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RESEARCH ARTICLE

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Survival of several *Rhizobium/Bradyrhizobium* strains on different inoculant formulations and inoculated seeds

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Abstract The effect of a variety factors on the survival of several rhizobia strains on inoculants and inoculated seeds has been evaluated. Since the rhizobia strains showed different cell-density-evolution patterns on peat-based inoculants and on inoculated seeds, several inoculant formulations with highly effective *Rhizobium/Bradyrhizobium* strains (for *Lupinus*, *Hedysarum, Phaseolus* and *Glycine max*.) were monitored under the following storage conditions: (a) the inoculants were kept refrigerated (at 4 °C), or (b) at room temperature (25 °C). The effect of water content (30–50%, w/w) in the inoculants as well as that of several seed-coating adhesives were also investigated. Alternative carriers including perlite and vermiculite were tested. For all of the strains, survival on sterile peat-based inoculants was higher than on the corresponding unsterile peat formulation; for the latter, refrigerated storage conditions are recommended to ensure high bacterial densities. The water content of the inoculants had a differential effect on strain survival depending on the sterility of the peat, such that a high water content was more detrimental when unsterilized peat was employed. The best adherent for rhizobia survival was a gum arabic/water solution. Perlite was as effective as peat in maintaining a high population of rhizobia, at least for 6 months of storage.

Keywords *Rhizobium* spp. · *Bradyrhizobium* spp. · *Sinorhizobium fredii* · Inoculants · Carriers

Introduction

Legume inoculants consist of rhizobial cultures usually mixed with solid-based carrier materials such as peat or other substrates rich in organic matter. Finely ground peat is the carrier of choice in legume inoculants world wide [2,17]. In our laboratory, we have used black neutral peat from Padul, Granada, Spain, as a carrier for commercial production of legume inoculants since 1975 [19]. In some regions of the world, however, peat is not always available or it is expensive [21]. In addition, in some countries that have programs to preserve wetlands, peat extraction is inadvisable if not forbidden. Thus, other materials able to support good growth and survival of bacteria have been developed, including different kinds of coal [5], bentonite and corn oil [10], mineral soils [4], vermiculite [8,20] and perlite [7,15]. The ability of rhizobia to survive on peat inoculants or preinoculated seed depends on different formulation characteristics, such as the carrier, moisture content, sterility of the carrier, and storage temperature, as well as the intrinsic traits of the chosen rhizobia strain. The inoculation of seeds with rhizobia dates from the end of nineteenth century [24]. The survival of rhizobia on the seed surface is usually lower than on solid carriers due to the lack of protection against desiccation, high temperature, and/or toxic compounds on the seed coat [3,6]. In this study, the effect of a variety of factors on the survival of several rhizobia strains on inoculants and inoculated seeds was evaluated.

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Materials and methods

Rhizobia strains and growth conditions

The ability of well-characterised rhizobia strains belonging to genera *Rhizobium* and *Bradyrhizobium* to survive on peat-based inoculants and/or on seeds of their host legumes was evaluated. Table 1 shows the origin and plant hosts of the bacterial strains. The bacteria were routinely grown in yeast extract-mannitol broth (YMB) [23]. When necessary, 15 g agar l^{-1} was added (YMA).

Carriers and inoculant production

Peat and perlite were used as carriers. Black neutral peat from Padul, Granada, Spain, has been used as a carrier for commercial

Table 1. Origin and plant host of the bacterial strains used in this work

Strain	Species	Plant host	Origin/reference
Hc3	*Rhizobium* sp.	*Hedysarum*	Spain/[12]
CIAT 899	*R. tropici*	*Phaseolus vulgaris*	Mejico/[11]
ISP23	*R. etli*	*Phaseolus vulgaris*	Spain/[14]
ISP42	*R. etli*	*Phaseolus vulgaris*	Spain/[14]
ISLU16	*Bradyrhizobium* sp.	*Ornithopus* sp.	Spain[a]
ISLU21	*Bradyrhizobium* sp.	*Lupinus hispanicus*	Spain[a]
ISLU40	*Bradyrhizobium* sp.	*Lupinus hispanicus*	Spain[a]
USDA110	*B. japonicum*	*Glycine max.*	USA/[9]
SMH12	*Shinorhizobium fredii*	*Glycine max.*	Vietnam[b]
A8425	*S. fredii*	*Glycine max.*	China, this work

[a]Temprano FJ (1990) Evaluación de la capacidad simbiótica de poblaciones nativas de *Bradyrhizobium* sp. (*Lupinus*) y de la necesidad de inoculación de altramuces (*Lupinus* sp.) en suelos españoles. Doctoral thesis, (ETSIA), Universidad Politécnica de Madrid
[b]Cleyet-Marel JC (1987) Dynamique des populations de *Rhizobium* et de *Bradyrhizobium* dans le sol et la rhizosphere. These d' Etat. University Claude Bernard-Lyon. Lyon (France)

production of legume inoculants in our laboratory since 1975 [19]. Perlite is a volcanic stone composed of little-hydrated aluminium silicate. Vermiculite is a hydrated, magnesium aluminium iron silicate exfoliated at extremely high temperatures. It is relatively inexpensive and widely available.

All inoculants were prepared by mixing the ground carrier (200 mesh) and saturated rhizobia liquid cultures ($>10^9$ cells ml^{-1}) in the proportions needed to obtain the desired final moisture content (30, 35, 40, 45, and 50%). When necessary, the peat was moistened (ca. 10 ml of water per 60 g of peat) and then autoclaved in flasks at 120 °C for 60 min. After packaging the inoculants in heat-sealed polyethylene bags, different storage conditions were imposed. Inoculants were sampled periodically in duplicate (independent bags), and viable bacteria were estimated by plating ten-fold serial dilutions on Congo-red/YMA agar.

Seed inoculation

In general, seed lots were mixed with the corresponding inoculant at a ratio of 1:100 (inoculant: seed-weight) and using 1% (v/w) of a 25% gum arabic/water solution as adhesive. Inoculated seeds (soybean, lupin and bean) were air-dried and kept at 25 °C. Other adhesives were evaluated, using *Hedysarum* seeds, at different concentrations (4% v/seed weight): 10% sucrose, 20% gum arabic, 2% carboxymethylcellulose (CMC), 2% hydroxypropylmethylcellulose-5000, and 2% hydroxypropylmethylcellulose-15000. Once the coated seeds had dried, they were kept at 4 °C. A subsample of gum-arabic-treated seeds was additionally coated with 20% (seed weight basis)calcium carbonate. Common bean (cv. Canellini) and soybean (cv. Williams) seed lots were mixed with 1% (w/w) peat- or perlite-inoculant (CIAT899/bean and USDA110/soybean) and 1% (v/w) of a 10% sucrose/water solution commonly used by farmers as adhesive. Additionally, seed lots of bean cv. Canellini were also inoculated with ISP42 peat- or perlite- inoculants using 20% gum arabic, 2% carboxymethylcellulose (CMC), 25% glycerol, or 50% polyethyleneglycol-1450 (PEG) as adhesive solutions at 1% (v/w), and kept at 25 °C.

Bacteria survival was measured periodically by transferring either 20 inoculated seeds or 2 g of inoculated seeds (in the case of *Hedysarum*) to 100 ml sterile saline buffer and plating 10-fold serial dilutions on Congo-red/YEM agar supplemented with 100 μg cycloheximide ml^{-1}. In order to compare the survival of slow- or fast-growing rhizobia-nodulating soybean, seeds of cv. Oshumi were mixed with peat inoculants containing either *Bradyrhizobium japonicum* USDA110 or *Shinorhizobium fredii* SMH12 and A8425 strains, to give a starting population of 10^6 rhizobia $seed^{-1}$, based on the viable counts on peat inoculants. Short-term survival of rhizobia on seeds were determined at 2, 6, and 24 h, 1 and 3 weeks after inoculation. Seeds lots were kept at room temperature.

Results and discussion

Survival of rhizobia strains on inoculants under different conditions

The effects of carrier, carrier sterilisation, moisture content and storage temperature on the survival of different rhizobia strains in inoculants were evaluated by the plate counting method. Figure 1 shows the survival of *Bradyrhizobium* sp. (*Lupinus*) ISLU40 strain in sterile and unsterilized peat inoculant with a water content of 30–50% (dry-weight basis). In all cases, survival values were higher for sterile than for unsterilized peat inoculants. By using sterile peat and 30% moisture, the initial population was reduced 1/8 after 56 weeks, whereas with non-sterile peat the reduction was higher than 1/5 of the initial density during the same storage period.

In sterile peat with moisture content of approximately 40%, the survival of the strain was close to 10^9 bacteria $(g\ peat)^{-1}$ after 1 year of incubation at 25 °C, whereas in non-sterile peat, at a moisture content of 40–50%, bacterial densities were below 10^6 cells $(g\ peat)^{-1}$, the slope of the decline being more pronounced when the moisture content increased. Our results agree with those obtained by Roughley [16]: high moisture concentrations inhibit the growth of rhizobia in unsterilized peat, whereas rizhobial growth is not inhibited in sterilized peat. Alternatively, the ratio of contaminants (mostly fungi) to rhizobia might be higher when moisture levels in the peat are increased.

The survival of *Rhizobium* sp. (*Hedysarum*) Hc-3 strain in sterile and non-sterile peat inoculants, kept either refrigerated (4 °C) or at room temperature (25 °C), was evaluated over 32 weeks (Fig. 2) [13]. In non-sterile and non-refrigerated inoculants (the most detrimental conditions), there was an initial increase of one order of magnitude; this was followed by a steady decrease to a final density of 10^7 rhizobia g^{-1}. When non-sterile peat inoculants were stored at 4 °C, the dynamics of the rhizobial population were similar to those in sterile peat at 25 °C (Fig. 2), the final population being larger than

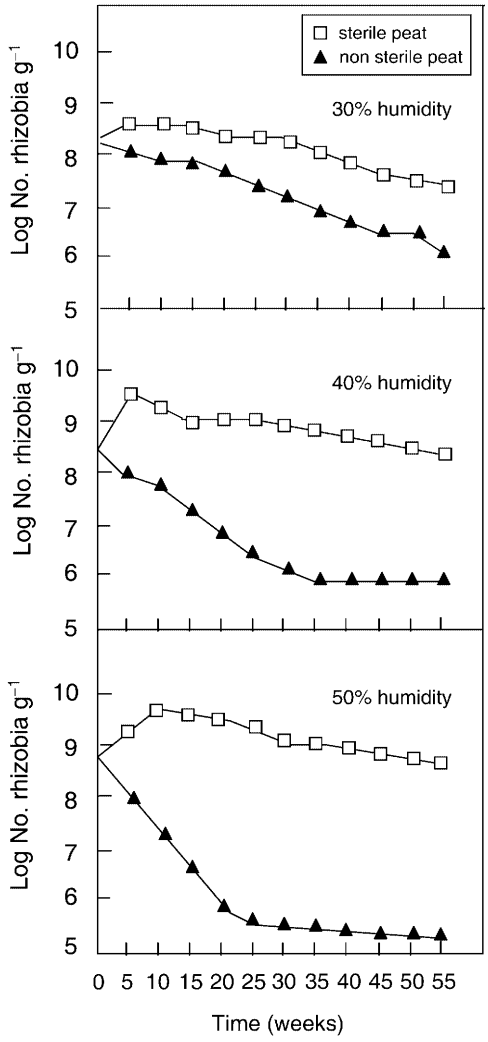

Fig. 1. Survival of *Bradyrhizobium* sp. (*Lupinus*) ISLU40 strain in sterile and unsterilized peat-based inoculants with different moisture contents

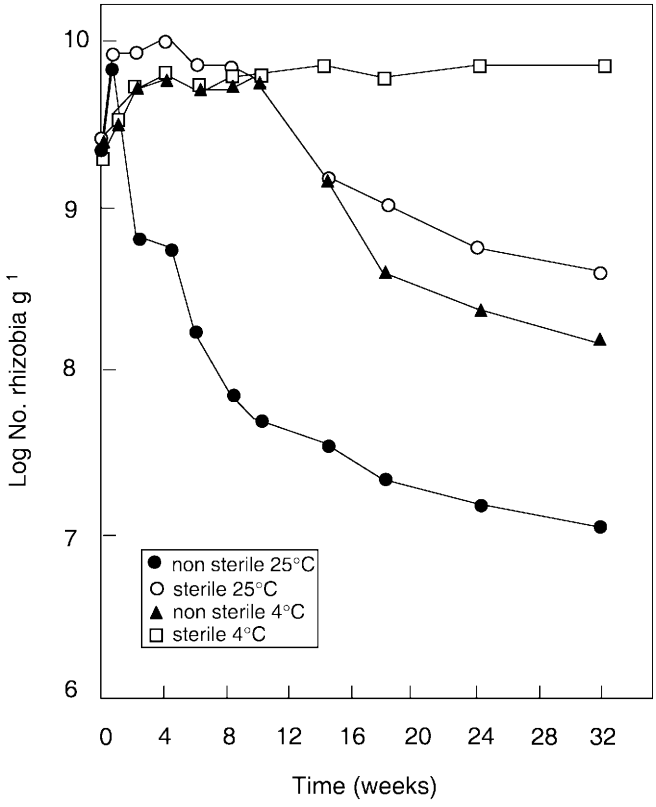

Fig. 2. Survival of *Rhizobium* sp. (*Hedysarum*) Hc3 strain in sterile and unsterilized peat-based inoculants stored at two different temperatures

that in non-sterile peat under non-refrigerated conditions.

At 25 °C there was an initial increase (2 weeks) in viable cells in both sterile- and non-sterile carriers. The viable counts in sterile peat leveled off during the following 8 weeks and, thereafter did not fall below 10^8 g^{-1} of peat. By contrast, in non-sterile carrier the viable cells sharply decline after the first 2 weeks of storage. The initial population in sterile peat inoculants kept at 4 °C increased over the first 4 weeks, then leveled off to about 10^{10} rhizobia and remained almost unchanged until the end of the assay. These data suggest that unsterilized peat inoculants kept refrigerated (4 °C) could be a good formulation; in fact, they can provide more than 10^8 bacteria (g peat)$^{-1}$ after more than 30 weeks. Thus, this can be a useful alternative approach when sterilisation facilities are not available.

The survival of *Rhizobium etli* ISP42 and *R. tropici* CIAT899 (common-bean nodulating strains) was followed in sterile peat-, perlite- and vermiculite-based inoculants, kept at 4 °C and 28 °C, for 6 months. Table 2 shows that, at 4 °C, all three carriers tested were equally effective in maintaining the initial rhizobial population almost unchanged or with small variations until the end of the assay.

Table 2. Survival of *Rhizobium etli* ISP42 strain in peat-, perlite-, and vermiculite-inoculants at two different storage temperatures. Decimal logarithm of viable cells g^{-1} of inoculant. Data are mean values of two independent counts

Time (days)	Peat		Perlite		Vermiculite	
	4 °C	28 °C	4 °C	28 °C	4 °C	28 °C
0	9.00	9.00	9.18	9.18	9.15	9.15
30	9.30	9.00	9.25	9.15	9.23	9.00
60	9.25	8.56	9.20	9.20	9.20	8.86
90	9.20	8.30	9.11	9.20	9.25	8.18
120	9.30	8.32	9.15	8.99	9.23	8.52
150	9.25	8.30	9.00	9.08	9.18	8.25
180	9.20	7.56	8.78	8.90	9.04	7.93

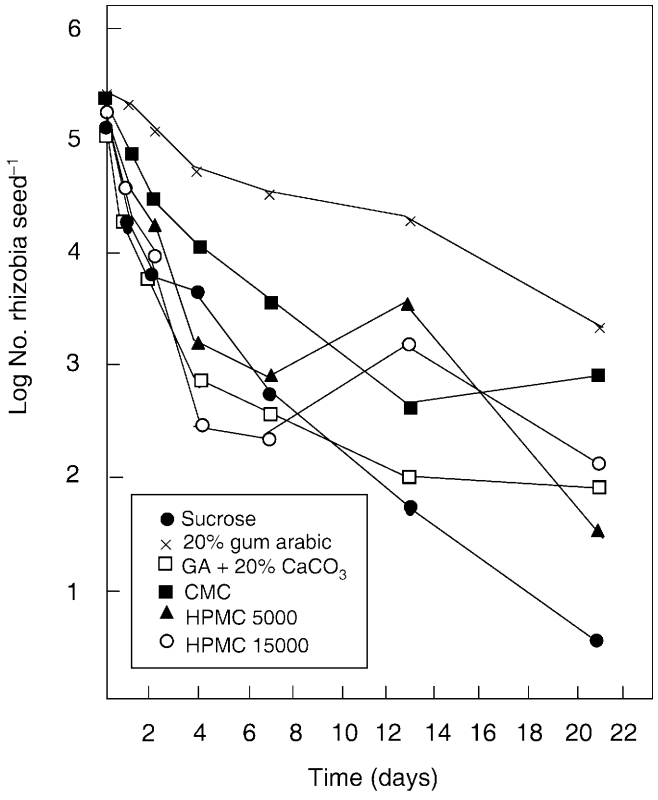

Fig. 3. Survival of *Rhizobium* sp. (*Hedysarum*) Hc3 strain peat inoculants on *Hedysarum coronarium* seeds using different adhesive solutions

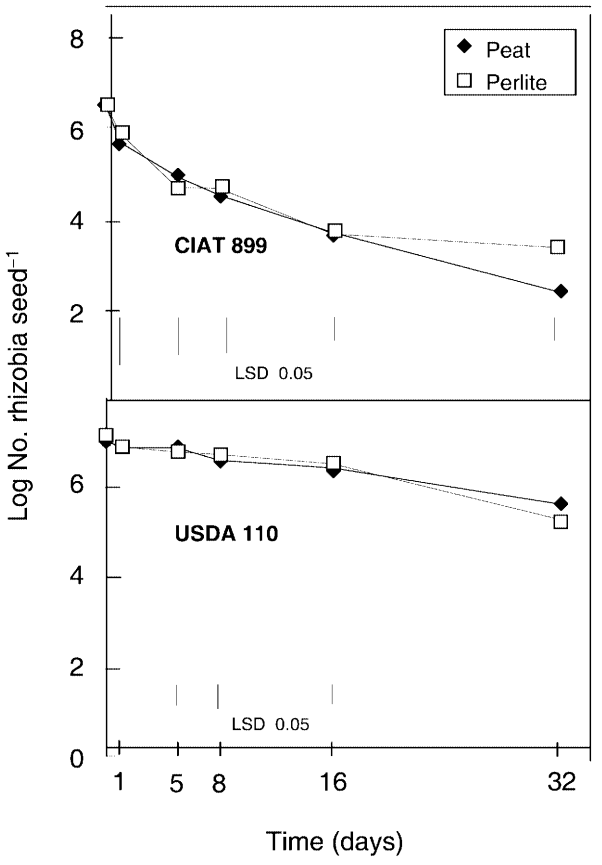

Fig. 4. Survival of *Rhizobium tropici* CIAT899 and *Bradyrhizobium japonicum* USDA110 peat and perlite inoculants on bean and soybean seeds [7]

Non-refrigerated conditions were more detrimental for rhizobia survival, especially in the case of peat and vermiculite inoculants, which gave final viable counts two order of magnitude lower than the starting population. The survival of *R. tropici* CIAT899 was similar to that of *R. etli* ISP42 (data not shown). Other sterile perlite inoculants with *Bradirhizobium japonicum* USDA110 and *Shinorhizobium fredii* SMH12 strains (slow- and fast-growing rhizobia nodulating soybean, respectively) were prepared to test the suitability of perlite compared to peat as a carrier. Long-term survival counts (carried out after 3 years under refrigerated conditions) showed that bacterial densities varied among strains but not among substrates. The viable-cell counts ranged from $>3\times10^8$ bacteria (g carrier)$^{-1}$ for strain USDA110 to 10^7 bacteria (g carrier)$^{-1}$ for strain SMH12.

Survival of rhizobia strains on seeds

A comparative study of the survival of two *Bradyrhizobium* sp. (*Lupinus*) ISLU16 and ISLU21 peat inoculants on *Lupinus albus* cv. Multolupa seeds was carried out for 1 month after inoculation. The results showed that the bradyrhizobia strains had similar dynamics on seeds (no significant differences throughout the sampling time were detected). There was a slow decline of one order of magnitude with respect to the initial popula-

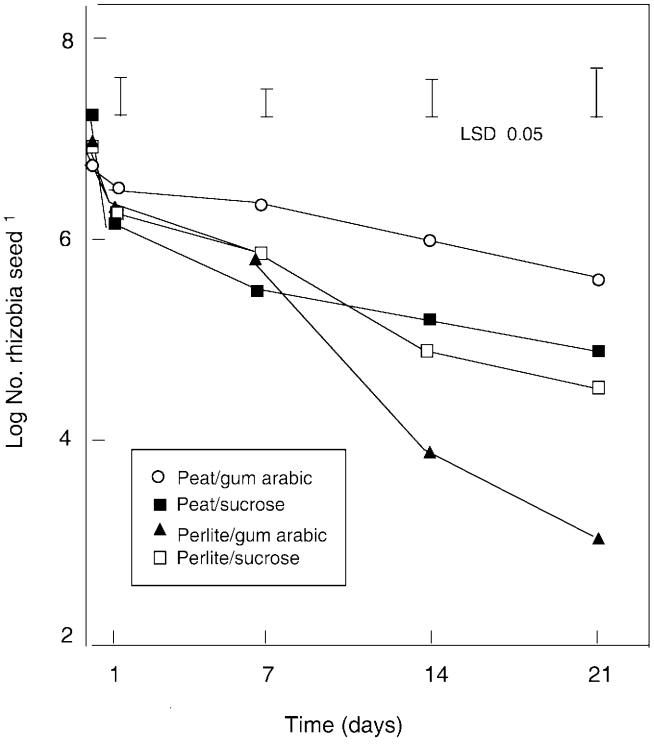

Table 3. Short-term survival of *Bradyrhizobium japonicum* USDA110 and *Sinorhizobium fredii* SMH12 and A8425 peat inoculants on soybean seeds (cv. Oshumi). Decimal logarithm of viable cells/seed. Data are mean values of two independent counts (based on 100 seeds). Data within a column followed by the same letter are not significantly different ($p < 0.05$)

	Hours					
	2	4	6	24	168	504
Strain						
USDA110	5.45 a	4.95 b	5.34 a	5.00 b	4.26 c	3.60 d
A8425	5.24 a	4.68 b	5.05 a	4.71 b	4.14 c	3.29 d
SMH12	4.79 a	4.73 ab	4.82 a	4.50 b	3.85 c	3.27 d

tion; one month after inoculation, however, viable-cell counts were still high enough to ensure good nodulation (10^6 bacteria seed^{-1}). In contrast to previous reports, our results suggest that lupin seed coats do not contain compounds inhibitory to bradyrhizobia [18,22].

The survival of *Rhizobium* sp. (*Hedysarum*) Hc3 strain peat-inoculant on seeds of *H. coronarium* using various adhesive solutions was monitored (Fig. 3) [13] to determine whether an alternative exists to gum arabic, which is expensive and difficult to manipulate. Furthermore, whereas gum arabic solutions need heating, other adhesives can be prepared at room temperature.

There were no significant differences ($p < 0.05$) in the recovery rates of rhizobia among treatments 6 h after inoculation, which indicated that the amount of peat inoculant that adhered to the seeds was similar. Of the five adhesives tested, gum arabic gave the best survival rates (> 1,000 rhizobia per seed) 3 weeks after inoculation. However, contrary to what we expected, the combination gum arabic/calcium carbonate pelleted treatment did not provide better protection; after 4 days of storage, the seed lot treated this way bore < 1,000 rhizobia per seed. Hence this method does not produce good nodulation, in contrast to the report by Brockwell et al. [1], who suggested that, in soils devoid of specific rhizobia, concentrations of 100–1,000 rhizobia per seed could allow satisfactory nodulations. Additionally, the survival of four peat cultures (rhizobia-nodulating *Hedysarum*) was followed on glass beads to demonstrate the absence of toxic substances in *Hedysarum* seed coats. The declining patterns of the applied rhizobial populations on glass beads were quite similar for the different strains and the decline was even greater than on seeds [13].

Both peat- and perlite-based inoculants provided similar protection to CIAT899 and USDA110 strains 16 days after the seeds were inoculated (Fig. 4). However, the slope of decline of strain CIAT899 was more pronounced than that of strain USDA110. The survival of *Rhizobium tropici* CIAT899 on seed was lower than that of other rhizobia strains [7].

The symbiotic effectiveness of pre-inoculated seeds with peat and perlite-based inoculants of *R. tropici* CIAT899 was assessed under greenhouse conditions. Non-stored inoculated seeds of bean cv. Canellini (ca. 10^6 rhizobia seed^{-1}) and 16-day-old pre-inoculated seeds (10^4 rhizobia seed^{-1}) were sown in 2.5-l sand pots. The nodulation response obtained with pre-inoculated seeds (either with peat- or perlite-based inoculants) stored at room temperature for 16 days before sowing was similar to that obtained with non-stored seeds. In previous field experiments using peat- or perlite-based inoculants of *B. japonicum* USDA110 and *S. fredii* SMH12 strains, we had obtained identical soybean yield and seed N content. We also found no differences in nodulation between the two types of inoculant carriers [7].

Perlite was less effective than peat in promoting rhizobial survival on seeds when gum arabic was used as adhesive; bacterial survival, however, was similar in both carriers when sucrose was employed as adhesive, providing more than 10^4 rhizobia seed^{-1} after 3 weeks (Fig. 5) [7]. Results obtained using perlite-based inoculants combined with CMC, glycerol or PEG as adhesive indicated that none of them was better than 10% sucrose (data not shown).

Large differences were detected between the applied inocula and the early counts (2 h after seed inoculation), mostly in the case of seed lot SMH12, which might indicate the loss of peat inoculant adhering to the seeds rather than a real decrease in viable bacteria. After 24 h, viable rhizobia populations ranged from 4×10^4 to 10×10^4 seed^{-1}, (half of the initial counts, but statistically different, $p < 0.05$) (Table 3). Cells counts 1 week after seed inoculation had declined by one order of magnitude and continued to decline gradually until the end of the assay. Although survival data for *S. fredii* SMH12 were lower, its survival pattern was similar to that of *B. japonicum* USDA110.

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Fig. 5. Survival of *Rhizobium etli* ISP42 peat and perlite cultures on bean seeds using different adhesive solutions [7]

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