Short communication. Influence of micronutrients on biological nitrogen fixation in bean (*Phaseolus vulgaris* L.) under greenhouse hydroponic culture conditions

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Abstract

Several microelements (Mo, Cu, Zn, Mn, B) had some degree of influence on nodule dry weight, plant dry weight and nitrogen content of *Phaseolus vulgaris* cv. Canellini inoculated with *Rhizobium tropici* CIAT899 under greenhouse hydroponic culture conditions. Nodule number was not significantly affected. The absence of boron produced the highest degree of inhibition. The absence of microelements affected uptake and assimilation of mineral nitrogen to a lesser extent than biological nitrogen fixation. A similar effect of microelements was observed on *Phaseolus vulgaris* (cvs. Canellini and Mutín)/*Rhizobium etli* ISP23 symbiosis.

**Key words:** *Rhizobium*, leguminous plants, micronutrients, nodulation, nitrogen content.

It has been known for several years that the association *Rhizobium-Phaseolus vulgaris* presents a poor efficiency of biological nitrogen fixation in field conditions (Graham, 1981; Piha and Munns, 1987; Santamaría *et al.*, 1997). Bean crops are very important in some countries such as Brazil and Mexico and in Spain there are three important areas of production: two of dry beans in the Autonomous Communities of Castille Leon and Galicia and another of green beans in Andalusia (MAPA, 2000). We, therefore, considered that it would be very interesting to enhance yields by symbiotic fixation of nitrogen. In this work, the influence of micronutrients on this symbiotic association was studied.

Strains of *Rhizobium* used in this work were *R. tropici* CIAT899 (Graham *et al.*, 1994) and *R. etli* ISP23 (Rodríguez-Navarro *et al.*, 2000). Both were grown in yeast extract–mannitol medium (YEM) (Vincent, 1970) and stored frozen at −20°C in a 20% glycerol solution. Greenhouse experiments were carried out in Leonard jars using a mixture of perlite and vermiculite as support (1:2, v:v). Seeds were surface-sterilized by soaking first in 95% ethanol for 1 min followed by 8 min in 10% sodium hypochlorite. Finally, they were washed successively with sterile distilled water to neutralize them. Initially, three pregerminated seeds were sown in each Leonard jar. One week later they were rinsed to leave two seedlings per container. The plants grew for 45 days at 18-20°C (night/day) with a 14 h photoperiod. The following nitrogen-free nutritive solution was used (g L−1): 0.1 PO₄H₂K₂, 0.1 MgSO₄.7H₂O, 0.1 KCl, 0.06 SO₄.2H₂O, 0.01 FeCl₃.6H₂O, that contained the following oligoelement composition (mg L⁻¹): 1.43 H₃BO₃, 1.04 MnSO₄.H₂O, 0.11 ZnSO₄.7H₂O,
0.04 CuSO₄·5H₂O, 0.06 Na₂MoO₄·2H₂O. To prevent the interference of possible trace oligoelements, the nutritive solution was prepared with distilled water with a low electrical conductivity (EC<4 µS cm⁻¹). Leonard jars and the substrate were also washed with this water. Occasionally, in addition to inoculated treatments, a non-inoculated control treatment and another non-inoculated treatment supplemented with mineral nitrogen (3 applications of 400 mg ammonium nitrate per container) were included. At the end of the experiment, plants were collected and shoot dry weight, nodule number and nodule dry weight were determined. Shoot nitrogen content was estimated by the Kjeldahl method (Vincent, 1970). Statistical analysis of variance (ANOVA) and the SNK comparison of means test (p<0.05) were done using the Statistix programme (NH Analytical Software, USA).

In a first experiment, the effect of the complete microelement solution on *Phaseolus vulgaris* (cv. Canellini)/*Rhizobium tropici* CIAT899 symbiosis was studied in greenhouse conditions. The results showed that in the absence of microelements, biological nitrogen fixation was strongly inhibited (Table 1). Shoot and nodule dry weights and shoot nitrogen content significantly decreased in the inoculated treatment when the nutritive solution did not contain microelements, although the number of nodules did not vary significantly. Although the absence of microelements also had a negative effect on dry weight and on the nitrogen contents of the plant in the treatment not inoculated and fertilized with mineral nitrogen, this effect was less than in the inoculated treatment, suggesting that the assimilation of mineral nitrogen was affected to a lesser extent than biological fixation of nitrogen.

A similar inhibitory effect of nitrogen fixation was observed in greenhouse hydroponic culture conditions with the strain *R. etli* ISP23 and the cultivars Canellini and Mutin (Table 2), suggesting that this inhibitory effect generally appears for other bean cultivars and other *Rhizobium* strains, although with different degrees of sensitivity.

The study of the effect of each microelement separately reveals that all the micronutrients affect, although to a different extent, the nodular mass and growth of bean cv. Canellini plants inoculated with *Rhizobium tropici* CIAT899. The biggest inhibition was caused by the absence of boron (Table 3).

Several works studied the effect of fertilization with micronutrients on vegetative growth, flowering and harvest yields of different legumes (Singh et al., 1992; Yanni, 1992; Singer et al., 1998), although most of them do not focus on the interaction of the microelements with biological fixation of nitrogen.

The data obtained in this work under controlled conditions indicated that boron was the microelement that most affected the bean/*Rhizobium* symbiotic process. This element seems to be involved in the synthesis and stability of the cell wall (Augsten and Eichorn, 1976).

### Table 1. Effect of microelements on *Phaseolus vulgaris* (cv. Canellini)/*Rhizobium tropici* CIAT899 symbiosis under controlled greenhouse conditions

| Treatment                          | Shoot dry weight (g) | Nodule dry weight (mg) | Nodule number | Nitrogen (%) |
|------------------------------------|----------------------|------------------------|--------------|--------------|
| Control (-N) with microelements    | 2.26 c               | 0 c                    | 0 b          | 0.97 c       |
| Control (-N) without microelements | 2.22 c               | 0 c                    | 0 b          | 0.99 c       |
| Control (+N) with microelements    | 8.37 a               | 0 c                    | 0 b          | 2.62 ab      |
| Control (+N) without microelements | 6.71 b               | 0 c                    | 0 b          | 2.55 b       |
| *R. tropici* with microelements    | 8.43 a               | 440 a                  | 1169 a       | 2.91 a       |
| *R. tropici* without microelements | 2.68 c               | 292 b                  | 1205 a       | 0.95 c       |
| LSD                                | 1.14                 | 65                     | 109          | 0.31         |

Data represent mean values of four replicates with two plants each and were estimated 45 days after sowing. Values followed by the same letter show no significant difference (p < 0.05). LSD: minimum significant difference.
and in the structure and function of membranes (Parr and Loughman, 1983). Lukaszewski et al. (1992) demonstrated that the symbiotic process rhizobium-legume requires boron both for indeterminate (Bolaños et al., 1994) and determinate nodule formation (Bonilla et al., 1997), and it has more recently been shown to affect nodulation of Frankia (Redondo-Nieto et al., 2000). It also plays an important role in mediating the surface interaction between bacteria and the plant cell and stabilizing the peribacteriodal membranes and the infection tube (Bolaños et al., 1996).

The results obtained in this work show that the nodules formed when boron is limiting are smaller and weigh less than those of the control treatment. However, we did not observe a significant reduction in the number of nodules formed. Bolaños et al. (1994) and Bonilla et al. (1997) also observed a significant reduction in nodule number but did not present any data on changes in this parameter, only showing those related with the reduced size and weight of nodules. Consequently, we consider that the absence of boron probably does not affect the number of root infection sites but hinders development of the nodules formed.

In spite of the clear results obtained under controlled conditions, in field experiments it was not possible to improve biological nitrogen fixation by foliar application of microelements (unpublished data). Although these results must be analyzed with caution, especially due to the different mobility of the oligoelements in the plant, they seem to suggest that limitation of microelements is not the main cause of poor nodulation and biological fixation presented by the Rhizobium/bean association in our field conditions. To elucidate the possible causes of this limitation, additional studies are required to study more in depth the interaction between boron and other nutrients such as calcium and phosphorus.

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### Table 2. Effect of microelements on the nodulation of *Rhizobium etli* ISP23 in two cultivars of *Phaseolus vulgaris*

| Cultivar | Nutritive solution | Nodule dry weight (mg) | Nodule number | Nitrogen (%) |
|----------|--------------------|------------------------|---------------|--------------|
| Canellini | Complete          | 1421 a                  | 2247 a        | 2.45 a       |
|          | Without microelements | 483 b                  | 2207 a        | 1.62 b       |
|          | LSD                | 724                    | 1075          | 0.81         |
| Mutin    | Complete          | 528 a                  | 878 a         | 2.51 a       |
|          | Without microelements | 274 b                  | 665 a         | 1.58 b       |
|          | LSD                | 191                    | 425           | 0.76         |

Data represent mean values of four replicates with two plants each and were estimated 45 days after sowing. Values followed by the same letter do not differ significantly (p < 0.05). LSD: minimum significant difference.

### Table 3. Effect of each microelement on the bean cv. Canellini/Rhizobium tropici CIAT899 symbiosis under controlled greenhouse conditions

| Nutritive solution | Shot dry weight (g) | Nodule dry weight (mg) | Nodule number |
|--------------------|---------------------|------------------------|---------------|
| Complete           | 11.91 a             | 739 a                  | 1027 a        |
| Cu                 | 7.83 b              | 579 ab                 | 886 a         |
| Mn                 | 6.96 b              | 716 ab                 | 1041 a        |
| Zn                 | 4.19 c              | 534 b                  | 1150 a        |
| Mo                 | 5.08 c              | 618 ab                 | 1230 a        |
| B                  | 2.01 d              | 312 c                  | 1244 a        |
| Without microelements | 1.73 d             | 316 c                  | 1047 a        |
| LSD                | 1.63                | 189                    | 610           |

Data represent mean values of four replicates with two plants each and were estimated 45 days after sowing. Values followed by the same letter do not differ significantly (p < 0.05). LSD: minimum significant difference.
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