Spanish Journal of Agricultural Research (2003) 1 (3), 77-80

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

A. Daza*, C. Santamaría, M. Camacho, D. N. Rodríguez-Navarro and F. Temprano

CIFA «Las Torres-Tomejil». Apdo. Oficial. 41200 Alcalá del Río (Sevilla). Spain

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 Mutin)/*Rhizobium etli* ISP23 symbiosis.

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

Resumen

Influencia de los microelementos en la fijación biológica de nitrógeno en judía *(Phaseolus vulgaris* L.) en condiciones de cultivo hidropónico en invernadero

En condiciones de cultivo hidropónico en invernadero se observó que varios oligoelementos (Mo, Cu, Zn, Mn, B) tuvieron algún grado de influencia sobre el peso seco de los nódulos y el peso seco y contenido en nitrógeno de la parte aérea de *Phaseolus vulgaris* cv. Canellini inoculado con *Rhizobium tropici* CIAT899. El número de nódulos no se vió afectado de una forma significativa. La ausencia de boro provocó la mayor reducción en los parámetros simbióticos. La ausencia de microelementos afectó menos a la absorción de nitrógeno mineral que a la fijación biológica de nitrógeno. Se observó un efecto similar de los microelementos sobre *Phaseolus vulgaris* cvs. Canellini y Mutin inoculados con *Rhizobium etli* ISP23.

Palabras clave: *Rhizobium,* leguminosas, micronutrientes, nodulación, contenido en nitrógeno.

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 –20ºC 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 18-20ºC (night/day) with a 14 h photoperiod. The following nitrogen-free nutritive solution was used (g L^{-1}): 0.1 PO_4HK_2, 0.1 $MgSO_4.7H_2O$, 0.1 KCl, 0.06 $SO_4.2H_2O$, 0.01 $FeCl_3.6H_2O$, that contained the following oligoelement composition (mg L^{-1}): 1.43 H_3BO_3, 1.04 $MnSO_4.H_2O$, 0.11 $ZnSO_4.7H_2O$,

* Corresponding author: antonio.daza.ext@juntadeandalucia.es

Received: 06-02-03; Accepted: 09-05-03.

0.04 $CuSO_4.5H_2O$, 0.06 $Na_2MoO_4.2H_2O$. 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.

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.

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.

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.

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.

Acknowledgements

The authors would like to thank I. Antonio, M. Andra and R. Cabezas for their technical assistance. This work was financed by INIA (Ministry of Science and Technology), Proyect SC 93-070, and by the DGIFA (Department of Agriculture and Fisheries of Andalusia Council), Project PIR CA-9526.

References

AUGSTEN H., EICHORN B., 1976. Biochemistry and physiology of the effect of boron in plants. Biol Rundsch 14, 268-285.

BOLAÑOS L., BREWIN N.J., BONILLA I., 1996. Effect of boron on *Rhizobium*-legume cell-surface interactions and nodule development. Plant Physiol 110, 1249-1256.

BOLAÑOS L., ESTEBAN E., DE LORENZO C., FERNÁNDEZ-PASCUAL M., DE FELIPE M.R., GÁRATE A., BONILLA I., 1994. Essentiality of boron for symbiotic dinitrogen fixation in pea (*Pisum sativum*) *Rhizobium* nodules. Plant Physiol 104, 85-90.

BONILLA I., PÉREZ H., CASSAB G., LARA M., SÁNCHEZ F., 1997. The effect of boron deficiency on development in determinate nodules: changes in cell wall pectin contents and nodule polypeptide expression. In: Boron in soils and plants, Kluwer Acad Publ, The Netherlands, pp. 213-220.

GRAHAM P.H., 1981. Some problems of nodulation and symbiotic nitrogen fixation in *Phaseolus vulgaris* L.: A review. Field Crop Res 4, 93-112.

GRAHAM P.H., DRAEGER K.L., FERREY M.L., CONROY M.J., HAMMER B.E., MARTÍNEZ, E., AARONS, S.R., QUINTO, C., 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium,* and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR 1899. Can J Microbiol 40, 198-207.

LUKASZEWSKI K.M., BLEVINS D.G., RANDALL D.D., 1992. Asparagine and boric acid cause allantoate accumulation in soybean leaves by inhibition of manganese-dependent allantoate amilohydrolase. Plant Physiol 99, 1670-1676.

MAPA, 2000. Anuario de Estadística Agroalimentaria. Ed. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.

PARR A.J., LOUGHMAN B.C., 1983. Boron and membrane function in plants. En: (Robb D.A., Pierpoint W.S., eds.). Metals and micronutrients: Uptake and utilitation by plants. Academic Press, London, UK. pp. 87-107.

PIHA M., MUNNS D.N., 1987. Sensitivity of the common bean (*Phaseolus vulgaris*) symbiosis to high soil temperature. Plant Soil 98, 183-194.

REDONDO-NIETO M., BOLAÑOS L., WALL L.G., BONILLA I., 2000. Effects of boron deficiency on growth, structure and functionality of a *Frankia* strain. In: Nitrogen fixation: from molecules to crop productivity (Pedrosa F.O., Hungria M., Newton W.E., eds.). Kluwer Acad Publ, Dordrecht, The Netherlands, p. 467.

RODRÍGUEZ-NAVARRO D.N., BUENDÍA A.M., CAMACHO M., LUCAS M.M., SANTAMARÍA C., 2000. Characterization of *Rhizobium* spp bean isolates from South-West Spain. Soil Biol Biochem 32, 1601-1613.

SANTAMARÍA C., RODRÍGUEZ D.N., CAMACHO M., DAZA A., LEIDI E.O., 1997. Selección de cepas de *Rhizobium leguminosarum* bv. *phaseoli* efectivas con cultivares comerciales de judía verde. In: Nutrición mineral de las plantas en la agricultura sostenible (Sarmiento R., Leidi E.O., Troncoso, A., eds.). Servicio de Publicaciones, Consejería de Agricultura y Pesca, Junta de Andalucía, Sevilla, Spain, pp. 48-54.

SINGER S.M., SAWAN O.M., ABDEL-MUOTY M.M., SALMAN S.R., 1998. Study of the effects of Delta mixTM and organic matter on growth and productivity of bean plants grown under calcareous soil conditions. Egypt J Hort 25, 335-347.

SINGH A., SINGH B.B., PATEL C.S., 1992. Response of vegetable pea (*Pisum sativum*) to zinc, boron and molybdenum in an acid Alfisol of Meghalaya. Indian J Agron 37, 615-616.

VINCENT J.M., 1970. A manual for the practical study of root-nodule bacteria. IBP Handbook No. 15, Blackwell, Edinburgh, UK.

YANNI Y.G., 1992. Performance of chickpea, lentil and lupin nodulated with indigenous or inoculated rhizobia micropartners under nitrogen, boron, cobalt and molybdenum fertilization schedules. World J Microbiol Biotecnol 8, 607-616.