflowing media was less than 20#M. Under S-deficiency cells derepressed an active S-uptake system and the enzyme alkaline sulfatase [38].

**EFFECT OF MICRONUTRIENTS ON NITROGEN FIXATION****Boron**

Boron (B) is one of the eight essential micronutrients, also called trace elements, required for the normal growth of most plants. Boron distribution in nitrogen-fixing pea plants. Yamagishi and Yamamoto [86] reported strong alterations in N<sub>2</sub> fixation in soybean plants with a low B supply. Bolanos et al. [15] made a study of the boron effect on Rhizobium-legume cell-surface interaction and nodule development in pea. In boron-deficient plants, the number of Rhizobia infecting the host cells and the number of infection threads were reduced and the infection threads developed morphological aberrations. The cell walls of root nodules of boron-deficient plants showing structural aberrations are reported to lack the covalently bound hydroxyproline/proline rich proteins [16], which contribute to an O<sub>2</sub> barrier, preventing inactivation of nitrogenase and associated decrease in N<sub>2</sub> fixation.

**Copper**

Apart from its role in respiratory proteins that are required for N<sub>2</sub> fixation in rhizobia [28], copper also plays a role in a protein that is expressed coordinately with the nif genes and may affect the efficacy of bacteroid function. Several rhizobial strains, particularly *R. leguminosarum* bv. phaseoli, make the pigment melanin. The genes for melanin production are on the same large Sym plasmid as the nod and nif genes [46]. The melA gene, specifying the copper-containing enzyme tyrosinase is expressed at high levels in bacteroids, this being under the control of the regulatory *R. leguminosarum* nifA gene [42]. Lastly, there is increasing interest in the phenomenon whereby bacteria enter a state that is 'viable but non-culturable'. There is a recent report that shows that, for reasons that are not clear, adding Cu to *Agrobacterium* or *R. leguminosarum* cells sends them to this state [3]. Cu deficiency decreased nitrogen fixation in subterranean clover [73].

**Zinc**

Zinc is a micronutrient needed in small amounts by crop plants, but its importance in crop production has increased in recent years. Weisany et al. [82] reported that zinc application on plants exposed to salinity stress caused a noticeable enhancement of photosynthesis (*Pn*), water use efficiency, mesophyll efficiency and quantum yield compared with plants exposed to salinity stress alone. Also Weisany et al. [83] reported that lipid peroxidation and hydrogen peroxide concentration under salinity treatments significantly reduced as a result of zinc application.

In addition to the possible role of zinc in the function of the Ros/MucR transcriptional regulators, there is a description of protein engineering by Chauhan and O'Brian [23], which relates to Zn and *B. japonicum*. In this bacterium, the enzyme S-aminolaevulinic acid dehydratase (the product of hemB) normally has Mg<sup>2+</sup> as a cofactor. In contrast, the corresponding enzyme in plants contains Zn<sup>2+</sup>. By site-directed mutagenesis of *B. japonicum* hemB, they substituted the N-terminal amino acids of the *B. japonicum* enzyme, and showed that this caused the engineered protein to bind Zn<sup>2+</sup> and not Mg<sup>2+</sup>. This did not affect symbiotic N<sub>2</sub> fixation, despite the known requirement for a functional hemB for N<sub>2</sub> fixation to occur [23]. Thus, the plant can supply the extra load of Zn<sup>2+</sup> that would be required by this novel inoculant strain.

**Iron**

Iron is required for several key enzymes of the nitrogenase complex as well as for the electron carrier ferredoxin and for some hydrogenases. A particular high iron requirement exists in legumes for the heme component of hemoglobin. Therefore, in legumes iron is required in a greater amount for nodule formation than for host plant growth, for example in lupins [77] and peanut. A reduction in specific rates of nitrogenase activity has been observed in Fe limited peanut nodules [57], indicating a possible direct limitation by Fe deficiency on nodule function. Leghaemoglobin is an oxygen-binding protein. The single most abundant protein that the plant host makes in the nodule is leghaemoglobin, an iron protein. In the bacteria, nitrogenase and nitrogenase reductase contain FeS clusters and the former has the cofactor FeMoCo at the active site for N<sub>2</sub> reduction. Further, bacteroids have a very high respiratory demand, requiring abundant cytochromes and other electron donors, each with their own Fe centers [28]. Although iron deficiency did not significantly affect shoot growth, it severely depressed nodule mass and particularly leghemoglobin content, number of bacteroids and nitrogenase activity, compared with those plants five days after a foliar spray of iron. In contrast to peanut, in lupin (*Lupinus angustifolius*) iron is not retranslocated into the nodules after a foliar spray, and direct iron supply at the infection sites at the roots required for effective nodulation [77]. In laboratory conditions, the lack of Fe has dramatic effects on nodule

development. In lupin and peanut, nodule development is much more susceptible to a shortage of Fe than are other parameters such as plant shoot and root weights [57].

### **Manganese**

Manganese plays a role in the synthesis of polyamines, which play important roles in plant growth and development, also in detoxification of active oxygen species [34]. In one of the earliest steps of the infection process, the binding of rhizobia to young root hairs is enhanced when *R. leguminosarum* is starved of Mn [48]. Whether this is because Mn affects the amounts and the type of rhizobial exopolysaccharide [7] remains to be seen.

### **Molybdenum**

Molybdenum is a micronutrient specifically for plants that form root nodules with nitrogen-fixing bacteria, though plants that do not form nodules also use trace amounts of it in a protein involved with nitrogen metabolism and uptake [84]. Its relevance to N<sub>2</sub> fixation is clear, given that the Mo in 'FeMoCo' cofactor is at the heart of the nitrogen reduction process - at least for most nitrogenases. The Mo-Fe protein contains two atoms of molybdenum and has oxidation-reduction centers of two distinct types: two iron-molybdenum cofactors called FeMoco and four Fe-S (4Fe-4S) centers. The Fe-Mo cofactor (FeMoco) of nitrogenase constitutes the active site of the molybdenum-containing nitrogenase protein in N<sub>2</sub>-fixing organisms [4]. Although at low supply, molybdenum is preferentially transported into the nodules [19], molybdenum deficiency-induced nitrogen deficiency in legumes relying on N<sub>2</sub> fixation is widespread, particularly in acid mineral soils of the humid and subhumid tropics. There are reports that foliar applications of Mo to grain legumes in field conditions increase levels of N<sub>2</sub> fixation and nodule mass, resulting in higher overall N content and seed yield [87,78]. It is also reported that a *B. japonicum* strain deficient in molybdenum transport showed impaired nitrogen fixation activity when inoculated to soybean roots (Delgado et al. 2006). In laboratory conditions, several different legumes that were severely starved of Mo showed more dramatic signs of deficiency [30].

### **Nickel**

Clear evidence that nickel application benefited the growth of nitrogen-fixing species of plant was demonstrated by Bertrand and DeWolf [13], who reported that soil-nickel application to field-grown soybean (*Glycine max* Merr.) resulted in a significant increase in nodule weight and seed yield. In some legumes, small amounts of Ni are essential for root nodule growth and hydrogenase activation. The efficiency of nitrogen fixation immediately depends on hydrogenase activity because the oxidation of hydrogen by the latter provides ATP required for N reduction to ammonia. Also is now well established with the determination that a nickel-dependent hydrogenase is active in many rhizobial bacteria [20]. On the contrary, when soybean plants grown in soil culture were irrigated, once in two weeks, with the nutrient solution containing 1mM NiCl<sub>2</sub>, at day 52 the hydrogenase activity of *Rhizobium japonicum* nodules exceeded that of the control plants by about 45%, although the promoting effect disappeared by day 100, apparently as a result of increasing Ni toxicity [26].

### **Cobalt**

Cobalt is essential for nitrogen-fixing microorganisms, including the cyanobacteria. Cobalt has been shown to be essential for symbiotic nitrogen fixation by legumes [2,31] and non legumes [43,45]. For example, soybeans grown with only atmospheric nitrogen and no mineral nitrogen have rapid nitrogen fixation and growth with 1.0 or 0.1µg Co ml<sup>-1</sup>, but have minimal growth without cobalt additions [2]. Role of cobalt in N<sub>2</sub> fixation is essentially attributed to its role as a cofactor of cobalamine (Vitamin b<sub>6</sub>) which functions as a coenzyme involved in N<sub>2</sub> fixation and nodule growth [31,50,47]. Wilson and Nicholas [85] produced evidence of cobalt requirement for nodule forming legumes and wheat. Cowles et al. [25] showed cobalt to be essential for rhizobial growth. Cobalt is also required as a part of a bacterial enzyme complex. Given that these elements play important structural roles in the proteins, they are critical nutrients for the nitrogen-fixing bacteria. Cobalt deficiency effects nodule development and function at different levels and to different degrees. Observations of B deficient beans and peas showed that under severe deficiency nodulation was totally inhibited [54] while under less severe B deficiency nodule development was affected [18].

## **CONCLUSIONS**

Mineral nutrients perform several functions. They participate in various metabolic processes in the plant such as proteins, nucleic acids, cell walls synthesis, maintenance of osmotic concentration of cell sap, electron transport systems, component of the chlorophyll molecule, enzymatic activity and

act as major constituents of macromolecules, co-enzymes and nitrogen-fixing. This review recognizes the role of some mineral nutrients in biological nitrogen fixation as a nonpolluting and more affordable way to improve soil fertility compared to other ways, such as chemical fertilizer-N and sewage sludge, with their high levels of toxic metals. Biological nitrogen fixation is one of the most important biological processes on this planet, and a continued improvement in the understanding of the role of mineral nutrients in biological nitrogen fixation will be necessary to sustain a food supply to its inhabitants.

Mineral nutrients can influence on nitrogen fixation in leguminous, for example; the presence of mineral nitrogen in the soil inhibits both nodule formation and nitrogenase activity [74]. The deficiency of phosphorous supply and availability remains a severe limitation on nitrogen fixation and symbiotic interactions. Calcium plays a key role in symbiotic interactions at the molecular level. Boron affects Rhizobium-legume cell-surface interaction and nodule development in pea [15]. Copper deficiency decreased nitrogen fixation in subterranean clover [73]. The nitrogen fixing enzyme, nitrogenase is composed of molybdenum and iron and without adequate quantities of these elements, nitrogen fixation can't occur. In some legumes, small amounts of Nickel are essential for root nodule growth and hydrogenase activation. Cobalt is essential for nitrogen-fixing microorganisms, including the cyanobacteria.

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