Evaluation of sol-gel silica matrices as inoculant carriers for *Mesorhizobium* spp. cells

G. S. Alvarez[1], F. L. Pieckenstain[2], M. F. Desimone[1*], M. J. Estrella[2], O. A. Ruiz[2] and **L. E. Díaz[1]**

[1] IQUIMEFA-CONICET. Facultad de Farmacia y Bioquímica. Universidad de Buenos Aires. Junín 956 Piso 3º (1113) Ciudad de Buenos Aires. Argentina. *Email: desimone@ffyb.uba.ar

[2] Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECh), Universidad Nacional de General San Martín - Consejo Nacional de Investigaciones Científicas y Técnicas (UNSAM-CONICET), Chascomús, Buenos Aires, Argentina.

The aim of this work was to evaluate the potential of sol-gel silica matrices as alternative supports for long-term storage of rhizobia at room temperature. For this purpose *Mesorhizobium* spp. cells, which develop nitrogen-fixing nodules in narrow leaf birdsfoot trefoil (*Lotus tenuis*) roots, were used as the experimental system. Sodium silicate was used for the immobilization of rhizobia. The effect of additives such as glycerol and TY medium over immobilized bacteria viability were also evaluated. Sol-gel immobilized bacteria were found to retain their viability, metabolic activity and nodulation capability after 360 days stored at room temperature. The number of viable cells in soil inoculated with immobilized bacteria was higher than that inoculated with a bacteria suspension. Moreover, silica matrices have the ability to protect entrapped bacteria against acid conditions. The immobilization of rhizobia in sol-gel silica matrices was explored for the first time in this work and has been proven to be an efficient technological tool for the protection and preservation of cells for long periods of time at room temperature and at various pHs. Moreover, this technology can be extended for the delivery of rhizobia or other beneficial bacteria to soils.

Keywords: immobilization; inoculant; *Lotus tenuis*; *Mesorhizobium* spp.; sol-gel.

1. Introduction

The formation of symbiosis between legumes and a group of soil bacteria collectively known as rhizobia, involves the exchange of specific chemicals signals that lead to the development of plant root nodules and their invasion by the nitrogen-fixing bacteria [1]. Inoculation of legume seed is an efficient and convenient way of introducing effective rhizobia to soil and subsequently to the rhizosphere of legumes. The efficacy of inoculation varies depending on several factors, all of which affect the number of viable rhizobia available for infection of legume roots. As there are several native species of rhizobia present in the soil, introduced strains must be present in extremely large numbers in order to compete with native rhizobia and have agricultural significance [2]. Alternatively, higher numbers of viable rhizobia per seed may be achieved by improving survival during seed inoculation. Thus, one of the main problems of the inoculation industry is how to keep rhizobial cells viable in large numbers in the inoculant. According to Herridge *et al.*, [3] an ideal inoculant should have the following characteristics: high water holding capacity, non-toxic to the rhizobia, easy to sterilize, readily available and inexpensive, sufficiently adhesive for efficient application to seed, high pH buffer capacity, cation and anion- exchange capacities.

The most common method for inoculation was for many years to apply a peat-based sterile inoculant with high numbers of rhizobia (e. g. 1 x 10^9 CFU per gram). Peat was the preferred inoculant for farmers because of its advantageous characteristics such as high moisture-holding capacity, the ability to foster rhizobia multiplication in the peat, and to protect rhizobia once they are applied to the seed coat. However, the problems associated to this solid carrier, including inaccessible deposits of true peat in certain areas, high costs of sterilization and difficulties in large-scale field application, soon led to the development of alternative carrier materials [4].

Nowadays, a more diverse range of inoculant products with different modes of application are available. Liquid formulations which use various broth cultures enriched with additives such as glycerol, polyvinylpirrolidone or arabic gum are nowadays extensively used. These additives can improve inoculant quality, provide better adhesion to the seed, stabilize the product, inactivate soluble seed coat toxins and enhance rhizobial survival during storage and after exposure to environmental conditions. Liquid formulations are easily applied to seeds because they pass through the planting machinery, have shown good field performance, are easily attainable by small producers and use low cost materials [5]. However, their expiration date is lower than peat inoculants, especially when they are stored without refrigeration.

Other inoculant products are granular materials and freeze-dried rhizobia [6]. While the former have short expiration dates and low yields, freeze-dried rhizobia, have shown to be able to maintain a high number of viable cells (e. g. 1 x 10^{12} CFU per gram) for as long as 24 months [7]. However, these are more expensive products and the lyophilization procedure can cause serious problems, such as the denaturation of sensitive proteins and cell death for some organisms. Several types of additives, such as sugars and other carbohydrates, have been used to try to alleviate the damage caused by freeze drying [8].

Soils are heterogeneous and unpredictable environments where the inoculated bacteria find it often difficult to establish a niche for survival among the competitors and predators. Thus, a progressive decline in the inoculated bacterial density occurs. Immobilization of microbial cells into polymer matrices has proved to be advantageous over direct soil inoculation [9]. The main goals of encapsulation of plant growth promoting bacteria are to protect them from harsh soil environment, reduce microbial competition and release them gradually to facilitate colonization of plant roots [10, 11]. Encapsulation of rhizobia in Ca-alginate beds has been proposed as a procedure to enhance heat and desiccation resistance of entrapped bacteria [12]. In this sense, microbial cells have been shown to remain physiologically active after being entrapped in the interstitial spaces of such gels [13]. This gel-like matrix allows the cells to remain viable and maintain their catalytic activity for longer time periods [14].

Other polymers have also been used as inoculant carriers such as polyacrylamide (PAM), xanthan, carob gum, ethylcellulose and methacrylic acid. According to Deaker *et al.*, [15], PAM entrapped rhizobia survived better than peat and liquid cultures after storage at 30º C.

Silica matrices, obtained through the sol-gel process, are highly porous hydrophilic polymers that provide chemical and thermal stability and, in some cases, mechanical strength. In contrast to organic polymeric matrices, silica matrices are inert and more resistant to microbial attack and contamination. Furthermore, silicate is a common and abundant component of most soil types which makes it suitable as an immobilization support.

Medicinal, biological and analytical applications of sol-gel chemistry were extensively reviewed [16, 17]. The encapsulation of bacteria in silica gels is rather recent [18]. Since then, many efforts have been made to take advantage of the biological activities of living cells to design functional materials [19]. Immobilized bacteria were preserved for long periods at high cell numbers and also conserve their enzymatic activity and their ability to produce recombinant proteins [20, 21]. Even though the sol-gel encapsulation of microorganisms has gained considerable interest during the past decade, this technology has not been applied to rhizobia so far.

The aim of this work was to evaluate the potential of silica gels as an alternative support for long-term storage of rhizobia for its possible application as a novel inoculant formulation. For this purpose, *Mesorhizobium* spp. cells, which develop nitrogen-fixing nodules in narrow leaf birdsfoot trefoil (*Lotus tenuis*) roots, were used as the experimental system.

2. Materials and methods

2.1 Chemicals

Sodium silicate was purchased from Riedel de Haën (Germany). TY media components and agar used for bacterial plate counts and cultures were obtained from Difco (Kansas, MO). Glycerol (99%) was purchased from Aldrich (Milwaukee, WI). All other reagents were of analytical grade and were used as received without further purification.

2.2 Bacterial strains and culture conditions

All the experiments were performed with *Mesorhizobium* spp. strain BA151, originally isolated from root nodules of *Lotus tenuis* plants growing in soils of the Salado River Basin. Bacteria were grown in TY medium (5 g Bacto Tryptone, 3 g Yeast Extract and 0.5 g $CaCl_2.2H_2O$ per litre) on a rotary shaker operating at 150 rpm and 28 ºC [22, 23]. The number of colony-forming units (CFU) per millilitre of this suspension was determined by the plate count technique.

2.3 Bacteria immobilization in silica matrices

Sodium silicate (1 g) was mixed with 6 ml water and heated at 80ºC. The sol obtained was allowed to reach room temperature and acidified to pH 6.5 with 0.75 M citric acid. Aliquots (0.15 ml) of bacterial cultures were diluted with an equal volume of pH 7.0 phosphate buffer. Afterwards, this bacterial suspension was mixed with an equal volume of the silicate suspension obtained in the previous step. The final mixture (pH 6.5) was left at room temperature for 5 minutes until gelation. Alternatively, sol-gel derived matrices with no additives, matrices in which glycerol was added to a final concentration of 2% previous to gelation, matrices with 1 ml liquid TY medium and matrices containing both additives (glycerol and TY medium) were obtained to evaluate their influence in bacteria viability preservation. Immobilized bacteria were then stored at 20ºC in hermetically closed tubes. In order to determine the number of viable bacteria in the sol-gel silica matrices, two gels of each formulation were washed three times with 1 ml 0.9% ClNa, and then disrupted in 10 ml of 0.9 % ClNa. Finally, serial dilutions were plated in solid TY medium by duplicate. Colony-forming units were counted after 72 h incubation at 28 ºC. The statistical analysis was performed by Bonferroni Multiple comparison post test if ANOVA $p<0.05$.

2.4 Silicate gel buffer holding capacity

The buffer capacity of matrices was evaluated by adding 0.1 N hydrochloric acid or sodium hydroxide solutions. After 30 minutes, the pH was measured with a pH electrode and gel buffer capacity was calculated as the number of acid or basic equivalents necessary to modify the pH in one unit per gram of wet silica gel. In parallel, immobilized bacteria were exposed to TY medium adjusted to different pHs (3.0 to 7.0) and their viability was analyzed after 24hs. In order to determine the number of viable bacteria in the sol-gel silica matrices, two gels of each formulation were washed three times with 1 ml 0.9% ClNa, neutralized and then disrupted in 10 ml of 0.9 % ClNa. Finally, serial dilutions were plated in solid TY medium by duplicate. The same experiment was performed with free bacteria exposed to pH from 3.0 to 7.0.

2.5. Bacteria release from silica carriers

The release of bacteria from silicate matrices was determined by incorporating each gel containing immobilized bacteria to a 10-ml flask, washing them with sterile 0.9% ClNa and then adding 10 ml 0.9% ClNa. They were gently shaken at 28ºC for one hour and the number of bacteria released to the saline solution was calculated by the plate count method on TY agar. After that period, silicate gels were washed again and the procedure was repeated with fresh saline solution for 5, 16, 24, 48 and 72 hs.

2.6. Survival of immobilized rhizobia in sterilized soil

Experiments were carried out using 20-ml flasks containing 2 g of sterile soil (water content 50 %, organic matter 7.5%, pH 5.0). Silica gels with 2 % glycerol and 0.15 ml of bacterial suspensions with 2% glycerol, both containing 7.5 x 10^9 cells were added to each flask and incubated at room temperature under natural sunlight. The number of viable cells was periodically determined in duplicate after suspending the whole tube content in 10 ml of 0.9% NaCl. The statistical analysis was performed by the unpaired t test.

2.7. Lysine decarboxylase activity

Lysine decarboxylase activity of immobilized rhizobia was determined *in vivo* by using radiolabeled lysine as a substrate. Gels (0.5 ml each) were suspended in 50 mM potassium phosphate buffer pH 6.8 to a final volume of 1 ml. Aliquots (0.5 ml) of the suspension were dispensed in glass tubes (14 x 880 mm) fitted with a rubber stopper and a filter paper disc soaked in 200 µl of 2M potassium hydroxide. Reaction was initiated by adding 0.1 µCi L-[1-^{14}C]-lysine and tubes were incubated at 28 ºC in a rotary shaker for 1h. Reaction was stopped and [^{14}C]CO_2 released by adding 200 µl of 5% perchloric acid. After allowing 2 h at 37 ºC for distillation of [^{14}C]CO_2, the radioactivity on the paper was determined using 200 µl scintillation fluid as described above. Reaction tubes to which perchloric acid was added simultaneously with the substrate were used as blanks.

2.8 Infectivity test

Root-nodule formation was analyzed in *L. tenuis* seedlings inoculated with suspensions of rhizobia obtained by mechanical disruption of the silica- immobilized bacteria, or with undisrupted gels containing bacteria. Seeds of *L. tenuis* cv Pampa INTA were scarified and surface disinfected with concentrated sulfuric acid during 15 min. After acid treatment, the seeds were washed with water until neutral pH. Seeds were distributed on the surface of 1% water-agar plates and incubated for 24-48 h at 25 ºC for germination. After germination, seedlings were transferred to square Petri dishes (10 seedlings each) containing Rigaud and Puppo agar [24]. Seedlings were inoculated by adding rhizobial suspensions obtained after gel disruption and suspension in liquid TY medium or undisrupted gels, which contain approximately the same number of bacteria (10^9 cells/ml). Non inoculated seedlings were used as negative controls and rhizobial cultures at the exponential growth phase (10^9 cells per seedling) were used as a positive control. Plants were cultivated in a growth chamber under a 16-8 h light / dark cycle and 20-24 ºC temperature. In order to evaluate infectivity, the number of nodules per plant was determined 45 days after inoculation.

3. Results

3.1 Survival of immobilized *Mesorhizobium* spp. cells in silicate matrices

The long-term survival of *Mesorhizobium* spp. in silica matrices, during storage at room temperature was analyzed. The effect of additives such as glycerol and liquid TY medium over immobilized bacteria viability were also evaluated. As shown in figure 1, at the onset of the experiment, 2.5 x 10^7 CFU/ml were present in each tube containing 260 mg of wet silica matrix. During the first 80 days, an increase in bacterial number was observed in all cases and there were no significant differences between the formulations under study.

The number of viable cells remained constant thereafter, except for control gels that contained no additives, in which case a significant decrease in the number of viable cells was evident 180 days after the onset of the experiment. Bacterial survival 180 days post-immobilization, was 1 log unit higher in those gels to which both glycerol and TY medium were added than in control gels. At this time the number of viable bacteria reached 1.9 x 10^9 CFU/ml in glycerol-TY medium containing gels (**Figure 1**) and this value remained constant for an additional 180-day period.
Comparing viability preservation for 180 days, no significant differences with control gels were found when glycerol was added, this is in concordance with our previous report for *E. coli* immobilization [25]. However, there were significant differences with control matrix when TY was added ($p<0.05$) and when both TY and glycerol were added ($p<0.01$). Glycerol and TY medium addition to the gels also resulted in enhanced survival when other *Mesorhizobium* strains, different to the one used in this experiment, were immobilized (data not shown). For this reason these gel matrices were selected to perform the following studies.

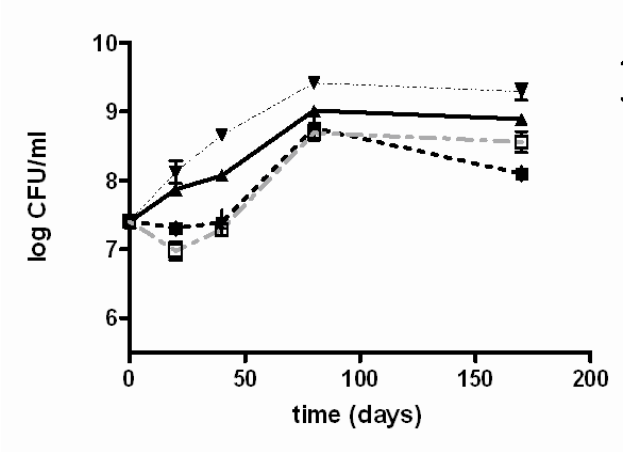

Fig. 1 Bacteria immobilization in silica matrices. Viability of immobilized *Mesorhizobium* cells stored at 20ºC in sealed tubes for 180 days with the addition of: glycerol (-□-), TY broth (-▲-), glycerol and Ty broth (-▼-) or without additives (-■-).

3.2 Sol-gel silica matrices buffer holding capacity

Following the methodology described in the Materials and Methods section, the buffer holding capacity of sol-gel matrices was evaluated and found to be 1.4 x 10^{-4} acid or basic equivalents per gram of wet gel. In order to evaluate the protective effect of silica gels over entrapped rhizobia exposed to different pHs, the survival of entrapped bacteria exposed to pHs ranging between 3.0 and 7.0, was evaluated. As it can be seen in **Figure 2**, the number of entrapped viable cells after exposure to acid pHs, did not differ in a significant manner with the number of viable cells exposed to neutral pH. On the other hand, the number of free bacteria exposed to low pHs (below pH 5.0) showed a significant decrease in comparison to pH 7.0 and no viable cells were found at pH 3.0.

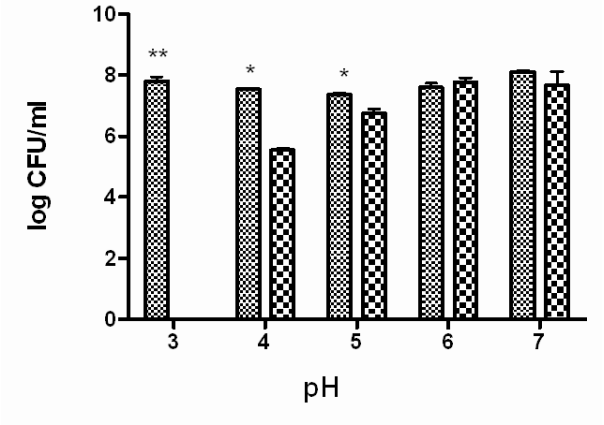

Fig. 2 Silica matrices buffer holding capacity. Viability of *Mesorhizobium* cells immobilized in silicate matrices exposed to different pHs. ▒ immobilized cells and ▓ free cells

3.3. Bacteria release from silica carriers

Silicate gels were used to study their ability to release entrapped bacteria in a sustained form. The initial number of entrapped cells was 1.07 x 10^9 CFU/ml. As shown in **Figure 3**, these silica carriers were able to release only a small number of bacteria during the first hour, approximately a hundred cells, which significantly increased after 5 hs to reach a number of 10^7 cells after 48 hs.

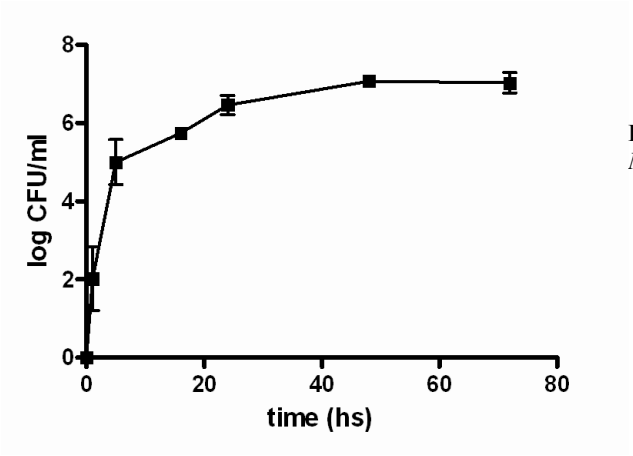

Fig. 3 Silica matrices buffer holding capacity. Release of *Mesorhizobium* cells immobilized in silica matrices.

3.4 Survival of immobilized *Mesorhizobium* spp. in sterile soil

As it is not only important to maintain rhizobia in high numbers during storage but also once it is incorporated to the field, their viability in sterile soil was studied. As it shows in **Figure 4**, after 45 days, the number of viable bacteria in the flasks inoculated with a rhizobia suspension, was approximately one log lower than that obtained in the soil flasks inoculated with entrapped rhizobia, showing a significant difference between means ($p = 0{,}0152$) according to the unpaired t test. A 45-day period after inoculation was chosen as it is the time between the seed or soil is inoculated and nodulation occurs. Previous studies indicate that most rhizobia cells died between inoculations and sowing because of adverse environmental conditions [26].

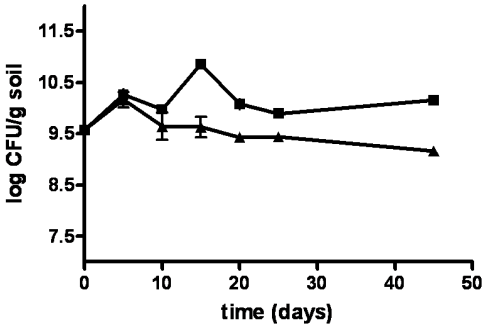

Fig. 4 Survival of immobilized *Mesorhizobium* cells in sterile soil. Viability comparison in sterile soil between *Mesorhizobium* cells immobilized in silicate matrices with 2 % glycerol (-■-) and a suspension of the bacteria with 2 % glycerol (-▲-) during 45 days.

3.5. Lysine decarboxylase activity of encapsulated rhizobia

Lysine decarboxylase activity was evaluated *in vivo*, as an indicator of the metabolic activity of rhizobia during storage of gels. An increase in enzyme activity was observed 57 days after encapsulation (**Figure 5**), which would be related to the increase in the metabolic activity during bacteria growth observed in the first 80 days post-immobilization.

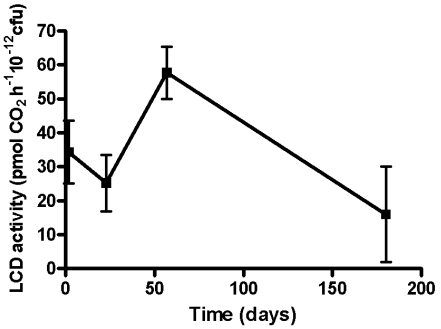

Fig. 5 In vivo determination of lysine decarboxylase activity in encapsulated *Mesorhizobium* spp. BA151cells. Gels (0.5 ml each) were suspended in 50 mM potassium phosphate buffer pH 6.8 to a final volume of 1 ml at different times after encapsulation. Then, 0.5 ml aliquots were mixed with 0.1 µCi L-[1-^{14}C]-lysine and incubated for 1 h at 28ºC. Radioactive CO_2 released as a consequence of lysine decarboxylation was determined as indicated in materials and methods. Results are the mean of three replicate gels ± standard deviation.

3.6. Infectivity of encapsulated rhizobia

Under the experimental conditions employed, *L. tenuis* seedlings inoculated with liquid cultures of *Mesorhizobium* spp. BA151 developed 2 to 3 pink-red nitrogen fixing nodules per plant. Inoculation of encapsulated bacteria onto seedlings,

either after gel disruption and suspension in culture medium or by gel application without previous disruption, led to the development of similar amounts of nodules to those found in seedlings inoculated with liquid cultures of *Mesorhizobium* spp. BA151. Nodules formed after inoculation with encapsulated bacteria also were pink-red, and the aspect of seedlings inoculated with encapsulated bacteria was similar to that of plants inoculated with liquid cultures of *Mesorhizobium* spp. BA151.

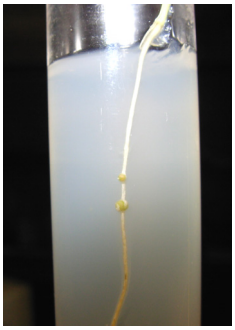

Fig. 6 *Lotus tenuis* seedlings inoculated with sol-gel encapsulated *Mesorhizobium* spp. BA151. The image shows the presence of 2 nitrogen fixing nodules in the plant.

4. Discussion

On the basis of a previous work [25], it was decided to use sodium silicate as a sol-gel precursor and citric acid as the catalyst (instead of other silica precursors and acids) for the immobilization process. Citric acid neutralizes the alkalinity of the silica precursors, it is an intermediary of the tricarboxylic acid cycle and may also act as a compatible carbon source for bacteria. Citrate could also allow growing of slow-growing *Rhizobium* strains [27]. Glycerol is also a carbon source for rhizobia, has a high water-binding capacity which may protect cells from the effects of desiccation and its flow characteristics appear to promote more rapid coating of seeds.

Silicate matrices have not been used for rhizobia immobilization until the present work. However, some results on long-term preservation of entrapped biocontrol bacteria in alginate beads have been published by Walker *et al*., [23] indicating that after 300 days of storage at 5 º C, most strains tested presented a <3-5 log $_{10}$ decline in bacterial number. Immobilization of rhizobia in silicate matrices has been proven in this work, to be an efficient technological tool for the protection and preservation of cells, for long periods of time (360 days) at room temperature with almost no decline in bacterial number.

Silica matrices buffer-holding capacity could be used as a further advantage to protect entrapped microorganisms against adverse environmental conditions such as acid soils. Soil acidity is an important subject for agricultural production in many areas. Forage species could give poor yields on acid soil because of their corresponding *Rhizobium* strain acid sensitivity. In order to counteract the acidity effects of soil and superphosphate fertilizers on the survival of the rhizobia, limestone was introduced as a neutralizer [15]. O'Hara *et al*., [28] found that acid-sensitive strains were unable to maintain their pH gradient and Howieson *et al*., [29] described acidity below pH 5.0 or alkalinity above pH 8.5 as primary stresses for most rhizobia strains.

In this work, entrapped rhizobia viability was not altered in more than one log unit when exposed to various pHs (3.0 to 7.0) indicating that sol-gel silica matrices are suitable for bacteria protection against this type of stress. Because of the nature of its components (citric acid, sodium phosphate, and sodium silicate), silica gels have the ability to regulate media pH.

Metabolic activity of entrapped bacteria was also studied to evaluate if any variation occurred during storage. Furthermore, an enzyme related to polyamine synthesis (lysine decarboxylase) was chosen for the experiments. These amines levels are generally five to ten times higher in root nodules than in other plant organs [30] and they might be related to nodule formation and nodule activity [31]. Some authors postulate that polyamines are substances which regulate plant growth. [32]. Moreover, polyamines could protect plants from osmotic stress [33]. Results indicate that lysine decarboxylase was active during the whole storage period thus immobilized rhizobia were still able to synthesized cadaverine, a type of polyamine, necessary for plant- bacteria interaction.

Root-nodule formation in *L. tenuis* seedlings inoculated with suspensions of rhizobia obtained by mechanical disruption of silica gels or with undisrupted gels containing bacteria, gave similar results indicating bacteria capability to spread out of the gel and reach seedlings. These results were confirmed by the bacteria release studies performed over entrapped bacteria.The number of nodules formed was similar to those obtained inoculating with liquid cultures of rhizobia, which demonstrates that immobilization did not alter the bacteria ability to nodule their specific hosts.

When tubes are hermetically closed, silicate gels are able to retain a high level of moisture (approximately 90%) and thus immobilized rhizobia are not affected by desiccation during storage. If adequate soil moisture content is maintained throughout inoculation and nodulation and rainfall incidence is high enough, then silicate gels can conserved their

structure and continue protecting bacteria once they face soil competitive environment. However, these inorganic gels tend to desiccate and collapse rapidly when exposed to air or arid conditions affecting rhizobia viability.

Acknowledgements. G.S.A is grateful for her doctoral fellowship granted by the Agencia Nacional de Investigaciones Científicas y Técnicas. The authors would like to acknowledge the support of grants from the CONICET (PIP 5740), Universidad de Buenos Aires (UBACYT B055 and B407) and Agencia Nacional de Investigaciones Científicas y Técnicas (BID 1728/OC-AR PICT 03/1783, 32310 and 33397). María Julia Estrella is a Researcher of the Comisión de Investigaciones Científicas (CIC-Argentina). F.L.P, M.F.D, O.A.R and L.E.D are members of the National Research Council (CONICET).

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