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ORIGINAL PAPER

Y. Bashan · L. E. Gonzalez

Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant

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Abstract Two plant-growth-promoting bacteria, *Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313, immobilized in 1983 in two types of alginate-bead inoculant (with and without skim-milk supplement) and later dried and stored at ambient temperature for 14 years, were recovered in 1996. The population in each type of bead had decreased, yet significant numbers survived (10^5–10^6 cfu/g beads). Population numbers depended on the bead type and the three independent bacterial counting methods: the conventional plate-count method, indirect enzyme-linked immunosorbent assay and the limited-enrichment technique. Both bacterial species retained several of their original physiological features. When inoculated onto wheat plants, both species colonized and produced plant-growth effects equal to those of the contemporary strain from a culture collection or to their own 1983 records. This study showed that bacteria can survive in alginate inoculant over long periods.

Introduction

Today, most marketed bacterial inoculants are peat-based formulations for seed coating or pellets for sowing (Smith 1992), performing satisfactorily primarily with rhizobia (Thompson 1980). During the last two decades several experimental formulations based on polymers have been evaluated (Bashan 1998), and demonstrated to be potential bacterial carriers (Jung et al. 1982). They offer advantages over peat, encapsulating and protecting the microorganisms against environmental stresses, and releasing them to the soil gradually when the polymers are degraded by soil microbes (Bashan 1998). They can also be stored dry at ambient temperatures, offer consistent batch quality and a better-defined environment for the bacteria, and can be manipulated according to species requirements. These inoculants may be amended with nutrients to improve short-term survival upon inoculation, especially with associatied plant growth-promoting bacteria (PGPB) (Bashan and Holguin 1998). However, they are more expensive and are more labor-intensive to produce than peat-based inoculants (Fages 1992). So far, there are no polymer-based inoculants on the market (Ogoshi et al. 1997).

Alginate is the most common polymeric material for encapsulation of microorganisms for commercial use (Cassidy et al. 1996). Macro-alginate beads (1–3 mm) containing bacteria are made by an easy and straightforward multi-step procedure (Bashan 1986a; Digat 1991) that may involve crushing or milling solid sheets of alginate into small particles and sieving them to the required size (Paul et al. 1993).

Several experimental alginate-based preparations were evaluated for agriculture, including the encapsulation of the VA-mycorrhizal fungi (Ganry et al. 1982), ectomycorrhizal fungi (Le Tacon et al. 1985), PGPB (Trevors et al. 1992), and fungi (DeLucca et al. 1990) and bacteria (Aino et al. 1997) used as biocontrol agents against soil-borne pathogens. This technology was also employed to encapsulate the PGPB *A. brasilense* and *P. fluorescens* (Bashan 1986a). *A. brasilense* survived for at least 4 months (Bashan 1986a; Paul et al. 1993) in dry alginate preparations. These preparations were later used successfully to inoculate wheat under field conditions (Bashan et al. 1987). Samples from these earlier studies were the source of the inoculant for the present study.

The aim of this study was to evaluate the capacity of *A. brasilense* and *P. fluorescens* for long-term survival, viability, and growth-promoting capacity after storage in dry alginate beads for 14 years.

Y. Bashan (✉)
Department of Microbiology, The Center for Biological Research of the Northwest (CIB), La Paz, P.O. Box 128, B.C.S. 23000, Mexico
e-mail: bashan@cibnor.mx
Fax: +52-112-54710 or -53625

L. E. Gonzalez
Department of Biology, Pontificia Universidad Javeriana, Apartado Aereo 56710, Santafe de Bogota, Colombia

Materials and methods

Bead production

The macrobeads used in this study were produced in 1983 (Bashan 1986a). The dry preparations were stored for 14 years at 10–28 °C in dark, screw-cap bottles containing about 4 g beads and about 5 g dry silica gel. Since the inoculant was conserved for archival purposes, no treatment was given and the bottles remained tightly closed during the entire period.

Bacteria and plant material

The PGPB *A. brasilense* Cd (DSM 7030) and *P. fluorescens* 313 were the encapsulated bacteria. Wheat plants (*Triticum aestivum* cv. Miriam, Hazera Co., Haifa, Israel), tomato plants (*Licopersicon esculentum* Mill. cv. M-82-1-8-VF), and cotton plants (*Gossypium barbadense* cv. Pima S-5, Hazera), all known to respond positively to *A. brasilense* inoculation (Bashan et al. 1989; Kapulnik et al. 1985), were used to evaluate the growth-promoting effect of the recovered bacteria.

Growth-promotion effects of the recovered bacteria

Plant growth-promoting activity was measured under gnotobiotic conditions by the method of Glick et al. (1995) with minor modifications. Seeds were surface-sterilized and inoculated with the respective PGPB as described earlier (Puente and Bashan 1993). Bacteria were isolated after the beads had been dissolved, as described later, and grown using standard methods (Bashan et al. 1993). Five inoculated seeds each were germinated in polyester growth pouches (Northrup King Co. Minneapolis, Minn.), covered with transparent plastic sheets and incubated at 22 ± 2 °C (wheat) or 28 ± 2 °C (tomato, cotton) in a growth chamber (Conviron, model TC 16, Winnipeg, Canada) at a light intensity of 120 μmol m^{-2} s^{-1} and a photoperiod of 12 h. Root length, root and shoot dry mass, root surface area, formation of root hairs, and root-colonization capacity (obtained by inoculating the recovered strains) were compared with the same parameters of plants inoculated with the same *A. brasilense* strain from a culture collection (stored in liquid N) and with noninoculated seedlings growing under identical conditions. Since a collection strain of the original *P. fluorescens* 313 was no longer available (the original strain, stored by the senior author at −40 °C in 1987 at the Weizmann Institute of Science, Israel, belonged to an Israeli commercial company, no longer existing, and is presumed lost), similar plant-promoting effects recorded in unpublished data from 1983 were included as reference.

Bacterial counts from beads

Bacteria entrapped in the beads were counted after ten bead samples were dissolved in 10 ml potassium phosphate buffer (0.25 M, pH 6.8 ± 0.1) in a test-tube for 16–24 h at 30 ± 2 °C. No bacterial multiplication occurred under these incubations (Bashan 1986a; Levanony et al. 1987). To facilitate solubility, the beads were shaken for 5 min at top speed on a Vortex-Genie-2 mixer (Scientific industries, Bohemia, N.Y.). For verification of results, the *A. brasilense* released from the beads was counted simultaneously by three different methods: a conventional plate-count method on nutrient agar (Difco, Detroit, Mich.), indirect enzyme-linked immunosorbent assay (ELISA) (Levanony et al. 1987), and by the limited-enrichment technique (Bashan et al. 1991). In the absence of immunomethods for *P. fluorescens*, its cell numbers were counted only by the plate-count method. Because the solubilization of the beads could not guarantee a single-cell suspension, recovery number should be considered a minimal population level.

Root-colonization capacity of the recovered bacteria

Bacterial counts from roots were taken after root segments had been homogenized and suspended in 5 ml 0.85% NaCl at 4000 rpm for 30 s at 22 ± 2 °C (Polytron Brinkmann Instruments, NY). The homogenate was serially diluted in the same saline solution and plated on nutrient agar (Difco, Detroit, Ill.), using the plate-count method for *P. fluorescens* and indirect ELISA for *A. brasilense*. The samples were then filtered through Whatman no. 1 filter-paper for the dry-mass determination (60 °C for 16 h).

Bacterial characterization and the effects on plants after inoculation with recovered *A. brasilense* Cd from dry inoculants

These were done using standard methods and are described in footnotes for Table 1 and Fig. 2.

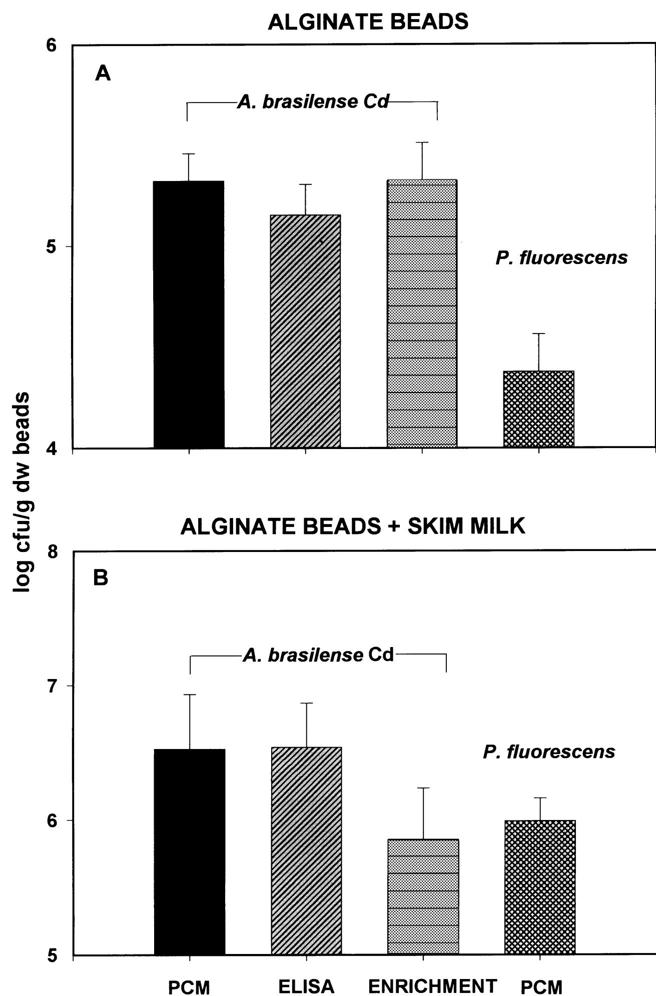

Fig. 1A, B Survival of *Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313 in dry alginate beads (**A**) and in dry alginate beads supplemented with skim milk (**B**) after 14 years, as measured by three independent bacterial counting methods: the plate-count method (*PCM*), indirect enzyme-linked immunosorbent assay (*ELISA*) and the limited-enrichment method (*Enrichment*). *Bars* Represent SE. The original bacterial concentration in the beads produced in 1983 were as follows: in alginate beads, 7.4×10^6 *A. brasilense* cfu/g beads and 6.3×10^6 *P. fluorescens* cfu/g beads; in alginate beads supplemented with skim-milk, 9.7×10^9 *A. brasilense* cfu/g beads and 8.3×10^9 *P. fluorescens* cfu/g beads

Table 1 Typical characteristics of *Azospirillum brasilense* Cd recovered from dried beads as compared to chracteristics of the same strain from a culture collection

Characteristic	*A. brasilense* Cd (recovered)	*A. brasilense* Cd (collection)
N_2 fixation in culture[a] (nmol C_2H_4 culture^{-1}h^{-1})	1200 ± 150	990 ± 180
Pink color of colony[b] (A_{500})	0.43	0.43
Motility in liquid[c]	+	+
Swarming on agar[d]	+	+
Capacity to form large aggregate[e]	+	+
Root colonization of noncereals (cotton)[f]	5.4 ± 0.8 × 10^5	3.8 ± 0.4 × 10^5
Root colonization of noncereals (tomato)[f]	6.6 ± 0.7 × 10^5	4.7 ± 0.9 × 10^5
Increased formation of root hairs and root-hair length[g]	+	+

[a] According to Holguin et al. (1992). [b] According to Eskew et al. (1977). [c] According to Reinhold et al. (1985). [d] According to Hall and Krieg (1983). [e] According to Del Gallo et al. (1989) in the presence of fructose and KNO_3. [f] According to Bashan et al. (1989), 14 days after inoculation. [g] According to Dubrovsky et al. (1994)

Experimental design and statistical analysis

Samples of ten dry beads each, in six replicates, were used for recovering the stored bacteria. Ten replicates were used for the growth-promotion effects and for all bacterial counts. A replicate consisted of five seeds per pouch, and three independent determinations of the bacterial population. Because of the shortage and irreplaceability of dry bead samples, recovery of bacteria from the beads was done only once. Growth-promoting tests were repeated. Results were analyzed by one-way analysis of variance and Student's *t*-test at $P < 0.05$ using Statgraphic plus (Statistical Graphics, Rockville, Maryland) and SigmaPlot (Jandel, San Rafael, Calif.) software.

Results

Taking advantage of an experimental dry inoculant preserved in 1983 for archival purposes, we were able to study how two PGPB survive for 14 years and

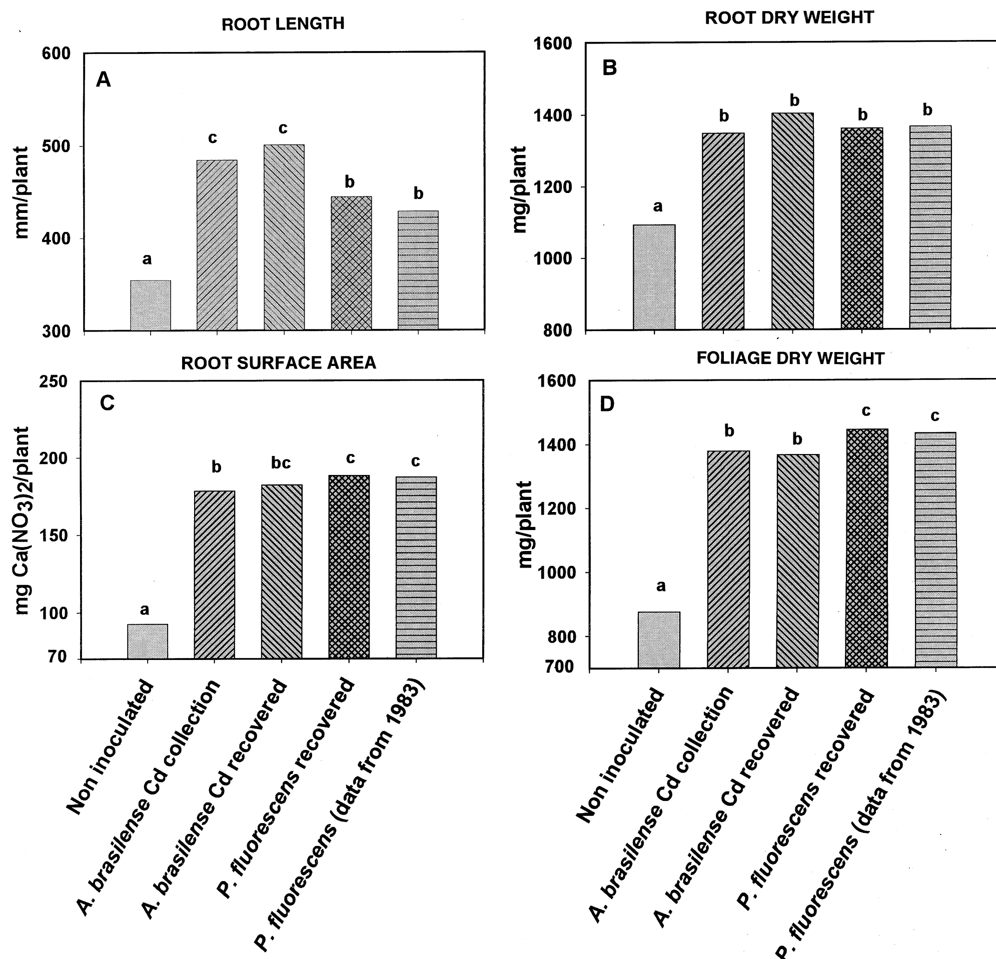

Fig. 2A–D The effect on variables of wheat plant growth after inoculating with recovered (skim-milk beads) and collection based *A. brasilense* Cd and recovered *P. fluorescens* 313. In the absence of *P. fluorescens* from a collection in 1996, the data on this strain's performance were taken from 1983 unpublished notes. Columns denoted by different letters in **A**, **B**, **C** or **D** differ significantly at $P < 0.05$ by one-way analysis of variance. Root lengths and dry weights were determined according to Bashan (1986b) and root surface area was measured according to Carley and Watson (1966)

maintain their beneficial plant-growth-promoting characteristics.

The numbers of *A. brasilense* decreased to 10% of the original population after storage in beads for 14 years, as measured by three independent methods, with results not significantly different among the methods (Fig. 1A). A greater decrease occurred in alginate beads supplemented with skim milk, where the population fell to 1/1000 of the original (Fig. 1B). However, since the original population in the skim-milk beads was higher than that in the milk-free beads (Bashan 1986a), its level was still over 10^6 cfu/g bead after 14 years, equivalent to the Canadian standard for freshly prepared *Rhizobium* inoculant (Bordeleau and Prevost 1981). The other PGPB, *P. fluorescens*, did not survive as well as *A. brasilense*, yet its population in both types of bead after 14 years was high (Fig. 1).

When eight typical physiological and environmental characteristics of the recovered *A. brasilense* Cd were compared to those of the same strain obtained from the German DSM culture collection, no differences between the strains were recorded (Table 1). The recovered *A. brasilense* Cd significantly affected plant development, similarly to the *A. brasilense* Cd from the collection. The 1996 results of *P. fluorescens* were insignificantly different from the results of the original strain tested in 1983 (Fig. 2). The root colonization capacity of the recovered *A. brasilense* Cd was superior to that of the original bacteria, whereas the root colonization of *P. fluorescens* in 1996 was inferior to that described in the 1983 records extant on this bacterium (Fig. 3).

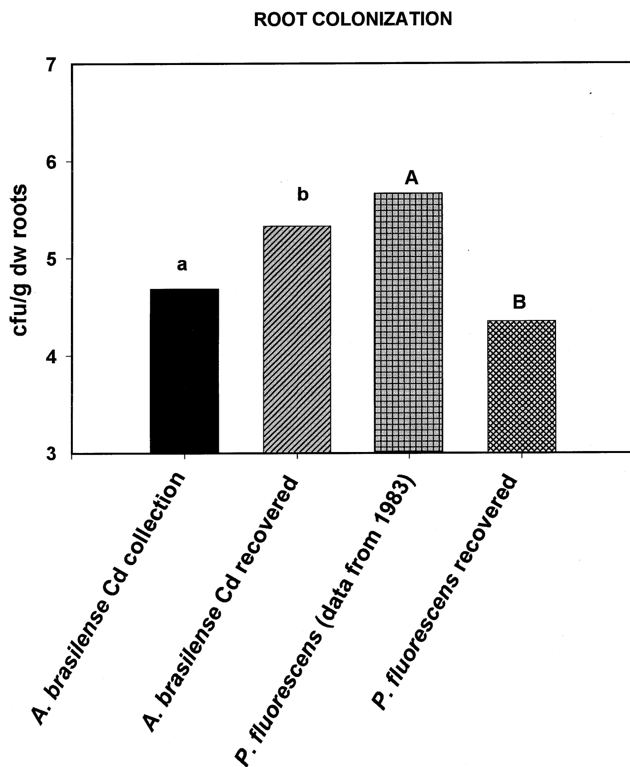

Fig. 3 Root colonization after inoculation of the wheat plants with recovered and collection-based *A. brasilense* Cd and recovered *P. fluorescens* 313. In the absence of *P. fluorescens* from a collection in 1996, the data on this strain's performance were taken from 1983 unpublished notes. Pairs of columns, for each bacterial species separately, denoted by different letters, differ significantly at $P < 0.05$ by Student's *t*-test

Discussion

The main advantages of alginate inoculants are their nontoxic nature, their degradation in the soil and, above all, their slow release of the entrapped microorganisms into the soil (Bashan 1998). Genetically engineered *P. fluorescens*, encapsulated and later released into soil microcosms, showed significantly longer survival rates than those of unencapsulated cells after 3 months (van Elsas et al. 1992). PGPB are known to survive for months in air-dried and lyophilized inoculants (Bashan 1986a; Paul et al. 1993). For commercialization, a long shelf-life is an advantage for any inoculant (Fages 1990, 1992). Currently, a shelf-life of about a year is mandatory for peat inoculants containing rhizobia (Bashan 1998). A longer shelf-life, with full preservation of strain characteristics, is desirable (Fages 1992; Smith, 1992). However, a very long inoculant survival period is not an industrial requirement.

Long-term survival of inoculants is a matter of commercial secrecy (Bashan 1998). To our knowledge, no information is available in the literature on very long survival periods of plant-growth-promoting *Azospirillum* in synthetic inoculants (Bashan and Holguin 1997a, b). We know of no published results for *P. fluorescens* 313.

The ultimate test for any PGPB is its ability to colonize its target plant roots and to produce growth effects (Bashan and Holguin 1997a; Glick and Bashan 1997). By inoculating a common host plant, such as wheat, and measuring the effects of PGPB on root length, root dry mass, root surface area and shoot dry weight, it was possible to compare and verify that plant responses to the recovered bacteria were similar to those obtained by control, culture-collection strains.

In summary, two PGPB survived for 14 years in dry alginate beads at ambient temperature. Upon recovery, both species retained the capacity to enhance plant growth.

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