Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2009 7(3), 665-671
ISSN: 1695-971-X

Role of *Bradyrhizobium japonicum* induced by genistein on soybean stressed by water deficit

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Abstract

The soybean (*Glycine max* (L.) Merr.) is a crop mainly grown under rain fed conditions although irrigation is increasingly being used. Water deficiency is the main factor limiting seed production. The symbiosis process is also negatively affected by water stress. The isoflavone genistein have been recognized as a powerful inducer of Nod factors production by *Bradyrhizobium* and its addition to inocula has been shown to increase nodule number and promote soybean nitrogen (N) fixation at low temperatures. This study looks for answers about the possible role of genistein in countering the stress on nodulation produced by water deficit in soybeans. *Bradyrhizobium japonicum* SEMIA 5079 was grown in culture media induced or not induced with genistein. Inocula were applied to plants growing at different moisture levels. The effect of the treatments on nodulation and N content was evaluated. An improved response to drought stress was observed when the bacteria were grown in presence of genistein as a Nod factors inducer. Nodulation values under moisture stress differed from 8.9 nodules plant^{-1} with genistein at 10 µM to 1.8 nodules plant^{-1} when no inducer was used. Genistein reduced the negative effect on nodulation caused by water deficiency.

Additional key words: humidity, inoculants, nitrogen fixation, nodulation, soil.

Resumen

Papel de *Bradyrhizobium japonicum* inducido por genisteína en soja estresada por déficit hídrico

El cultivo de la soja (*Glycine max* (L.) Merr.) se realiza fundamentalmente bajo condiciones de lluvia, pero la irrigación, específicamente el riego suplementado, es cada vez más utilizado. El proceso de simbiosis también se daña con el estrés hídrico. La isoflavona genisteína ha sido reconocida como un potente inductor de la producción de factores de nodulación por *Bradyrhizobium* y su adición a los inoculantes ha mostrado incrementos en el número de nódulos y la fijación del nitrógeno en soja a bajas temperaturas. Este estudio busca respuestas sobre el posible papel de la genisteína frente al estrés en la nodulación producido por el déficit de agua en plantas de soja. Se cultivó *Bradyrhizobium japonicum* SEMIA 5079 en medios conteniendo genisteína o no como inductor, y se inoculó posteriormente en plantas sometidas a diferentes niveles de humedad. Se evaluó el efecto sobre la nodulación y el contenido de nitrógeno en los diferentes tratamientos. Se obtuvo una mejor respuesta a la sequía cuando las bacterias fueron multiplicadas en presencia de genisteína como inductor de los genes de nodulación. Los valores de nodulación en condiciones de estrés fueron tan diferentes como 8,9 nódulos por planta con el empleo de genisteína 10 µM con respecto a 1,8 nódulos por planta cuando no se utilizó ningún inductor. Se detectó un efecto importante de la genisteína en disminuir la caída de la nodulación por la deficiencia de agua.

Palabras clave adicionales: fijación de nitrógeno, humedad, inoculantes, nodulación, suelo.

Abbreviations used: DAS (days after sowing), LCO (lipochitooligosaccharides), LSD (least significant difference), *nod* (nodulation genes), RWC (relative water content), YMA (yeast mannitol agar).

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Received: 14-11-07. Accepted: 29-06-09.

Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important world crops. It is mainly cultivated for oil and protein and is a major food and feed source. As with other grain legumes, soybean is very sensitive to drought stress which leads to reduced yield and seed quality (Bosniols *et al*., 1986; Frederick *et al*., 2001; Purcell *et al*., 2004). Negative effects of water stress on growth, photosynthesis, and photoassimilate translocation in soybean were demonstrated by Ohashi *et al*. (2000) and Fulai *et al*. (2004).

Because of their ability, as a legume, to interact with rhizobia, soybean forms root nodules where the bacteria fix atmospheric nitrogen (N) to ammonia, the substrate for protein biosynthesis (Gresshoff, 1993; Morgan *et al*., 2005). The rhizobia and the plant cooperate to develop a symbiosis controlled by both partners. However, environmental conditions such as low temperature, high salinity, low pH and drought can negatively affect the rhizobia–legume symbiosis.

Drought stress is a major factor affecting symbiosis and leads to decreased nodule formation, reduced nodule size and N fixation (Serraj *et al.,* 1999; King and Purcell, 2001; Serraj, 2003; Streeter, 2003; Tajima *et al*., 2004). Several mechanisms have been reported to be involved in the physiological response, carbon (C) shortage and nodule C metabolism, oxygen limitation, and feedback regulation by the accumulation of N fixation products, which results in poor nodulation and reduced amounts of fixed N (Zahran, 1999; Kurdalai *et al*., 2002; Serraj, 2003). Modifications in the activity of key nodule enzymes such as sucrose synthase and isocitrate dehydrogenase and in nodular malate content also occur. The decline in nodule water potential results in a cell redox imbalance (Marino *et al*., 2007). Considerable work has been conduced aimed at understanding the physiological mechanisms involved in the soybean-*Bradyrhizobium* symbiosis when subjected to drought stress. Ladrera *et al*. (2007) demonstrated that drought reduced C flux and N accumulation in nodules, but not in shoots.

In the molecular exchange between the plant and the microorganism, legume roots are known to exude flavonoid and isoflavonoid molecules that induce expression of *nod* (nodulation) genes in the rhizobia (Long, 2001). Genistein and daidzein are the major isoflavones in soybean exudates, responsible for *nod* gene induction in *Bradyrhizobium* (Kosslak *et al*., 1990). As a result, the bacteria produce lipo-oligosaccharide Nod factors, whose precise structure determines the host range and specificity of the association and induces several plant responses until complete nodule formation (Vijn *et al*., 1993; Stokkermans and Peters, 1994; Heidstra *et al*., 1997; Spaink, 2000; Geurts *et al*., 2005). The structure and function of the lipo-chitooligosaccharides as essential signal for symbiosis with legumes have been extensively reported (Spaink *et al*., 1998; Broughton *et al*., 2000; Perret *et al*., 2000; Gage, 2004; Jones *et al*., 2007).

The addition of genistein to *Bradyrhizobium* inoculants has been shown to increase nodule number and dry matter (DM) and N fixation in soybean at low temperatures (Zhang and Smith, 1996, 1997). The biological activity of Nod factor, from *B. japonicum,* added to soybean roots under abiotic stress was also evaluated by Duzan *et al*. (2004). They found that addition of high levels of Nod factor was able to overcome the effects of low pH and temperature stress, but not salinity. The impact of a lipochitinoligosaccharide spray application on the physiology and productivity of "water stressed" soybean plants was evaluated by Atti *et al*. (2005).

This work studied the influence of three inoculants of *B. japonicum*, two of them induced with genistein, at different concentrations, on soybean nodulation at different water supply levels.

Material and methods

Bacterial culture

Bradyrhizobium japonicum SEMIA 5079 was obtained from the FEPAGRO culture collection. The strain was selected because it is widely used in commercial *B. japonicum* inoculants in Argentina and Brazil. The culture for inoculation was grown at 28°C in 100 mL of Jap medium (Cozzi and Benintende, 1989) and the determination of cellular concentration in yeast mannitol agar (YMA, Vincent, 1970). The Jap medium was supplemented in two treatments by the addition of 5 and 10 µM genistein respectively, to induce Nod factor synthesis. Flasks were shaken at 150 rpm for 60 hours on an incubator orbital shaker. Bacterial cultures ($1.4\text{-}1.8 \times 10^9$ CFU mL^{-1}) were used to inoculate seeds at rate of 25 mL 100 g^{-1} seed.

Plant growth conditions

Soybean seed cv. DM 4600 RR was inoculated with the three inoculants, one non-induced culture and two induced with genistein. A fourth treatment was non-inoculated seed as a control.

A soil in which soybean had not been grown was used for the assay. Plants were cultivated in a growth chamber with 5000 lux of illumination, temperature of 25-30°C and 60-70% of relative humidity (Montero and Sagardoy, 2005) for 31 days.

Application of water stress

Plants were grown at three soil moisture levels: 30, 60 and 90% of field capacity. During the experiment water was supplied every two days to maintain these moisture levels. The relative water content (RWC) was measured at 12, 20 and 31 days after sowing (DAS), 48 hours after irrigation. The RWC was determined on three plants treatment^{-1}, with three repetitions using the method of Pomper and Breen (1997).

Nodule biomass and plant nitrogen content

Plants were harvested at stage V3 (31 DAS); corresponding to the vegetative stage with at least three fully developed trifoliate leaves (Fehr *et al.*, 1971). The number and DM of nodules on main, secondary and total root, were determined. Shoot total N content was by the Kjeldahl method.

Experimental design and statistical analysis

The experiments were conducted twice. Results from the second assay are shown. The treatments were a completely randomized design with 20 replicates. The InfoStat program (InfoStat, 2003) was used for two-ways analysis of variance. Where a significant treatment effect ($p \leq 0.05$) was observed by ANOVA, a least significant difference (LSD) test was conducted to determine differences among means at $p \leq 0.05$.

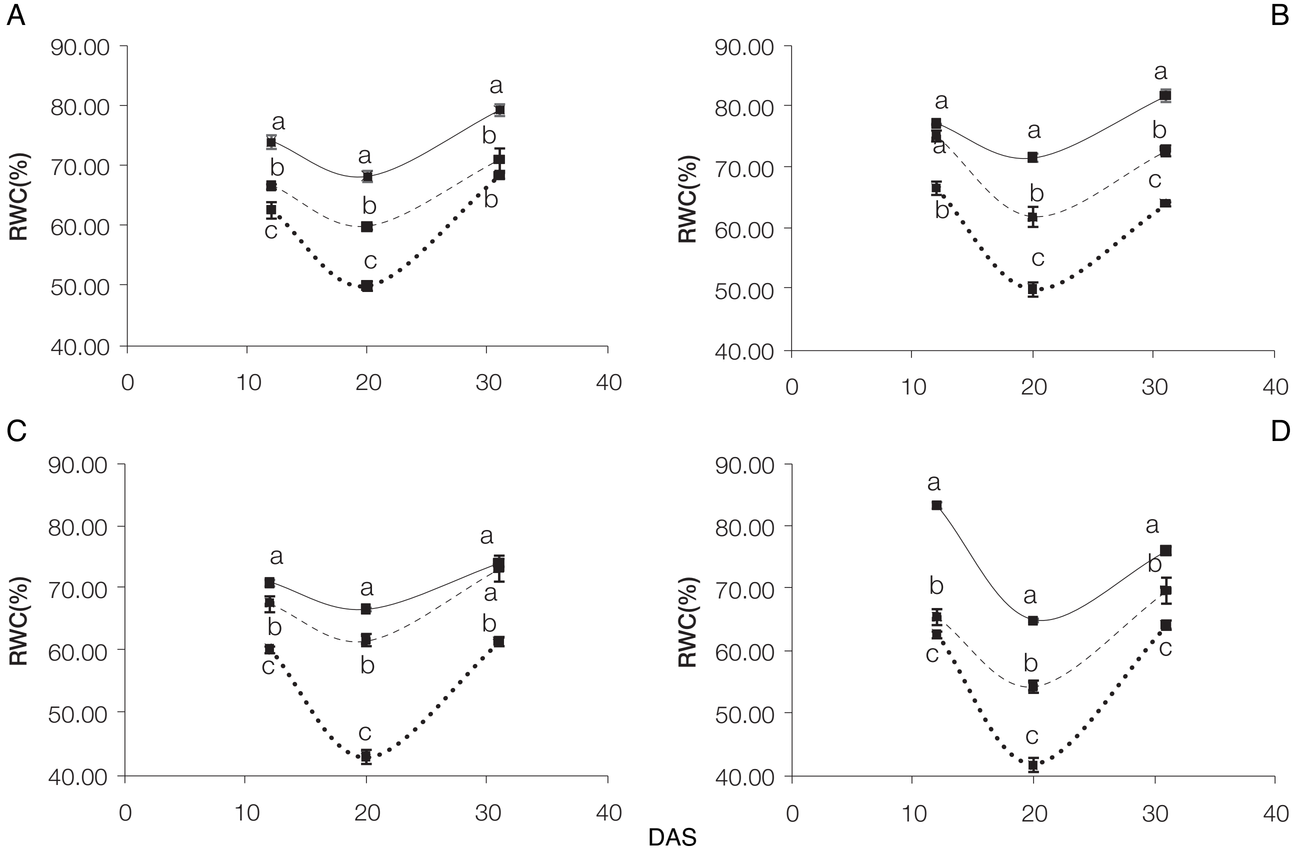

Figure 1. Relative water content (RWC) in inoculated plants (A: medium Jap without induction, B and C: medium Jap supplemented with genistein at 5 and 10 μM, respectively) and (D) non inoculated at three irrigation levels (line in points: 30% field capacity, suspense line: 60% field capacity, continuous line: 90% field capacity) at 12, 20 and 31 days after sowing (DAS). Vertical bars indicate ± standard error (SE). Treatments with different letters are significantly different, ANOVA ($p < 0.05$) $LSD_{0.05}$.

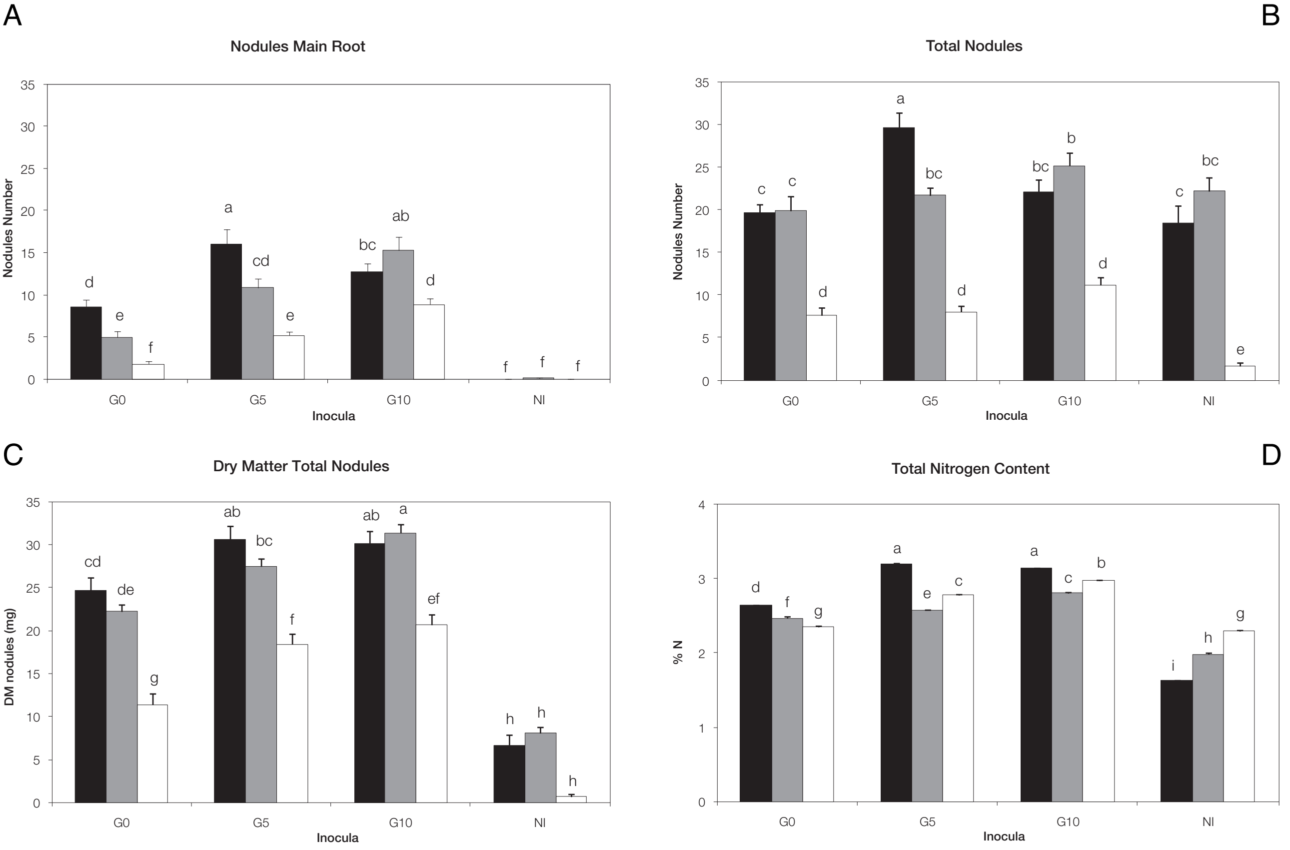

Figure 2. Effect of different inocula on plants grown at three soil moisture levels (90% black, 60% grey, 30% white bars) on nodulation and nitrogen accumulation. G0: uninduced medium, G5 and G10: medium Jap supplemented with genistein at 5 and 10 µM, respectively. NI: un-inoculated control. Treatments with different letters are significantly different, ANOVA ($p < 0.05$) $\mathrm{LSD}_{0.05}$.

Results

Effect of irrigation level on relative water content

Evaluation of the RWC showed the effect of soil moisture level on the plant water relationship (Figure 1). The RWC fell considerably in all treatments after 12 days. After 20 days, plants had values of 40 to 50% RWC at 30% field capacity indicating moisture stress. However, there was some recovery from stress by the end of the assay. The RWC showed significant differences in treatments between each stress level, and over time 48 hours after irrigation.

Effect of irrigation level on nodulation and plant nitrogen content

Generally, genistein increased nodulation and plant N content (Figure 2). The number of nodules on the main root (Figure 2A), total nodule DM (Figure 2C) and N content (Figure 2D) of stressed plants (30 and 60% field capacity) were increased by use of genistein. There was no difference in total nodulation between genistein and uninduced inoculum for 10 µM at 60% field capacity (Figure 2B), but with greater water availability (60 and 90% field capacity), the number and weight of nodules increased considerably in all treatments (Figure 2A, B and C).

The uninduced inoculum produced 8.6 nodules plant^{-1} at 90% field capacity 5.0 at 60% and to 1.8 at the most severe stress level (Figure 2A). Nevertheless, the induced media decreased to 5.2 and 8.9 nodules plant^{-1} in the most stressed conditions with 5 and 10 µM of genistein, respectively. Similar results were observed for nodule DM and N content, which indicates a correlation between the effect on nodulation and its efficiency.

At 90% of field capacity, genistein at 5 µM was enough to obtain the best results, while under stress (30 and 60% of field capacity) more genistein was required (10 μM).

Discussion

Both nodulation and N fixation in soybean are sensitive to drying soil and this can have a negative effect on yield (Serraj and Sinclair, 1998). Nodule number, total plant dry weight and shoot-N pool declined as drought stress increased. Serraj *et al.* (1999) established that drought stress leads to decreased N fixation, mainly as a result ureide accumulation in shoots and asparagine in nodules.

In all treatments lowest RWC values were observed at 20 days (Figure 1). This suggests that the period when nodules were formed was the most critical stress phase.

The results indicate that the flavonoid genistein positively influences nodulation, its efficiency and N fixation.

Although no differences were observed in total nodulation between genistein and the uninduced medium (Figure 2B), the abundant nodulation on lateral roots in response to medium Jap without genistein, the N content and nodule weight demonstrated that they were less effective.

The highest soil moisture level guaranteed better nodulation and a higher efficiency of this process was modulated by the inducer. Williams and De Mallorca (1984) demonstrated that the magnitude of stress effects and the rate of inhibition of symbiosis usually depended on the growth and development phase, as well as stress severity. In their results, mild water stress only reduced nodule number on soybean roots. Moderate and severe water stress reduced both nodule number and size.

An effect on N content in induced treatments at 60% field capacity was rare.

The results showed a positive effect of genistein on nodulation, its efficiency and contribution to plant N nutrition at all soil moisture levels and was specially marked under the adverse conditions of drought stress.

Extensive research has focused on decreasing yield losses during soybean crop production. Atti *et al.* (2005) found that foliar application of lipochitinoligosaccharides on soybean gave a positive effects on growth under moderate stress. Their results agree with ours, considering that they used LCO direct. In our work it was used as a *nod* gene inducer, which lead to synthesis of Nod factors in the inocula.

The effect of water lack on nodulation has been extensively documented (Franson *et al.*, 1991; Sellstedt *et al.*, 1993; Serraj *et al.*, 1999). It is important to produce inoculants which have been obtained from induced media, because they will not only increase nodulation and N fixation, but can also help under adverse conditions of water stress. Other factors may be considered, such as plant growth stage. Peña-Cabriales and Castellanos (1993) found that water stress during vegetative growth was more detrimental to nodulation and N fixation than at the reproduction stage.

In conclusion, after evaluating the effect of genistein as an inducer of *Bradyrhizobium japonicum* inoculants under water stress it was possible to show an important influence of this isoflavonoid on reducing the effect of water stress on nodulation.

Acknowledgements

This work was supported by Rizobacter Argentina S.A. The authors also thank Fernando Incerti for his help in the experiments.

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