

***Sinorhizobium fredii* cultured in media supplemented with *Amaranthus cruentus* L. seed meal and bacterial cell survival in liquid and peat based inoculum**

L.B. Videira¹, M.D. Pastor², G. Lorda², L. Iriarte³ and P.A. Balatti^{1,*}

¹Instituto de Fisiología Vegetal, Area de Microbiología Agrícola, Departamento de Biología y Ecología, Facultad de Ciencias Agrarias y Forestales Universidad Nacional de La Plata CC31 La Plata 1900, Argentina

²Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Uruguay 151, Santa Rosa 6300, Argentina, E-mail: exanpam@cpsarg.com

³Chacra Integrada BurrowINTA, Ministerio de Asuntos Agrarios de la Provincia de Buenos Aires, Argentina

*Author for correspondence: Tel./Fax: +54-221-4233698, E-mail: pbalatti@ceres.agro.unlp.edu.ar

Received 15 May 2001; accepted 4 January 2002

Keywords: Inoculant, nitrogen fixation, nodulation, *Sinorhizobium*, soybean

Summary

Nine different growth media were evaluated to determine the best growth conditions to achieve cultures of a high cell number of fast-growing rhizobia to produce inoculants. We found that *Sinorhizobium fredii* strains have complex nutritional requirements that were fulfilled by adding to the media 4 g *Amaranthus cruentus* L. seed meal/l. The survival of fast growing strains is a variable trait, but those strains that survived at high levels even after 6-month storage, hypernodulated soybeans and fixed atmospheric nitrogen at levels as high as those of *Bradyrhizobium japonicum*.

Introduction

Based on the area of production, the amount of protein that it contains and the extent that it is used in the diet of many people in several countries, soybean is one of the most important cultivated plants in the world. Like other legumes, soybean can establish a symbiotic association with Gram-negative soil bacteria known as *Sinorhizobium*, *Bradyrhizobium* or *Rhizobium* spp., that results in the fixation of atmospheric nitrogen. These associations provide combined nitrogen to the plant, thus reducing the use of nitrogenous fertilizers and contributing to a sustainable agriculture.

Bradyrhizobium japonicum, *B. elkanii*, *B. liaoningense*, *Sinorhizobium fredii* and *Rhizobium* sp. nodulate and fix nitrogen efficiently on soybean (Fred *et al.* 1932; Kuykendall *et al.* 1992; Gao & Yang 1995; Xu *et al.* 1995). *Bradyrhizobium japonicum*, a slow growing organism that alkalifies culture media, has been considered the only symbiont of soybean for many years (Fred *et al.* 1932). Furthermore, *B. japonicum* strains isolated from different places have been the main and probably only source of nitrogen-fixing bacteria that were used as the main genetic resource to produce soybean inoculants. Fast-growing rhizobia *S. fredii* (Scholla & Elkan 1984; Chen *et al.* 1988;) and *Rhizobium* sp. were also found to nodulate and fix atmospheric nitrogen in association with soybean (Keyser *et al.* 1982; Dowdle & Bohool 1985; Gao & Yang 1995).

Manjanatha *et al.* (1992), Buendía-Clavería *et al.* (1994) and Videira *et al.* (2000) found that *S. fredii* soybean interactions were as efficient as those of *B. japonicum* in fixing nitrogen. Furthermore, it was also found that *S. fredii* strains tend to form a higher number of nodules on soybean (Buendía-Clavería *et al.* 1994; Videira *et al.* 2001), although this did not necessarily result in a higher nitrogen fixation activity.

The best way to establish a new strain of rhizobia amongst a resident population is to apply a heavy dose of an effective and persistent inoculum (Brockwell & Bottomley 1995). Even the most promiscuous legume growing in a soil hosting a large population of suitable rhizobia will always respond to inoculation provided the rate of application is sufficiently high (Streeter 1994). However rhizobia isolates have different growth requirements when the purpose of cell culture is to produce high density cell cultures of 1×10^{10} for inoculant production. One inexpensive way to provide additional nutrients to the media at the industrial level may be by supplementing the media with extracts or seed meals rich either in specific nutrients or cofactors. Therefore, different culture media should be tested for their ability to sustain active cell multiplication so that highly concentrated cell cultures can be grown to produce inoculants. Survival of rhizobia on the inoculant is another very important characteristic of inoculants, which is evaluated by plate count of viable

rhizobia, being this an accurate index of the potential inoculant success (Olsen *et al.* 1994; Brockwell & Bottomley 1995).

Fast-growing rhizobia *S. fredii* and slow growing *B. japonicum* may have different nutritional requirements and growth responses. *Sinorhizobium fredii* uses a greater assortment of carbohydrates than *B. japonicum*, which may be related, at least in part, to the high levels of β -galactosidase found in *S. fredii* (Sadovsky *et al.* 1983; Stowers & Eaglesham 1984).

The purpose of this paper was to establish the nutritional requirements of fast-growing rhizobia in order to obtain highly concentrated cell cultures to formulate inoculants and also to test the ability of *Amaranthus* seed meal to provide additional nutrients for the culture of *S. fredii*. In addition, we formulated liquid- and peat-based inocula and tested the survival of fast-growing rhizobia and their nitrogen-fixing ability.

Materials and methods

Bacterial strains

Sinorhizobium fredii strains were kindly provided by Dr Steven G. Pueppke (University of Missouri, Columbia, USA) and Dr José Ruíz Sainz (Universidad de Sevilla, Sevilla, Spain). Stock cultures were maintained at -70°C in 7% glycerol. Cultures were initiated from agar slants kept at 4°C . Bacteria were grown in the desired culture media in an orbital shaker at 150 rev min^{-1} . The compositions of the growth media used are presented in Table 1.

Bacterial cell cultures

Culture starters consisted of 50-ml cultures of rhizobia grown in those media shown in Table 1. The cultures

were grown in 250-ml Erlenmeyer flasks, stirred in an orbital shaker at 250 rev min^{-1} until the number of cells reached $1 \times 10^9\text{ cells ml}^{-1}$. Cultures were inoculated by adding two loops from agar slant cultures kept at 5°C . Cultures of microorganisms were studied in 500-ml Erlenmeyer flasks filled with 100 ml of the desired media to keep a 5:1 ratio of air to liquid volume, so that oxygen was provided at non-limiting rates. In all cases the inoculum consisted in the addition of 10% of the flask volume, of a cell culture with 10^8 viable rhizobia cells ml^{-1} . Purity of the cultures was determined at the beginning and at the end of each growth culture by Gram staining and also by plating a dilution in YEM (Vincent 1970).

Amaranthus cruentus L. seed meal was added as a provider of additional nutrients when required as described in the media presented in Table 1. *Amaranthus cruentus* L. is a native crop plant of America whose seed meal is rich in minerals, amino acids and cofactors (Table 2).

Peat-Based Inoculant formulation

Peat-based inoculum was prepared on peat from Tierra del Fuego (an island in the southern part of Argentina). The 200 mesh peat was neutralized by adding 10% CaCO_3 and dried at 60°C until the peat had 12% humidity. After sterilization in the autoclave for 3 h the peat was placed on pans to allow vapour diffusion and then it was placed in polypropylene bags that again were sterilized at 120°C for 30 min. *Rhizobium* cell suspensions were added to the bags in a 1:1 relation. The inoculants were stored in the dark at $20\text{--}25^{\circ}\text{C}$ and at regular time intervals a sample was taken and a serial dilution was plated to count the number of colony-forming units (c.f.u.) to assess cell survival. Cell biomass was estimated by cell dry weight and optical density unit (U.D.O.) at 625 nm.

Table 1. Compositions of the growth media that were used to multiply *S. fredii*.

Components (g/l)	Media no.								
	1	2	3	4	5	6	7	8	9
Mannitol	10.00			10.00	10.00			10.00	
Glucose	—	10.00	—	—	—	10.00	—	—	
Yeast Extract	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
NaCl	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mg $\text{SO}_4 \cdot 7\text{H}_2\text{O}$	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
K_2HPO_4	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
KH_2PO_4	—	—	0.50	0.50	—	—	0.50	0.50	0.50
KNO_3	—	—	0.80	0.80	—	—	0.80	0.80	0.80
FeCl_3	—	—	0.06	0.06	—	—	0.06	0.06	
MnCl_2	—	—	0.06	0.06	—	—	0.06	0.06	
Amaranth flour	—	—	—	—	4.00	4.00	4.00	0.40	
$(\text{NH}_4)_2\text{HPO}_4$									0.30
Glycerol									10.00

Media 1–8 were used to grow *S. fredii* strains and media no. 9 was used to grow *B. japonicum* strain E109. The media were sterilized in an autoclave at 120°C for 20 min. The pH of the media was adjusted to 6.8 before sterilization.

Table 2. Amino acid, vitamin and mineral content of *A. cruentus* L.

Amino acids	
Cysteine	2.0–3.8
Isoleucine	3.4–3.7
Leucine	4.8–5.9
Lysine	4.8–5.8
Methionine	1.8–1.6
Phenylalanine	3.4–4.5
Threonine	3.2–4.2
Tryptophan	0.4
Tyrosine	2.4–4.0
Valine	3.9–4.3
Vitamin	
Thymine	0.07
Riboflavin	0.19
Niacin	1.00–1.45
Biotin	42.5
Folic Acid	43.80
Ascorbic Acid	4.50–4.90
Mineral	
Phosphorus	556.00
Potassium	525.00
Calcium	242.00
Magnesium	344.00
Sodium	25.00
Iron	26.00
Copper	1.69
Manganese	3.40
Zinc	4.20

Amino acid content is expressed in g of Aa/100 g of protein. Vitamin content is expressed as mg/100 g of dry weight and mineral nutrient content is expressed as mg/100 g of dry weight (Bressani 1990; Pastor 1999).

Liquid inoculants

Liquid inoculants were prepared by stabilizing the high cell number culture media by the addition of 8.5 g NaCl/l; 0.3 g KH₂PO₄/l; 0.6 g K₂HPO₄/l and 0.1 g meat peptone/l.

Symbiotic evaluation

Soybean [*Glycine max* L. (Merr)] seeds, cultivar RR4100, were kindly provided by Ing. Rossi (Nidera Semillas Company S.A., Venado Tuerto, Argentina). This is a glyphosphate-resistant transgenic soybean that is being commercialized in Argentina by Nidera S.A. One hundred grams of seeds were inoculated with 1 g of peat-based inoculum or 2 ml liquid inoculum. In both cases, a solution of 10% sucrose was added as adhesive. Control uninoculated plants were treated with 10% sucrose alone. Three seeds were planted per pot in 5-l pots filled with soil, and after germination only one seedling per pot was left. Plants were kept in the greenhouse under a temperature of 15–25 °C, until maturity.

Nitrogenase activity was assayed by the acetylene reduction assay as described before (Chatterjee *et al.* 1990). The number of plants of each treatment screened for nitrogenase activity was six and the experiment was performed twice.

The data were statistically analysed by means of ANOVA test and least significant difference was established at the 5% level.

Results

Growth kinetics of *S. fredii* strains in different liquid media

The analysis of the rhizobia growth kinetics on different media allowed us to group *S. fredii* strains according to their growth rate or generation time and nutritional demands. Strains USDA191 and SMH12 grew fairly well on most of the tested media, which was shown both by the high cell number achieved in only 36 h of growth on all the media tested and by the generation time, which was only 2.47 h for USDA191 and SMH12 (Table 3). The most obvious difference between the strains was their preference for the carbon source since SMH12 and USDA191 grew better in glucose and mannitol, respectively.

The other group of *S. fredii* strains included sibling strains HH102 and HH103. They did not grow on four of the eight media tested that coincidentally were those lacking amaranth seed meal (Table 3). Furthermore, the number of cells per ml of media on three of the four cultures tested was well below 10⁶ cells g⁻¹ or ml⁻¹. The generation times of strains HH102 and HH103 were longer than those of the other strains, though still much shorter than for *B. japonicum*, suggesting that the growth potential of these strains is lower than that of USDA191 or SMH12. Both strains HH102 and HH103, grew better on glucose than on mannitol as the sole carbon source and were completely dependent on the addition of amaranth seed meal.

Survival of *S. fredii* as peat-based or liquid-based inoculants

We tested the ability of fast-growing rhizobia to survive on liquid- or peat-based inoculants by counting the number of viable colonies that arose after plating serial dilutions of the inoculants. In Figure 1A and B the log numbers of colonies recovered from each one of the tested strains are presented. We introduced as an internal control *B. japonicum* E109 inoculants, which is the strain that is currently being used in commercial inoculants in Argentina. The responses of the strains were very clear. Two Chinese strains closely related, HH102 and HH103, showed a low survival rate either on peat- or liquid-based inoculants. On the other hand, strains USDA191 and SMH12 showed a normal survival rate either on peat or liquid inoculants. Bacterial cells were recovered at numbers as high as 7 × 10⁷ after a storage period of 180 days.

Table 3. Growth kinetics of *S. fredii* as affected by the composition of the growth media.

Time (h)	Strain	Media	Dry weight (g/l)	pH	No of live cells/ml	μ (1/h)	T_g (h/g)
48	HH102 ^a	5	5.80	6.29	7.0×10^8	0.18	3.85
		6	4.00	5.69	9.5×10^5	0.15	4.62
		7	3.00	7.05	4.4×10^4	0.14	4.95
		8	3.20	5.87	7.3×10^4	0.14	4.95
48	HH103 ^a	5	6.00	6.29	3.0×10^{10}	0.17	4.07
		6	4.80	5.65	7.6×10^4	0.13	5.17
		7	3.00	7.02	5.5×10^4	0.13	5.17
		8	3.20	5.15	1.2×10^4	0.14	4.95
36	SMH12	1	4.34	5.84	6.2×10^9	0.22	3.15
		2	5.20	5.70	2.6×10^{10}	0.22	3.15
		3	6.14	7.06	2.9×10^{10}	0.26	2.56
		4	5.34	7.26	2.4×10^{10}	0.24	2.88
		5	8.00	6.90	2.5×10^{10}	0.26	2.66
		6	8.00	6.88	2.0×10^{10}	0.26	2.56
		7	9.10	7.72	3.8×10^{10}	0.27	2.47
		8	6.42	7.97	2.4×10^{10}	0.26	2.66
36	USDA191	1	4.38	6.60	3.4×10^9	0.18	3.85
		2	1.18	5.07	1.0×10^6	0.18	3.85
		3	4.14	7.68	5.0×10^9	0.18	3.85
		4	1.64	5.27	2.0×10^7	0.17	4.07
		5	4.65	6.37	1.6×10^{10}	0.28	2.47
		6	4.42	5.54	2.4×10^9	0.23	3.01
		7	4.56	7.05	3.9×10^9	0.23	3.01
		8	4.54	6.55	4.6×10^9	0.23	3.01

μ is the specific growth rate calculated as the variation in biomass at different time intervals; T_g is the generation time, which was measured as hours per generation. pH was measured at the end of the growing period. No of live cells was calculated by plating a dilution series of the culture on YEM plates. Dry weight was calculated by drying the centrifuged cells from the culture.

^a Strains HH102 and HH103 did not grow in media 1–4.

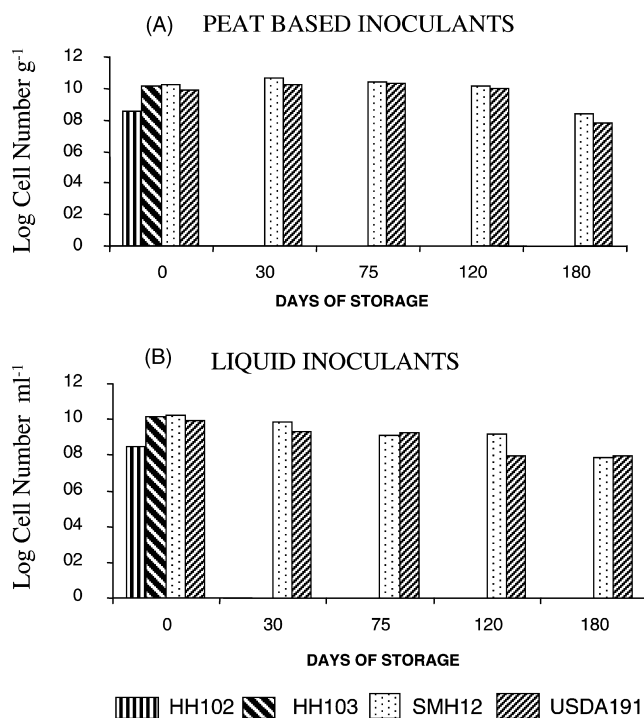


Figure 1. Survival of *S. fredii* strains HH103, USDA191 and SMH12 as commercial inoculants. (A) Peat-based inoculants; (B) Liquid-based inoculants. Serial dilutions of a 1 g suspension of inoculum diluted in 100 ml of sterile distilled water. The dilutions were plated on YEM plates and c.f.u. were counted after 96 h of incubation at 30 °C. Inoculants were stored at 20 ± 5 °C room temperature.

Symbiotic performance of liquid-based and peat-based inoculants on soybean

Soybean seeds were inoculated with liquid- or peat-based inoculants made up of strain HH103 or SMH12 or *B. japonicum* E109. The plants were grown in the greenhouse under controlled conditions and at maturity stage R4, acetylene reduction and dry weight of the fruit, shoot and whole plant were determined. Nodulation and nodule dry weight were also assessed. The results obtained with liquid- and peat-based inocula were identical, therefore we present the results obtained with peat-based inoculum only (Tables 4 and 5). Plants inoculated with fast-growing rhizobia yielded similar amounts of whole plant and fruit dry weight to those inoculated with *B. japonicum* E109. These results represent the nitrogen fixation ability of the interaction along soybean growth until they reach maturity stage R4. In contrast, the acetylene reduction assay though controversial, suggests the actual state of nodule nitrogen fixation (Table 4). The analysis of the acetylene assay indicates a higher level of fixation for fast growing inoculated plants. This is surprising since the whole plant dry weight was not statistically significant. *Sinorhizobium fredii* strains hypernodulated soybean (Tables 4 and 5). The number of nodules induced by the *S. fredii* strains was well above the nodule number induced by *B. japonicum*.

Table 4. Nodulation and nitrogen fixation of soybean plants inoculated with *S. fredii* or *B. japonicum* strains.

	<i>S. fredii</i> HH103	<i>S. fredii</i> SMH12	<i>B. japonicum</i> E109
Plant dry weight (g)	5.70 a	6.80 a	6.50 a
Fruit dry weight (g)	2.10 a	2.20 a	2.09 a
Nodule dry weight (g)	0.560 a	0.444 a	0.262 b
Nodule number	83.00 b	171.8 a	39.8 c

Plants were inoculated with peat-based inoculants and harvested at maturity stage R4. The ANOVA test was performed and differences were established by the LSD at the 5% probability level. E109 is a derivative from *B. japonicum* strain USDA138. The number of replicates per treatment was 15 pots with one plant per pot. Values followed by the same letter indicate no significant difference.

Table 5. Nitrogen fixation activity as measured by the acetylene reduction assay.

	Ethylene ($\mu\text{m/plant}^{-1}$)	Nodule number	Nodule dry weight (mg)
<i>S. fredii</i>			
HH103	21.9 b	26.3 b	607.5 mg b
SMH12	71.2 a	102.6 a	824.0 mg a
<i>B. japonicum</i>			
E109	5.2 c	18.2 b	421.8 mg c

The data are means of six replicates per experiment. Plants grown in the greenhouse were harvested at maturity stage R4. Roots were incubated in 250-ml air-tight flasks in the presence of 10% acetylene for a period of 15 min.

Discussion

Sinorhizobium fredii strains have been isolated from different places in Asia and it has been demonstrated that though they share several characteristics, there is considerable diversity within the species (Heron & Pueppke 1984; Krishnan & Pueppke 1994; Rodriguez-Navarro *et al.* 1996; Saldaña & Balatti 2000). Because of this, we performed the experiments with four representatives isolated from three different places. Strain USDA191 and HH102 and HH103 isolated from the soils of China were described by Keyser *et al.* (1982) and Dowdle & Bohlool (1985), respectively. Strain SMH12 isolated from the soils of Vietnam was also included and has been described by Cleyet-Marel (1987).

The generation time found for strain USDA191 was shorter than previously reported by Keyser *et al.* (1982) and slightly longer for SMH12 than the 2 h reported by Cleyet-Marel (1987). These differences most probably were due to different culture conditions like media composition, aeration rate, inoculum size, etc. It should also be mentioned that in our experiments the pH values of the media after bacterial growth were higher. The stress exerted by the changes in the pH of the media might have well stopped bacterial growth earlier, in the experiments performed by Keyser *et al.* (1982). The generation time found for strain SMH12, under the conditions of growth of our experiments, was slightly

longer than the generation time of 2 h reported by Cleyet-Marel (1987). This is surprising since this 2 h generation time was reported when the bacterial cells were fed with mannitol as the sole carbon source, while we found that this strain grew better on glucose. Obviously, other conditions of growth might have been responsible for the differences found.

The fact that SMH12 and USDA191 strains achieved high cell numbers in most of the media suggests that they did not have specific additional requirements for growth. Patelli *et al.* (1994) and Ronchi *et al.* (1997) described a similar cell behaviour while working with isolates of a closely related organism to *S. fredii* like *S. meliloti*, the symbiont of alfalfa (de La Judie *et al.* 1994).

SMH12 and USDA191 grew better in media provided with glucose or mannitol respectively, as the carbon source. This suggests that the strains should have differences at the metabolic level. Although both strains grew to fairly high cell numbers, growth of both strains was improved by adding *Amaranthus* seed meal to the media. This suggests that additional growth factors that may improve microorganism growth can be supplemented to the media by adding seed meals. Ronchi *et al.* (1997) also found that *B. japonicum* and *S. meliloti* reached higher cell density numbers when *Amaranthus* seed meal was added to the culture media and also this resulted in a better survival rate in stored inoculants.

The generation time of strains HH102 and HH103 was longer than that of the other strains USDA191 and SMH12, though still much shorter than for *B. japonicum*, suggesting that there are some differences between these strains. Dowdle & Bohlool (1985) found a similar generation time and final pH when they grew strain HH103. Both strains HH102 and HH103 grew better on glucose than on mannitol as the sole carbon source and were completely dependent on the addition of *Amaranthus* seed meal. These similar requirements of carbon source confirmed the close relationship of the strains and the response to *Amaranthus* seed meal suggests that they require additional growth factors that are not provided by yeast extract. To our knowledge these results and those from Ronchi *et al.* are the first reports to suggest the use of *Amaranthus* seed meal to provide additional nutrients required by rhizobial growth at an industrial level.

De O. Chueire & Hungria (1997) studied both compatibility and competitive ability of fast-growing rhizobia against Brazilian genotypes and slow-growing rhizobia. They concluded that fast growers were poor competitors and also that the rate of growth was unrelated to competition. However, the authors did not study the survival ability of the fast-growing rhizobia in the inoculants or the seed surface. The low survival ability of HH102 and HH103 found in our experiments suggests that these cells might be fragile and the environmental conditions encountered by fast-growing rhizobia both on peat or liquid media were lethal. In our experiments both peat and liquid inoculants were

amended to prevent bacterial cell damage. Peat was neutralized to prevent the effect of extreme pH and in liquid inoculants cells were stabilized to prevent their osmotic damage. On the other hand USDA191 and SMH12 showed survival rates similar to the one observed under similar conditions for *S. meliloti* (Ronchi *et al.* 1997). It appears that survival is a highly variable trait of fast-growing rhizobia. Therefore it may be possible that the competitive deficiencies observed by De O. Chueire & Hungria (1997) must have been the result of inoculating fragile cells or cells unable to survive when introduced into soils of new cultivated areas.

Those plants inoculated either with *S. fredii* or with *B. japonicum* yielded similar amounts of whole plant and fruit dry weight. This is surprising since the number of nodules was different and the acetylene reduction analysis suggested that those plants inoculated with fast-growing organisms fixed nitrogen at a higher level. It has already been reported that fast-growing rhizobia nodulate soybean more profusely than *B. japonicum* (Buendía-Clavería *et al.* 1994; Videira *et al.* 2001) although nitrogen fixation remained at similar levels. One possible explanation might be that acetylene reduction activity of *B. japonicum* and *S. fredii* strains peaked at different moments of plant development probably due to differences in the development of nitrogen-fixing nodules. We are currently testing this hypothesis.

We conclude that high cell number cultures of *S. fredii* can be achieved on media with mannitol as a carbon source, provided that growth factors are supplemented by means of adding *Amaranthus* seed meal to the media. *Sinorhizobium fredii* strains did not show the same survival ability either on peat- or liquid-based inoculants and the composition of the broth culture did not alter the strains' ability to survive and nodulate soybeans.

Acknowledgements

Pedro Balatti was supported by CICBA and the Universidad Nacional de La Plata. This work was supported by a grant from FONCYT SCyT de la Nación and Consejo Nacional de Investigaciones Científicas y Técnicas y CICBA.

References

- Balatti, A.P. & Jardim, J.R. Freire. 1996 *Legume inoculants. Selection and Characterization of Strains. Production, Use and Management*. Kingraf, La Plata Argentina 148 pp.
- Bressani, R. 1990 Grain Amaranth. Its chemical composition and nutritive value. *Proceedings of the fourth National Amaranth Symposium: Perspectives on Production, Processing and Marketing*. Minnesota, pp. 23–25.
- Brockwell, J. & Bottomley, P.J. 1995 Recent advances in inoculant technology and prospects for the future. *Soil Biology and Biochemistry* **27**, 683–697.
- Buendía-Clavería, A.M., Rodríguez-Navarro, D.N., Santamaría-Linaza, C., Ruiz Sainz, J.E. & Temprano Vera, F. 1994 Evaluation of the symbiotic properties of *Rhizobium fredii* in European soils. *Systematic and Applied Microbiology* **17**, 155–160.
- Cleyet-Marel, J.C. 1987 Thesis: *Dynamique des populations de Rhizobium et de Bradyrhizobium dans le sol et la rhizosphere*. Université Claude Bernard, Lyon, France
- Chatterjee, A., Balatti, P.A., Gibbons, W. & Pueppke, S.G. 1990 Interaction of *Rhizobium fredii* USDA257 and nodulation mutants derived from it with the agronomically improved soybean cultivar McCall. *Planta* **180**, 303–311.
- Chen, W.X., Yan, G.H. & Li, J.L. 1988 Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *International Journal of Systematic Bacteriology* **38**, 392–397.
- de Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M.D., Dreyfus, B., Kersters, K. & Gillis, M. 1994 Polyphasic taxonomy of Rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov. *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *International Journal of Systematic Bacteriology* **44**, 715–733.
- De O. Chueire, L.M. & Hungria, M.A. 1997 N₂-fixation ability of Brazilian soybean cultivars with *Sinorhizobium fredii* and *Sinorhizobium xinjiangensis*. *Plant and Soil* **196**, 1–5.
- Dowdle, S.F. & Bohlool, B.B. 1985 Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the Peoples Republic of China. *Applied and Environmental Microbiology* **50**, 1171–1176.
- Fred, E.B., Baldwin, F.L. & McCoy, E. 1932 *Root Nodule Bacteria and Leguminous Plants*. University of Wisconsin Studies in Science, vol. 5, Madison.
- Gao, W.M. & Yang, S.S. 1995 A *Rhizobium* strain that nodulates and fixes nitrogen in association with alfalfa and soybean plants. *Microbiology* **141**, 1957–1962.
- Heron, D.S. & Pueppke, S.G. 1984 Mode of infection, nodulation specificity and indigenous plasmids of 11 fast-growing strains of *Rhizobium japonicum*. *Journal of Bacteriology* **160**, 1061–1066.
- Keyser, H.H., Bohlool, B.B., Hu, T.S. & Weber, D.F. 1982 Fast-growing rhizobia isolated from rot nodules of soybean. *Science* **215**, 1631–1632.
- Krishnan, H.B. & Pueppke, S.G. 1994 Host range, RFLP and antigenic relationships between *Rhizobium fredii* strains and *Rhizobium* sp. NGR234. *Plant and Soil* **161**, 21–29.
- Kuykendall, L.D., Saxena, D.B., Devine, T.E. & Udell, S.E. 1992 Genetic diversity in *Bradyrhizobium japonicum*. Jordan 1982 and a proposal for *B. elkanii* sp. nov. *Canadian Journal of Microbiology* **38**, 501–505.
- Manjanatha, M.G., Loynachan, T.E. & Atherly, A.G. 1992 Efficiency, competitiveness and persistency of Chinese *Rhizobium fredii* in Iowa soils. *Agronomy Journal* **84**, 676–681.
- Olsen, P.E., Rice, W.A., Bordeleau, L.M. & Biederbeck, V.O. 1994 Analysis and regulation of legume inoculants in Canada: the need for an increase in standards. *Plant and Soil* **161**, 127–134.
- Pastor, M.D. 1999 Thesis. *Utilización del amaranto en procesos fermentativos como factor de crecimiento*. Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Pcia de Lapampa, Argentina.
- Patelli, G.C., Gomez, H.E. & Balatti, A.P. 1994 Cultivos de *Rhizobium meliloti* y *Bradyrhizobium* en medios con levaduras de panificación. *Biotam* **6**, 17–22.
- Rodríguez-Navarro Dulce, N., Ruiz-Sainz, J.E., Buendía-Clavería, A.M., Santamaría, C., Balatti, P.A., Krishnan, H.B. & Pueppke, S.G. 1996 Characterization of fast-growing rhizobia from nodulated soybean (*Glycine max* L. Merr) in Vietnam. *Systematic and Applied Microbiology* **19**, 240–248.
- Ronchi, A.L. Grassano, A. & Balatti, A.P. 1997 Perlite as a carrier for legume inoculants. *Agrochimica* **61**, 186–195.
- Sadowsky, M.J., Keyser, H.H. & Bohlool, B.B. 1983 Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. *International Journal of Systematic Bacteriology* **33**, 716–722.
- Saldaña, G. & Balatti, P.A. 2000 Genetic diversity of fast growing rhizobia isolated from different geographical areas. In *Nitrogen Fixation: From Molecules to Crop Productivity*, eds. Pedrosa, F.O., Hungria, M., Yates, G. & Newton, W.E. *Proceedings of the 12th*

- International Congress on Nitrogen Fixation Foz do Iguazu, Paraná, Brazil. September 1999*, 614 pp, Dordrecht, Kluwer ISBN 0-79236233-0.
- Scholla, M.H. & Elkan, G.H. 1984 *Rhizobium fredii* sp. nov. a fast growing species that effectively nodulates soybeans. *International Journal of Systematic Bacteriology* **34**, 484–486.
- Stowers, M.D. & Eaglesham, A.R.J. 1984 Physiological and symbiotic characteristics of fast-growing *Rhizobium japonicum*. *Plant and Soil* **77**, 3–14.
- Streeter, J.G. 1994 Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Canadian Journal of Microbiology* **40**, 513–522.
- Videira, L.B., Pastor, M.D., Lorda, G., Pastorino, G., Martinez-Alcántara, V. & Balatti, P.A. 2000 Nitrogen Fixation, competitiveness and survival on liquid and peat based inoculant of fast growing strains *Sinorhizobium fredii*. In *Nitrogen Fixation: From Molecules to Crop Productivity*, eds. Pedrosa, F.O., Hungria, M., Yates, G. & Newton, W.E. *Proceedings of the 12th International Congress on Nitrogen Fixation Foz do Iguazu, Paraná, Brazil. September 1999*. 615 pp, Dordret, Kluwer ISBN 0-79236233-0.
- Videira, L.B., Pastorino, G.N. & Balatti, P.A. 2001 Incompatibility may not be the rule in the *Sinorhizobium fredii*-soybean interaction. *Soil Biology and Biochemistry* **33**, 837–840.
- Vincent, J.M. 1970 *A Manual for the Practical Study of Root Nodule Bacteria*. Blackwell Scientific Publications, Oxford. ISBN 0-63206410-2.
- Xu, L.M., Ge, C., Cui, Z., Li, J. & Fan, H. 1995. *Bradyrhizobium liaoningense* sp. nov. isolated from the root nodules of soybean. *International Journal of Systematic Bacteriology* **45**, 706–711.