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ORIGINAL PAPER

Peat-based inoculum of *Bradyrhizobium japonicum* and *Sinorhizobium fredii* supplemented with xanthan gum

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Abstract The addition of xanthan to high water retention capacity peat (HWRC) inoculants did not show differences on the survival of *Bradyrhizobium japonicum* E109. In low water retention capacity peats (LWRC) however, xanthan increased the survival of *B. japonicum* significantly. Xanthan showed the best effect at 0.1 g/l for *B. japonicum*, in contrast to *Sinorhizobium fredii* USDA205 where the concentrations evaluated (0–1.0 g/l) did not affected significantly its survival. Nevertheless, when the symbiotic performance on soybean was evaluated, the presence of 0.1 g xanthan/l increased the nodule number for both strains.

Keywords Inoculant · *Bradyrhizobium japonicum* · Xanthan · *Sinorhizobium fredii* · Nodulation · Soybean

Introduction

Soybean (*Glycine max*) is one of the most important crops worldwide with respect to both cultivated area and protein content. It can establish a symbiotic association with Gram-negative soil bacteria, which results in fixation of atmospheric nitrogen. This association provides combined nitrogen to the plant, reducing the need of nitrogenous fertilizers and contributing to a sustainable agriculture. In particular, soybean plants can establish symbiotic association with high growth rate bacteria, like *Sinorhizobium* or *Rhizobium* sp. as well as with the slower growing genus, *Bradyrhizobium* sp. (Chen et al. 1988). These microorganisms, have been shown different nutrient requirements for scaled up production of inoculants, and the survival was clearly affected during storage (Balatti and Freire 1996).

Host-plant carbohydrate-binding proteins have been shown to bind specifically to various *Bradyrhizobium* and *Sinorhizobium* cell surface compounds like polysaccharides, exopolysaccharides (EPS), capsular polysaccharides (CPS) and lipopolysaccharides (LPS) (Puvanesarajah et al. 1987). Other acidic EPS could also improve the symbiosis in other bacteria–plant system (Martin et al. 2000). Thereby, certain strains of *Bradyrhizobium* sp. produce a polysaccharide within the root nodules of soybean as well as extracellular polysaccharides, the structure of these polymers has a major ß-linked monosaccharide with a general motif with L-rhamnose-rich backbone and a single branching glucuronosyl residue (An et al. 1995).

The xanthan molecular structure is a backbone of β-1-4-D-glucosyl residues, where every alternate glucose residue has a three-sugar side chain consisting of the L-rhamnose epimer (mannose) with a glucuronic acid residue between them. It forms aqueous homogeneous dispersions exhibiting high viscosity and an unusual insensitivity to salt and heat effects (Lorda et al. 1999). The aim of this work was to evaluate bacterial survival and infection performance of peat-based soybean inoculants supplemented with xanthan gum.

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Materials and methods

Bacterial strains

Bradyrhizobium japonicum E109 and *Sinorhizobium fredii* USDA205 strains were kindly provided by INTA Castelar, Argentina. Stock cultures were maintained at –15°C in 7% (w/v) glycerol and agar slants at 4°C.

Bacterial cell cultures

Inoculum medium for *B. japonicum* E109 contained (g/l): glycerol 5; yeast extract 2; NaCl 0.1; K_2HPO_4 0.5; KH_2PO_4 0.5; $(NH_4)_3PO_4$ 0.5; $MgSO_4{\cdot}7H_2O$ 0.2; KNO_3 0.8; $MnSO_4$ 0.06; $FeCl_3$ 0.06. And for *S. fredii* USDA 205 (g/l): mannitol 5; yeast extract 2; NaCl 0.1; K_2HPO_4 0.5; $MnSO_4$ 0.2 and amaranth flour 4. Agar slants for storage used the same media supplemented with 15 g/l agar.

Optimized fermentation conditions, previously performed in our laboratory, were used to reach high cell density (2×10^{10} c.f.u./ml) of *S. fredii* and *B. japonicum* after culturing for 36 and 72 h, respectively (Videira et al. 2002). Cell inoculant production medium for *B. japonicum* E109 contained (g/l): glycerol 30; yeast extract 4; NaCl 0.1; K_2HPO_4 0.5; KH_2PO_4 0.5; $(NH_4)_3PO_4$ 0.5; $MgSO_4{\cdot}7H_2O$ 0.2; KNO_3 0.8; $MnSO_4$ 0.06; $FeCl_3$; 0.06. And for *S. fredii* USDA 205 (g/l): mannitol 10; yeast extract 4; NaCl 0.1; K_2HPO_4 0.5; $MnSO_4$ 0.2; amaranth flour 4. The pH was adjusted to 6.8 before sterilization (20 min at 120°C). Inoculum cultures (50 ml) were grown in an orbital shaker (250 rev/min) at 28°C for 24 h for *S. fredii* and 48 h for *B. japonicum*. Ten ml were used to inoculated the production media (100 ml) incubating in the same condition until reaching 2×10^{10} c.f.u./ml. Cell counting was performed according to Daniels et al. (1981).

Peat-based inoculant formulation

Peat-based inoculant was prepared using peats with different water retention capacity: 44% v/w low water retention capacity (LWRC) (Brazilian origin) and 56% v/w high water retention capacity (HWRC) from Tierra del Fuego, Argentina. The 200 mesh peat was neutralized by adding 10% w/w $CaCO_3$ and dried at 60 °C until the peat had 10% humidity and sterilized at 120°C for 3 h. It was placed into polypropylene bags (50 g) and sterilized again at 120°C for 30 min. The peat was impregnated with *B. japonicum* and *S. fredii* suspension at initial concentrations of 6×10^6 and 6×10^9 c.f.u./g. Xanthan gum was added to the bacterial suspensions at a concentration of 0.1–1 g/l. The inoculant was stored in the dark at 20–25°C. At regular time intervals samples were processed for measuring moisture and colony-forming units (Daniels et al.1981).

Symbiotic evaluation

Soybean (*Glycine max)* cultivar Donmario 4800 seeds, (Donmario Semillas, www.donmario.com), were kindly provided by Dr. Maria I. Cervellini. Four soybean seeds were planted per pot containing Haplustol entico soil (17.3 g/kg carbon, 0.9 g/kg nitrogen, 11.2 mg/kg phosphorus, 0.83 mS/cm conductivity and pH 6.8). After germination, only two seeds per pot were left and, each pot was inoculated with 10 ml of slurry containing 1% w/v peat based inoculum and 10% w/v sucrose. Plants were kept for 45 days in the greenhouse under temperature of 15–25°C, until maturity. They were watered with Sandman solution (CIAT Manual). Symbiotic performance was evaluated by the following parameters: plant dry weight, nodule size and number and nitrogen content quantified by Kjeldahl method using Tecator 1030 analyzer. Five pots for every condition were processed and the data were statistically analysed by means of MANOVA (Statistic 4.5 software).

Results and discussion

Survival of *B. japonicum* E109 on peats with high and LWRC

The high rainfall and high temperatures are perhaps the most important features distinguishing tropical peat areas from those of temperate regions. The nature of the organic soils is different, because in the tropics, trees are frequently involved, as opposed to sedges and sphagnum moss in temperate regions (Andriesse 1988). The differences found between the water retention capacities of peats, could be related to the different nature of the organic matter between bog peats from Ushuaia, in comparison to the woody peats from Brazil.

The influence of 0.1 g/l w/v xanthan on peat with different water retention capacity, was evaluated for two concentrations of *B. japonicum* E109 (10^6 and 10^9 c.f.u./g) (Fig. 1). It shown, that no remarkable differences were found in the survival of *B. japonicum* and humidity content by the presence of the biopolymer, independently of the initial cell concentration, although the moisture, during the incubation, for both peats was significantly different, ranging 10–26% for

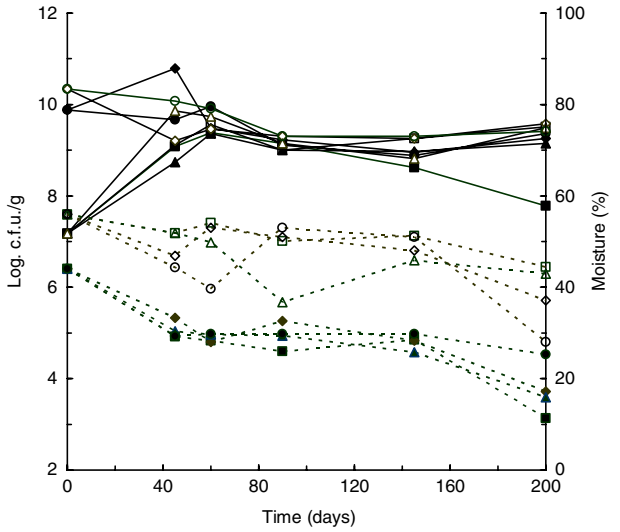

Fig. 1 Survival of *B. japonicum* E109 in peat-based inoculants containing 0.1 g/l xanthan gum. Two different water retention capacity peats were impregnate with *B. japonicum* cells containing xanthan: ■ 10^6 c.f.u./ml on peat LWRC, ♦ 10^9 c.f.u./ml on peat LWRC, ▲ 10^6 c.f.u./ml on peat LWRC + 0.1 g/l of xanthan gum, ● 10^9 c.f.u./ml on peat LWRC + 0.1 g/l of xanthan gum, □ 10^6 c.f.u./ml on peat HWRC, ◇ 10^9 c.f.u./ml on peat HWRC, Δ 10^6 c.f.u./ml on peat HWRC + 0.1 g/l of xanthan gum, ○ 10^9 c.f.u./ml on peat HWRC + 0.1 g/l of xanthan gum. (—) c.f.u./g and (- - -) moisture%

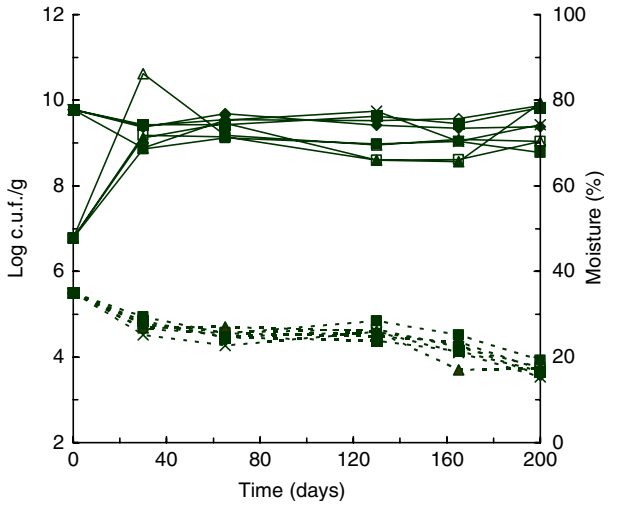

Fig. 2 Xanthan gum concentration effect on the survival of *B. japonicum* E109 in peat LWRC-based inoculants: ■ 10^6 c.f.u./ml control, ♦ 10^9 c.f.u./ml control, ▲ 10^6 c.f.u./ml + 0.1 g/l xanthan, ● 10^9 c.f.u./ml + 0.1 g/l xanthan, □ 10^6 c.f.u./ml + 0.5 g/l xanthan, ◇ 10^9 c.f.u./ml + 0.5 g/l xanthan, Δ 10^6 c.f.u./ml + 1 g/l xanthan, * 10^9 c.f.u./ml + 1 g/l xanthan. (—) c.f.u./g and (- - -) moisture%

LWRC and 28–44% for HWRC peat, after 6 months incubation.

Notwithstanding this, a lower viable cell value was found after 6 months with LWRC peat in the absence of xanthan. When HWRC peat (56% w/w) was used, no significant differences on bacteria survival were found in presence of xanthan. This suggests that HWRC peat itself gave the conditions for survival of inoculant cells during 6 months and, most probably the major water retention capacity masked the effect of xanthan on the improving viability of bacterial cells (Fig. 1).

Effect of xanthan concentration on *B. japonicum* E109 and *S. fredii* USDA205 survival

Usually, microorganisms produce EPS in the stationary phase, which increases the tolerance to stressing conditions (Lorda et al. 1999). The survival of *B. japonicum* at different xanthan concentrations (0.1–1.0 g/l) using LWRC peat, showed a slight increase of c.f.u./g at 0.1 g/l, while humidity content remain unchanged among the samples during the 6 months incubation (Fig. 2).

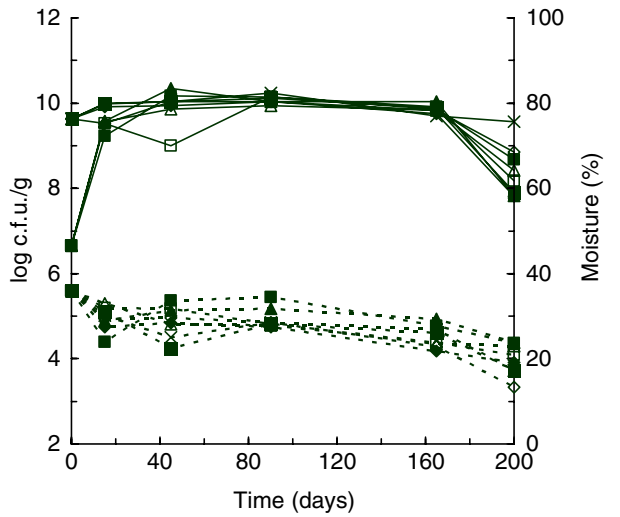

Fig. 3 Effect of xanthan gum concentration on the survival of *S. fredii* USDA 205 in peat LWRC-based inoculants: ■ 10^6 c.f.u./ml control, ♦ 10^9 c.f.u./ml control, ▲ 10^6 c.f.u./ml + 0.1 g/l xanthan, ● 10^9 c.f.u./ml + 0.1 g/l xanthan, □ 10^6 c.f.u./ml + 0.5 g/l xanthan, ◇ 10^9 c.f.u./ml + 0.5 g/l xanthan, Δ 10^6 c.f.u./ml + 1 g/l xanthan, * 10^9 c.f.u./ml + 1 g/l xanthan. (—) c.f.u./g and (- - -) moisture%

On the other hand, in contrast with *B. japonicum*, the c.f.u./g of *S. fredii* USDA205 on peat LWRC remain constant after 6 months incubation (Fig. 3).

Although both strains began to grow at lower initial cell concentration (10^6 c.f.u./ml), this increased in the first 15 days up to 10^9 c.f.u./ml, independently of the presence of xanthan gum (Figs. 1–3).

Symbiotic performance of xanthan-supplemented peat inoculants

Six-month-old peat LWRC inoculants of *B. japonicum* E109 and *S. fredii* USDA205 supplemented with different xanthan concentrations, were assayed in greenhouse to evaluate the symbiotic capability. *B. japonicum* E109 did not show significant differences for most of the variables studied, giving the follow average values: 3.09 g aerial part of the plant dry weight; 1.21 g root dry weight and 2.67% w/w nitrogen. The size of nodules was homogeneous and they also showed the typical reddish colour feature of the pigment leghaemoglobin. But regarding the number of nodules per plant, significant differences were found (Fig. 4). Higher amount of nodules were observed for 0.1–0.5 g/l xanthan concentrations. This suggests that the presence of xanthan at low concentration (0.1–0.5 g/l) improves the infection capability of *B. japonicum*, due probably to a lower stress of the cells after 6-month incubation at room temperature.

On the other hand, *S. fredii* USDA205 was not able to infect the soybean plants for the samples without xanthan, although for the different concentrations of xanthan assayed not significant differences were exhibited on total nitrogen percentage, dry weight in the aerial part as well as the root. The average values for each variable were: 1.78% w/w, 2.49 and 3.25 g, respectively. However, as also with *B. japonicum*, a significant increment in the number of nodules was detected for *S. fredii* with 0.1 g xanthan/l (Fig. 4). All the nodules exhibited a red colour; they were 3–10 mm in length, a great proportion being of 6 mm length.

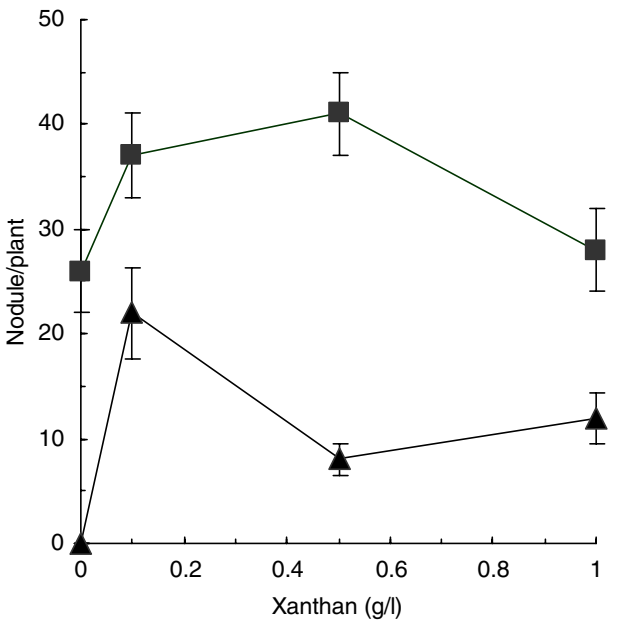

Fig. 4 Effect of xanthan concentration on the infection nodules number per soybean plant. Plants were incubated for 45 days in the greenhouse: ■ *B. japonicum* E109, ▲*S. fredii* USDA 205

Concluding remarks

The use of polymeric additives for improving the performance of inoculants still a seeking area of research (Diouf et al. 2003). Thereby, the develop of a more stable inoculant preparation with the addition of 0.1 g/l xanthan to the cell suspension, improves survival of *B. japonicum* and the number of nodules produced for both strains, and for *S. fredii* the symbiotic performance was clearly improved by the presence of the polymer. These results in combination with optimized fermentation conditions, reported previously by our laboratory, dealing with shortening the fermentation times, yield improvement and generating physiological and infective high cell densities (Videira et al. 2002) could facilitate the scaling up for the production of high quality inoculants.

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References

An J, Carlson RW, Glushka J, Streeter J (1995) The structure of a novel polysaccharide produced by *Bradyrhizobium* species within soybean nodules. Carbohydr Res 269: 303–317

Andriesse JP (1988) Nature and management of tropical peat soils. FAO, Rome Italy, ISBN 92-5-102657-2

Balatti A, Freire J (1996) Legume inoculants. Selection characterization of strains. Production, use and management. Kingraf. La Plata. Argentina. ISBN-987-96116-08

Chen WX, Yan GH, Li JL (1988) Numerical taxonomic study of fast-growing soybean rhizobia and proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int J Syst Bacteriol 38:392–397

Claus D, Berkeley RCW (1984) Bergeýs Manual of Systematic Bacteriology. In: Noel R. Krieg (ed) vol 2, , pp 1104–1208. Baltimore: Williams & Wilkins. ISBN 0-683-0408-8

Daniels L, Hanson R, Phillips J (1981) Manual of methods for General and Molecular Bacteriology. In: Gerhardt P (ed) pp 514–554. American Society for Microbiology. Washington DC. ISBN 1555810489

Diouf D, Forestier S, Neyra M, Lesueur D (2003) Optimisation of inoculation of *Leucaena leucocephala* and *Acacia mangium* with rhizobium under greenhouse conditions. Ann For Sci 60:379–384

Dowdle SF, Bohlool BB (1985) Predominance of fast growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. App Environ Microbiol 50:1171–1176

Fred EB, Baldwin FL, McCoy E (1932) Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science, Vol. 5, Madison

Lorda G, Pastor M, Balatti A (1999) Stabilization and survival of *Rhizobium meliloti* in relation to exopolysaccharide and biomass content. In: International Congress on Nitrogen Fixation. 12th (1999: Paraná, Brazil): Nitrogen fixation : from molecules to crop productivity. In: Pedrosa F, Hungria M, Yates M, Newton W (eds) pp 616, Kluwer Academic, Dordrecht ISBN 0792362330

Martin M, Lloret J, Sanchez Contreras M, Bonilla I, Rivilla R (2000) MucR is necessary for galactoglucan production in Sinorhizobium meliloti EFB1. Mol Plant Microbe Interact 13:129–135

Moorhouse R, Walkingshaw M, Arnott S (1977) Xanthan gum: molecular conformations and interactions. Am Chem Soc Symp 45:90–102

Puvanesarajah V, Schell F, Gerhold D, Stacey G (1987) Cell surface polysaccharides from *Bradyrhizobium japonicum* and a non-nodulating mutant. J Bacteriol 169:137–141

Videira L, Pastor MD, Lorda G, Iriarte L, Balatti P (2002) *Sinorhizobium fredii* cultured in media supplemented with *Amaranthus cruentus* L. seed meal and bacterial cell survival in liquid and peat based inoculum. World J Microbiol Biotechnol 18:193–199