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ORIGINAL PAPER

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Alginate microbeads as inoculant carriers for plant growth-promoting bacteria

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Abstract A method of inoculating wet and dry seeds with plant growth-promoting bacteria (PGPB) using alginate microbeads as a substrate and *Azospirillum brasilense* as the model PGPB was developed. The microbeads were produced by low pressure spraying of an alginate solution mixed with liquid bacterial culture suspended in a very rich medium through a small nozzle resulting in small-diameter droplets. These droplets, when sprayed into a slowly stirred solution of $CaCl_2$, immediately hardened into microbeads at diameters ranging between 100 and 200 µm. Although the process killed part of the entrapped bacteria, the remaining bacteria residing in the microbeads were sufficient [$>10^{11}$ colony-forming units (CFU) g^{-1} inoculant] for seed inoculation. Further, it was found that the bacterial population in the inoculant could be enhanced by secondary multiplication in the same medium for an additional 16 h. It was found that the microbeads could be used either wet or dry. Dry inoculant was produced using dry air at 38°C, creating a powdery substance loaded with >109 CFU g^{-1} beads. Alternatively, dry microbeads were produced using a standard freeze-drying procedure. This dry preparation was easily attached to dry seed surfaces with the addition of 1% alcohol-diluted lecithin or with 0.5% synthetic paper adhesive (Resistol). The bacteria were slowly released from the microbeads in amounts ranging from 10^4 to 10^6 CFU g^{-1} depending on the type (wet or dry, with or without skim milk) and the time of incubation (the longer the incubation period, the smaller the amount of bacteria released with time). The wet and dry inoculants enhanced the development of wheat and tomato seedlings growing in unfertile soil, and biodegraded within 15 days in moist soil.

Keywords Alginate - *Azospirillum* - Bacterial inoculants Beneficial bacteria - Bacterial immobilization

This paper is in memory of the late Mr Avner Bashan and the late Mr Uzi Bashan of Israel

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Introduction

To date, most marketed bacterial inoculants are peat-based formulations used to coat seeds or pellets for sowing in furrows (Bashan 1998; Kenny 1997; Smith 1992). They usually produce satisfactory results primarily with rhizobia (Burton 1976; Thompson 1980), but the technological limit of their development has almost been reached. They have several severe drawbacks because of the nature of peat, and the unavailability of peat in many countries. During the last decade, several experimental formulations based on polymers have been evaluated (Bashan 1998). These polymers were demonstrated as potential bacterial carriers (Jung et al. 1982) offering substantial practical advantages over peat (Amiet-Charpentier et al. 1998, 1999). These formulations encapsulate the living cells, protect the microorganisms against many environmental stresses and release them to the soil gradually when soil microorganisms degrade the polymers. They can be stored dry at ambient temperatures for prolonged periods, offer consistent batch quality and a better-defined environment for the bacteria, and can be manipulated easily according to the needs of specific bacteria or the crop. These inoculants can be amended with nutrients to improve the short-term survival of the bacteria upon inoculation, especially with associative plant growth-promoting bacteria (PGPB) (Bashan 1986a, 1998). However, they are rather expensive relative to peat-based inoculants and require more biotechnical handling by the inoculant industry (Fages 1990, 1992). There is no commercial agricultural inoculant so far that uses this technology, but environmental applications for the clean-up of hazardous materials are entering the market (Cassidy et al. 1996; Stormo and Crawford 1992).

Alginate is the most common polymer material for the encapsulation of microorganisms for various industrial microbiological purposes (Chen and Huang 1988; Fenice et al. 2000; Paul et al. 1993; Smidsrod and Skjak-Braek 1990; Walker and Connick 1983), although other algal polysaccharides have been used (Hammill and Crawford 1997). The raw material, the kelp macroalga (*Macrocystis pyrifera*), is a renewable marine resource of great abundance in the Pacific Ocean (Lada et al. 1999); it is harvested regularly and processed (Hernández-Carmona et al. 1999) mainly for the food industry. The most common experimental formulation for bacterial inoculants is macrobeads with a diameter of 1-4 mm either for agricultural or environmental use (Bashan 1986a, 1998; Gonzalez and Bashan 2000). Nonetheless, their relatively large size is disadvantageous for agricultural uses. To produce smaller size beads, two possibilities exist: (1) to mechanically crush large beads or solid alginate sheets and then to sieve the powder to the desired size, and (2) to produce micro beads directly using an appropriate technology.

The aims of this study were to demonstrate: (1) the technical feasibility of direct alginate microbead production; (2) that these microbeads can be dried, thus conserving the majority of the bacterial population; (3) that these microbeads can be attached to seed prior to sowing; and (4) that these preparations can release the bacteria and enhance plant growth. This study employed the wild type bacteria, *Azospirillum brasilense* Cd, commonly used for agricultural inoculation (Bashan and Holguin 1997) as a model microorganism and wheat and tomato as model plants.

Materials and methods

Equipment for microbead production

The production of microbeads (encapsulated bacteria in an alginate matrix) has been accomplished using a prototype device described earlier (Fig. 1; Carrillo and Bashan 1997) after incorporating minor modifications. These include using stainless steel for its construction to allow sterilization by autoclave, and positioning the entire equipment at 45° inclination allowing production of a stream of droplets instead of vertical output of spray. It was built of commonly used materials by the CIB Technical Directorate's workshop. The basic design can be modified or modularly enlarged to produce a larger amount of microbeads without compromising the basic features. This prototype produces microbeads ranging in size from 100 to 200 µm.

Bacteria and bacterial growth conditions

Azospirillum brasilense Cd (ATCC 29710) served as a model bacterium for this study and was cultivated according to the standard procedure for this genus (Bashan et al. 1993). Briefly, the preinoculum was prepared on a nutrient agar (Difco, Mich.) plate at 30±2°C for 48 h. Several identical developing colonies were transferred into a liquid nutrient broth medium and were grown for 12 h at 30±2°C in a rotary shaker at 120 r.p.m. The inoculum was harvested by centrifugation (7,000 g, 10 min), washed twice in potassium phosphate buffer (pH 7.0, 0.06 mol l^{1}) and supplemented with 0.15 mol NaCl l^{1} (Bashan 1986b). To obtain a higher yield of bacteria, a trypton-yeast extract-glucose (TYG) medium was used (Prabhu et al. 2000). This medium contains: 5 g trypton l^{-1} (Difco), 5 g yeast extract l^{1} (Sigma), and 5 g D-glucose l^{-1}. This basic medium was improved by supplementing it with the following salts: 4.8 g KOH l^{1}; 1.2 g NaCl l^{1}; 0.25 g $MgSO_4 \cdot 7H_2O$ l^{-1}; 0.13 g K_2HPO_4 l^{-1}; 0.22 g $CaCl_2$ l^{-1}; 0.17 g K_2SO_4 l^{-1}; 2.4 g Na_2SO_4 l^{-1}; 0.5 g $NaHCO_3$ l^{-1}; 0.09 g Na_2CO_3 l^{-1}; 0.07 g Fe(III) EDTA l^{-1}; the pH was adjusted to 7.0 after sterilization. The N-free OAB medium (Bashan et al. 1993) supplemented with 8 mM fructose and 0.5 mM KNO_3 (Sadasivan and Neyra 1985) was used as a reference medium. The growth conditions of bacteria in all media were similar and all cultures were similarly washed after growth.

Microbead formation

Ten millilitres of bacterial suspension obtained after cultivation in improved TYG medium was mixed under aseptic conditions with 2% sodium alginate (CICIMAR, La Paz, Mexico) under low stirring at ambient temperature for 1 h when all the ingredients were thoroughly mixed. The bacterial concentration at this stage was 1010 colony-forming units (CFU) ml-1 solution. Alternatively, skim milk without Ca (Difco; 0.75%) was added to the alginate-bacterial suspension to produce beads that are more biodegradable (Bashan 1986a). Each of these suspensions was then placed in an Erlenmeyer flask or a beaker that was attached to the microbead-producing device and the device was pressurized at 10-15 psi using a commercial air compressor. The bacterial suspension sucked from the Erlenmeyer flask was then forced to pass through a 222-µm-diameter capillary exit, which created a fine spray of miniature droplets. The mist was sprayed into a 25x40-cm stainless steel flask rotating at 40 r.p.m. and containing 0.1 M $CaCl_2$. Microbeads were formed instantly upon contact of the droplets with the solidifying solution. The microbeads were allowed to cure in the $CaCl_2$ solution for 30 min. This procedure produced microbeads ranging in size from 100 to 200 µm. At this stage, the bead bacterial concentration was $2x10^{11}$ CFU g^{-1} microbeads on average (fresh concentration; for more details see the Result section). After extracting the wet beads from the $CaCl_2$ solution, they were rinsed in 500 ml saline solution (0.85% (w/v) NaCI) 4 times under aseptic conditions and transferred into fresh TYG medium. Bacteria were multiplied in this mixture for an additional 12 h at 30±2°C and 100 r.p.m. Then the microbeads were separated from the suspension by filtration using Whatman no. 4 filter paper, and rinsed 3 times with 500 ml saline solution. The population in fresh beads after this secondary multiplication always reached a concentration of at least $1x10^{12}$ CFU g^{1} microbeads. Usually, wet preparations of microbeads at this stage were used for plant inoculation immediately or after overnight storage at 4±1 °C, which did not affect the number of bacteria in the preparation.

Drying procedures

Ten grams of microbeads was placed in a thin layer on a filter paper in a Petri dish and dried at 38±l°C for 48 h. Then the dry microbeads were collected and stored in hermetically sealed containers with silica gel until usage. Additionally, dry beads were prepared by standard lyophilization (Virtis 5L, MY).

Seed coating with microbeads and microbead counts from seeds

Dry or wet microbeads were manually mixed with dry seeds of wheat and tomato. Alternatively, a very dilute solution of adhesive (described later) was applied to the seeds. This solution created a sticky surface but did not allow the seeds to stick to one another. Four sticky substances were used: (1) lecithin (solidified fatty acids derived from soybean, 0.1% and 0.5% dissolved in ethanol that was evaporated immediately by exposure to ambient temperature and dried with an unheated hair dryer); (2) 0.5% Agrex-f (Laboratorios Agroenzymas, Tlalnepantla, Mexico City) dissolved in water; (3) synthetic paper adhesive (0.1%, 0.5% and 1% Resis-

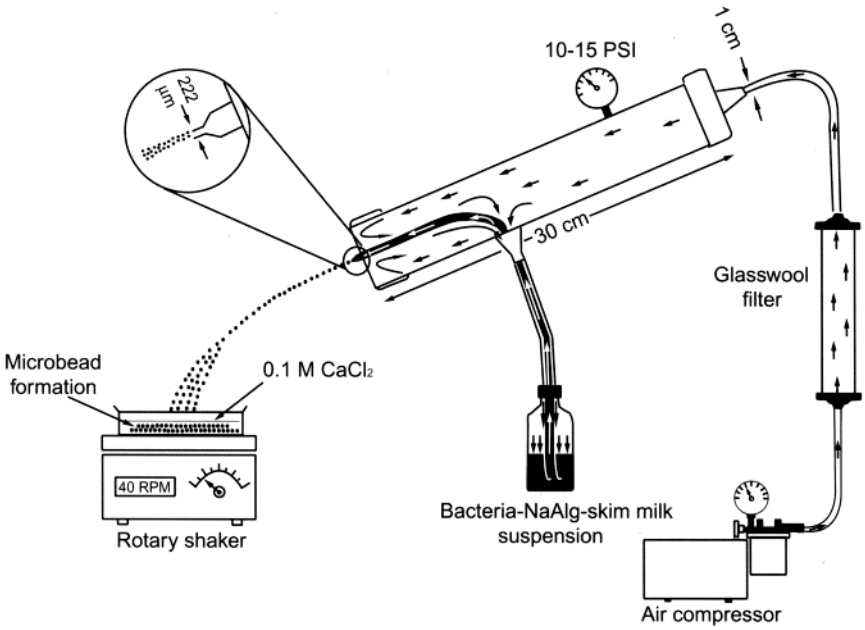

Fig. 1 Equipment for the production of microbeads. The original drawing is reproduced from Carrillo and Bashan (1997), by permission of the publisher

tol, Mexico) dissolved in water; and (4) undiluted mineral oil. All these sticky substances were first mixed, separately, with the seeds. Then the microbeads were mixed with the sticky seeds manually. Microbeads adhering to seeds were counted under stereoscopic microscope or by taking images of seeds by image analyzer (Image Pro Plus 4.1, Dell, USA, connected to an Olympus BX41 microscope) and allowing automatic counting of microbeads by the software. Data are presented using the following indices: 1=<50 microbeads seed^{-1}; 2=50-100 microbeads seed^{-1}; 3=100-150 microbeads seed^{-1}; 4=150-200 microbeads seed^{-1}.

Bead and root bacterial counts and slow release of bacteria from beads

The beads were dissolved in 0.2 M, pH 7.0 phosphate buffer for 1-2 h at 30°C in a rotary shaker at 100 r.p.m. which released the bacteria entrapped in or covering the microbeads. The released bacteria were counted using the conventional plate count method on nutrient agar (Difco). The slow release of bacteria from the microbeads was accomplished as follows: 0.5 g washed microbeads containing immobilized bacteria was transferred into 10 ml sterile saline solution [0.85% (w/v) NaCl] and gently shaken at 30±1°C for 16 h. Then, triplicate 0.1-m1 samples of saline solution were collected, and the number of released bacteria was determined by the plate count method on nutrient agar plates. The microbeads were rinsed twice with sterile water and transferred into fresh saline solution, and the procedure was repeated after 24 h. Afterwards, the microbeads were kept at 4±l °C under a thin layer of water, and the procedure was repeated after 5 days and again after 10 days (Bashan 1986a). After the inoculated plants were allowed to grow, they were harvested and root colonization by *A. brasilense* strains was determined as described before (Bashan and Levanony 1989).

Transmission and scanning electron microscopy

Over 25 of each type of wet and dry microbead, taken randomly from the production line of microbeads, were prepared for observation by two types of electron microscopy using methods described earlier for scanning electron microscopy (SEM) (Puente et al. 1999) and for transmission electron microscopy (TEM) (Lebsky et al. 2001).

Biodegradation of microbeads in desert

Four types of beads (alginate alone, alginate with 0.75% skim milk, and each with and without immobilized bacteria) were placed in 5x5-cm very fine mesh (10-20 µm) nylon bags, with 40 microbeads bag^{-1}, and were buried 5 cm below the soil surface in the natural soil described below for plant cultivation. The soil was maintained at 30°C for 15 days and was kept slightly below water saturation by adding distilled water as necessary. Every 3 days the bags were pulled out from the soil and each of the beads was examined under a stereoscopic microscope. The following indices were used to score the biodegradation of beads: 0=microbead not degraded; 1=little degradation with small holes and deformations in the bead structure; 2=completely degraded and the microbead was absent from the bag. Data presented are from all the beads examined of each type.

Inoculation of wheat plants with microbead inoculant containing *A. brasilense* as PGPB

It was desirable to check the microbead carriers under the realistic soil conditions prevailing in desert area agriculture. At the same time, it was desirable to avoid masking the effect of *A. brasilense* sp. inoculation by too rich a desert soil where the PGPB is less effective (Carrillo-Garcia et al. 2000). All experiments described in this study were carried out in pots. Pot experiments were conducted in a 1:1 (v:v) mixture of 500-mesh-sieved poor desert soils obtained from barren areas where perennial plants usually do not grow, and sieved rich desert soils found under the canopies of old mesquite trees in the same areas supporting exuberant perennial growth (Carrillo-Garcia et al. 1999). The detailed analysis of each soil was published earlier (Bashan et al. 2000; Carrillo-Garcia et al. 2000). The soil mixture contained the following: 3,750 mg Ca kg^{-1} dry soil; 790 mg Na kg^{-1} dry soil; 750 mg Mg kg^{-1} dry soil; 2,300 mg N kg^{-1} dry soil; 1,000 mg total P kg^{-1} dry soil; 100 mg orthophosphate kg^{-1} dry soil; 500 mg K kg^{-1} dry soil; 0.74 mg Cu kg^{-1} dry soil; 0.77 mg Zn kg^{-1} dry soil; 3.85 mg Fe kg^{-1} dry soil; 55 mg Mn kg^{-1} dry soil; 12,000 mg total dissolved solids, kg^{-1} dry soil; conductivity, 2,400 µS cm^{-1}; 35.3 g total C kg^{-1}; 18.6 g inorganic C kg^{-1}, canon exchange capacity, 20.6 mEq 100 g soil^{-1}; pH 6.78. Wheat (*Triticum aestivum* cv. Batequis F-97), and tomato (*Lycopersicon esculentum* Mill. cv. APT-127; Asgrow, Mexico) seeds were coated with microbead-encapsulated bacteria as follows. The growth substrate was placed in round, white, opaque

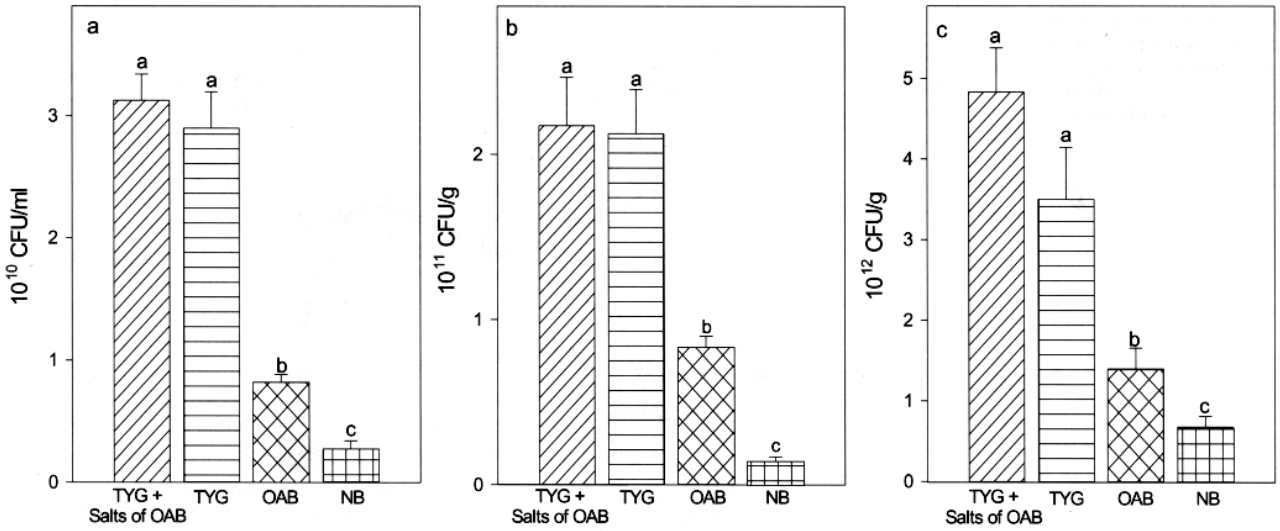

Fig. 2 a Development of *A. brasilense* Cd in various growth media. **b** The number of encapsulated bacteria in microbeads using the same media as part of the bead structure. **c** The level of bacteria in various microbead after an additional growth period inside the bead supported by the same media. Bars denoted by a *different letter* differ significantly at $P \leq 0.05$ according to one-way ANOVA. Error bars represent SE. TYG Trypton-yeast extract-glucose medium, OAB N-free medium supplemented with fructose and NO_3 , *NB* nutrient broth, CFU colony-forming unit

100-m1 pots of 5.5 cm diameter and 4.5 cm effective soil height. The substrate was saturated with distilled water before sowing. The pots were immediately sown with inoculated seeds. The level of inoculation was 200 microbeads seed-^{1} on average, corresponding to $2x10^{5}$ CFU seed-1. Pots containing wheat were incubated in a growth chamber at 22±2°C, for 14 h under illumination of 200 µmol m^{-2} s^{-1} (Conviron TC 16; Controlled Environments, Winnipeg). To prevent the collapse of wheat leaves during longer experiments, each pot was equipped with a 30-cm-high mechanical support made of galvanized 1-mm wire and three plastic rings of 5.5 cm diameter spaced vertically every 10 cm. Tomato plants were inoculated similarly to wheat but were incubated at 30±2°C. No fertilization was applied, as the plants did not show any deficiency during the course of the experiment. Distilled water was added only when needed to keep the pots moist without exceeding the water holding capacity. Plant height was evaluated during the growth and the dry weight of roots and leaves was measured at the end of the experiments, usually 21-30 days after germination.

Dry weight determination

Dry weight was measured by placing the shoots and roots, separately, into small, pre-weighed brown paper bags for large root systems or pre-weighed aluminium foil pouches for small root systems and drying them in a forced-draught oven at 75±2°C for 16 h.

Experimental design and statistical

Six experiments for wheat and three for tomato were carried out. Four of the wheat experiments and the three tomato experiments were set up in a randomized design in identical manner; ten replicates each, containing eight seedlings pot^{-1} (i.e. a total of 80 plants per treatment). The experimental design of the other two wheat experiments was a randomized block design (five blocks, four treatments, four pots per treatment, ten plants per pot). Treatments included: plants inoculated with live *A. brasilense* in microbeads, heat-killed *A. brasilense* in microbeads, microbeads alone and non-inoculated plants. Results of all experiments for each plant species were combined for statistical analysis. For the laboratory experiments, experiments for the selection of the best medium were done in triplicate where one Erlenmeyer flask served as a replicate. Adhesion of microbeads to seeds was done in triplicate where ten seeds served as a replicate. For the biodegradation of microbeads in soil, ten replicates per treatment were used. One pot containing three bags of fine nylon mesh, each bag containing 40 microbeads, served as a replicate. Over 100 photomicrographs of microbeads were taken under electron microscopy in this study. All these laboratory experiments were repeated 3 times and results presented are the averages of all experiments. Statistical analysis was done by ANOVA or Student's t-test at $P \leq 0.05$ and data were analysed by Statistics software (Statsoft, Tulsa, Okla.). All graphical data are shown with SEs.

Results

Mass production of cells for immobilization and bacterial concentration in microbeads

Four different culture media were screened for their capacity to sustain high growth rates of *A. brasilense* and their suitability for immobilization of the bacteria in microbeads. The media, organized in order of greater bacterial growth were: TYG plus salts, TYG, OAB supplemented with fructose and NO_3, and nutrient broth. The TYG medium with salts of the OAB medium was found to be the superior medium, and the commonly used nutrient broth yielded the lowest bacterial population (Fig. 2a). After immobilization, the population in each type of bead significantly declined (Fig. 2b). However, the relative differences in the populations supported by each media persisted. Secondary multiplication increased the populations but the order stated above remained unchanged (Fig. 2c). Drying of the beads, both by lyophylization and by low heat, yielded dry microbeads loaded with significant amounts of bacteria; $3±0.9x10^{9}$ CFU g^{-1} heat-dried microbeads and $2.4+0.5x10^{9}$ CFU g^{-1} freeze-dried microbeads. SEM and

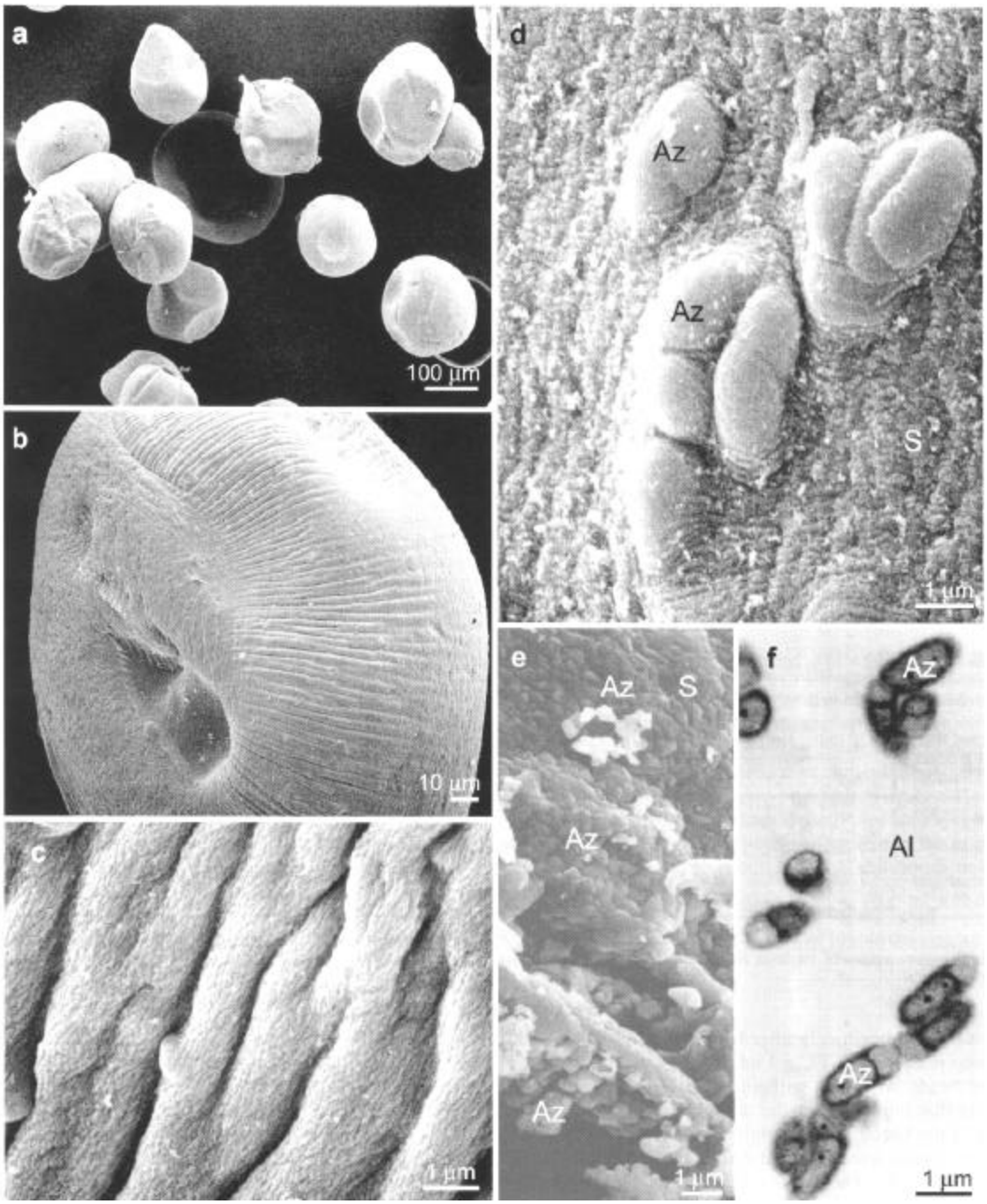

Fig. 3a-f Scanning electron microscopy of microbeads. **a** Microbeads after formation and curing with or without bacteria appear similar at this magnification; **b** a microbead without bacteria; **c** a close up of the surface of a microbead without bacteria; **d** microcolonies of *A. brasilense* Cd on the surface of wet microbead; **e** microcolonies of *A. brasilense* Cd on the surface of a dry (lyophilized) microbead; **f** a transmission electron microscopy of *A. brasilense* Cd inside a wet microbead. *Al* Alginate, *Az A. brasilense* Cd, S surface of the microbead

TEM revealed that the surfaces of the beads, as well as their interiors, were populated with bacteria (Fig. 3).

Slow release of bacteria from dry and wet microbeads

Wet and dry microbeads containing bacteria were tested for their ability to release the entrapped bacteria. Table 1

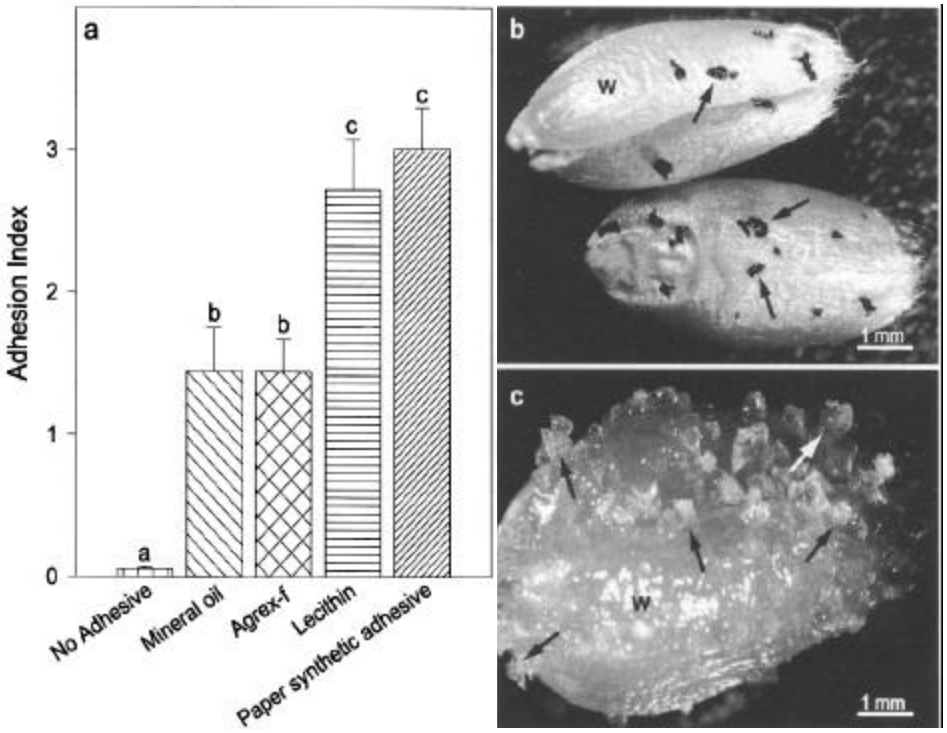

Fig. 4 a Attachment of dry microbeads to the surface of seeds using four adhesive agents. Bars denoted by a *different letter* differ signify-cantly at $P \leq 0.05$ according to one-way ANOVA. *Error bars* represent SE. **b** Attachment of heat-dried microbead with lecithin to wheat seeds, dyed by 1% methylene blue for better visualization of micro-beads. **c** Attachment of a lyophilized dry bead with lecithin to wheat seed (W). *Arrows* indicate microbeads. *1* <50 Microbeads seed^{-1}, *2* 50-100 microbeads seed^{-1}, *3* 100-150 microbeads seed^{-1}, *4* 150-200 microbeads seed^{-1}

Table 1 Slow release of *A. brasilense* Cd from wet and dry microbeads. *CFU* Colony-forming units

Microbead concentration (CFU g^{-1})								
Elapsed time after production of the beads	10 min		24 h		144 h		240 h	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
Type								
Alginate	$9.2\pm0.3\times10^{5a}$	25 ± 12^{c}	$2.2\pm0.3\times10^{5}$	$3.7\pm0.6\times10^{3}$	$2.1\pm0.6\times10^{5}$	$2.3\pm0.4\times10^{5}$	$7.2\pm0.2\times10^{4}$	$6.3\pm0.1\times10^{5}$
Alginate plus skim milk	$7.8\pm0.3\times10^{7b}$	17 ± 6^{d}	$1.7\pm0.4\times10^{6}$	$4.62\pm0.5\times10^{4}$	$9.1\pm0.7\times10^{5}$	$5.2\pm0.2\times10^{5}$	$4.2\pm0.9\times10^{5}$	$2.7\pm0.6\times10^{5}$

[a] Initial concentration of bacteria in wet alginate bead was 2×10^{11}
[b] Initial concentration of bacteria in wet alginate plus skim milk bead was 2.2×10^{11}
[c] Initial concentration of bacteria in dry alginate bead was 3×10^{9}
[d] Initial concentration of bacteria in dry alginate plus skim milk bead was 3.9×10^{9}

shows that immediately after formation, wet beads were able to release 10^5 CFU g^{-1} microbeads and 10^7 CFU g^{-1} microbeads with and without skim milk, respectively; levels that remained for the next 24 h. To avoid starvation of the bacteria, the sampling was stopped after 24 h and the beads were stored at 4±1 °C for additional 5- and 10-day periods. The beads continued to release bacteria when heated to 30°C after those periods. The numbers of released bacteria were comparable to the initial measurement after 5 days and were a little lower after 10 days. A similar trend was observed with beads containing skim milk. However, the released number of bacteria was higher after each period of sampling (Table 1). Dry microbeads did not release significant numbers of bacteria upon formation, and even after 24 h the number released was lower than for wet beads. However, after longer periods of storage and after being transferred to wet conditions, they released the bacteria equally as well as wet microbeads (Table 1).

Attaching microbeads to seeds using adhesives

Four types of adhesives were used to attach the microbeads to wheat seeds, since only zero to five dry microbeads clung to each seed without adhesive. Wet microbeads, in contrast, stuck well at over 100 microbeads seed^{-1} without adhesive. Of the four adhesives, 0.5% and 1 % lecithin and 0.5% Resistol were the best and equal in adhesiveness (Fig. 4a). Image analysis of the surface indicates random distribution of the microbeads, either wet or dry, over the surface of the seeds (Fig. 4b).

Biodegradation of microbeads in soil

Biodegradation of microbeads in soil was followed for 15 days and was influenced by the presence of *A. brasilense* and skim milk in the bead construction. The observed level of biodegradation of beads containing bacteria or skim milk after 15 days (Fig. 5) was much greater than for beads containing no skim milk and no bacteria (Fig. 5).

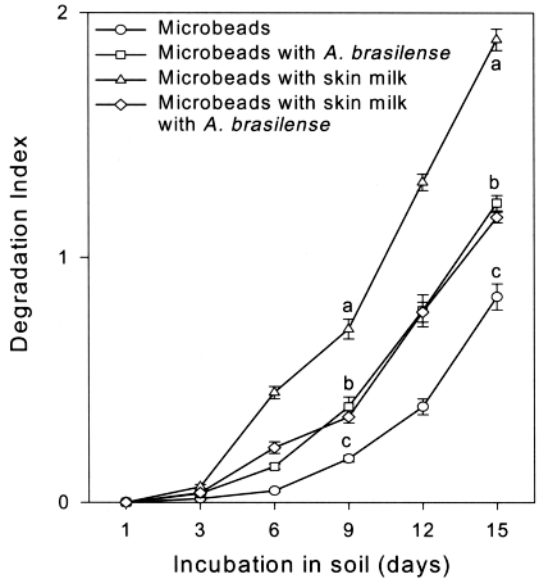

Fig. 5 Biodegradation of various types of bead in poor soil with time. Data points at the same time denoted by a *different letter* differ significantly at $P \leq 0.05$ according to one-way ANOVA. *Error bars* represent SE. *0* microbead not degraded, *1* little degradation with small holes and deformations in the bead structure, *2* completely degraded and the microbead was absent from the bag

Inoculation of wheat and tomato plants with microbead inoculants containing *A. brasilense*

Nine independent inoculation experiments were conducted in a growth chamber environment to evaluate the efficiency of the microbead inoculant (six with wheat and three with tomato). Microbeads by themselves did not exhibit any improvement of plant growth when compared to non-inoculated controls or to heat-killed bacteria controls (data not shown). Similarly, inoculation with unloaded microbeads or with those loaded with live or heat-killed bacteria did not affect the germination of wheat and tomato seeds, which was at the commercially acceptable market level of >85% (data not shown). However, inoculation of both wheat and tomato plants with *A. brasilense* encapsulated in the alginate microbeads described in this study, in both wet and dry formulations, significantly increased plant height (Fig. 6a, b). Similar results were obtained with wheat plants. The dry weight of both shoots (Fig. 7a-c) and roots (Fig. 7c, d) of both tomato and wheat plants increased. The average concentration of *A. brasilense* Cd in the roots at the end of the experiments was 1.1×10^5 CFU g^{-1} dry root.

Discussion

The main advantages of alginate preparations are their non-toxic nature, degradation in the soil, their slow release of microorganisms into the soil (Bashan 1998; van Elsas and Heijnen 1990) and almost unlimited shelf life (Bashan and Gonzalez 1999). The preparation of macro-alginate beads 1-3 mm in diameter containing bacteria is fairly easy and is a mufti-step procedure (Bashan 1986a; Digat 1991). Several alginate-based preparations were evaluated for agricultural purposes including the encapsulation of biocontrol agents against soil-borne patho-

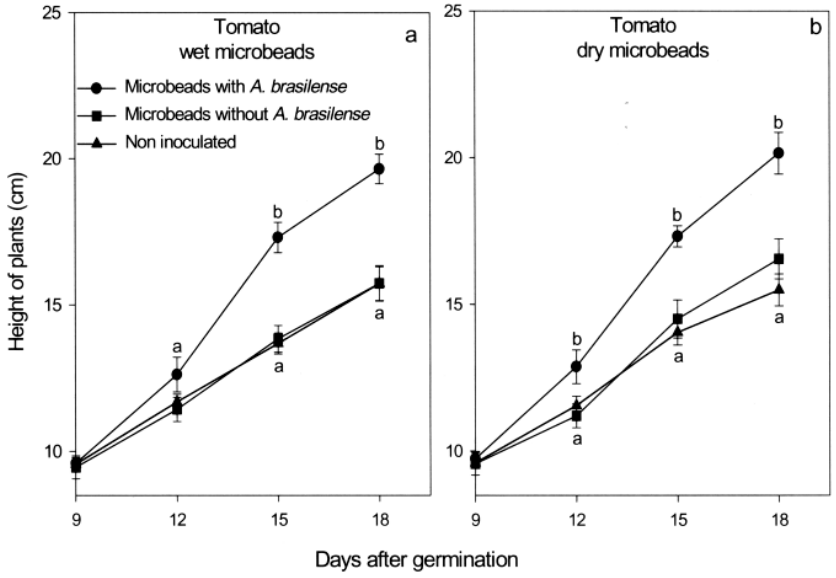

Fig. 6 The effect of inoculation with *A. brasilense* in microbeads on the height of wheat and tomato seedlings. **a** Wet microbeads; **b** dry microbeads. Data points at the same time denoted by a *different letter* differ significantly at $P \leq 0.05$ according to one-way ANOVA. *Error bars* represent SE

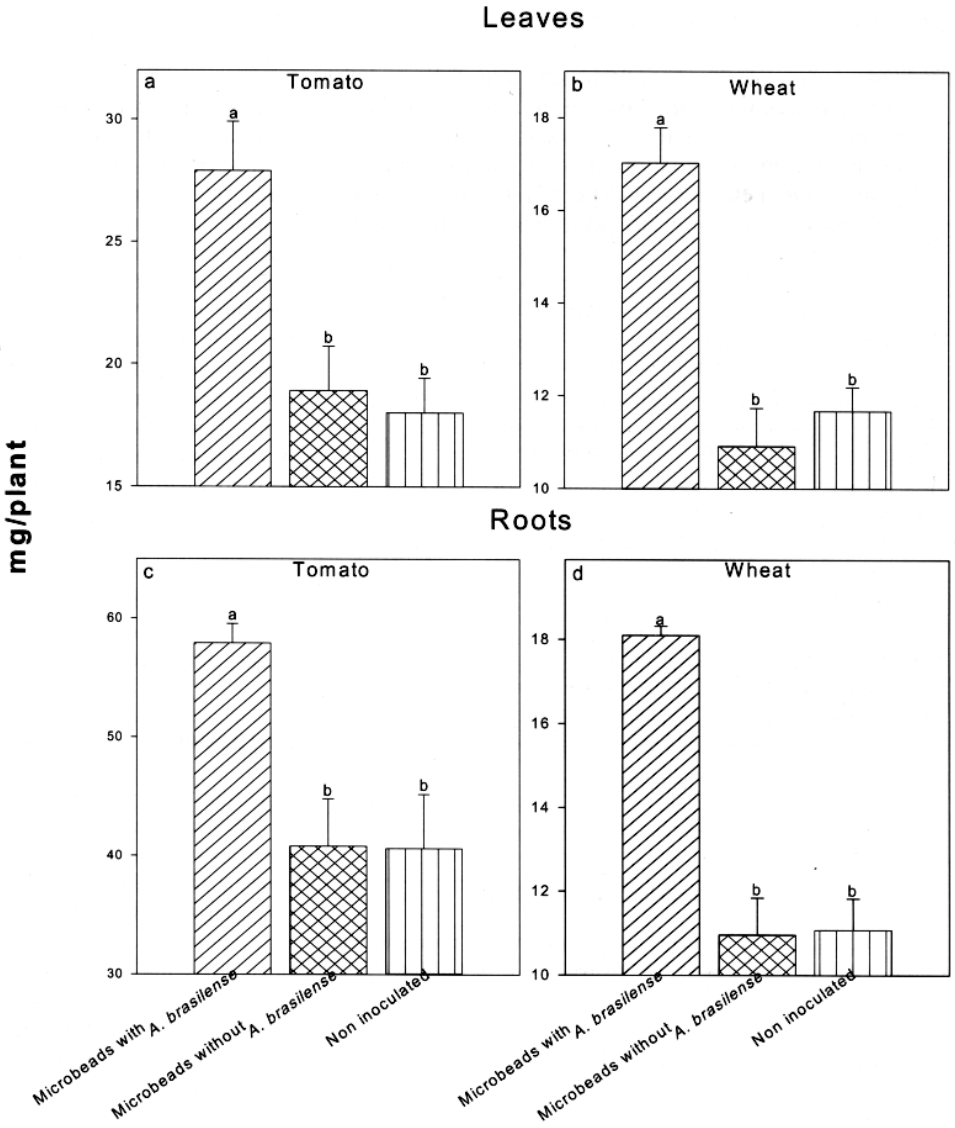

Fig. 7a-d The effect of inoculation with *A. brasilense* in microbeads on the dry weight of leaves and roots of wheat and tomato seedlings after 21 days. Bars denoted by a *different letter* differ signifycantly at $P \leq 0.05$ according to one-way ANOVA. *Error bars* represent SE

gens (DeLucca et al. 1990; Fravel et al. 1985; Lewis and Papavizas 1985; Russo et al. 1996) and phosphate-solubilizing bacteria (Vassilev et al. 1997). This technology was also employed to encapsulate the PGPBs *A. brasilense* and *Pseudomonas fluorescens* (Bashan 1986a) that were successfully used to inoculate wheat plants under field conditions (Bashan et al. 1987). Encapsulated genetically engineered *P. fluorescens*, released later into soil microcosms, showed significantly increased survival rates over non-encapsulated cells after 3 months (Trevors et al. 1992; van Elsas et al. 1992).

The use of macro-alginate beads has three major disadvantages:

1. O_2 diffusion into the bead is limited, making about 80% of the bead volume uninhabitable for aerobic PGPB (Sun et al. 1989).
2. The bacteria must move through the soil from the bead towards the plants (Bashan and Holguin 1994). Using conventional agricultural practices, the beads containing the bacteria might fall up to several centimetres from the seeds when beads are loosely mixed with seeds and sown together. The bacteria released from the beads would then have to migrate through the soil, facing competition from the native microflora. Sometimes, the absence of a continuous film of water would temporarily block their movement. These distances might be proven prohibitively large for many PBPGs, even those with proven soil motility like *A. brasilense* (Bashan and Holguin 1994). Root colonization might then not occur and no beneficial effect would be observed on the plants.
3. There is need for an additional, specialized treatment during sowing (Bashan 1998).

To overcome these problems, the microbead concept as conceived. If the beads were small but capable of encapsulating sufficient bacteria, it would be possible to pro-

duce a powder-like formulation that could be used in seed coating.

Microbeads made of various compounds are produced by the pharmaceutical industry (Broadhead et al. 1992; Matulovic et al. 1986; Wan et al. 1992), and can be produced in various diameters. Wet formulated beads of 2-50 µm in diameter were previously proposed for the degradation of pentachlorophenol by *Flavobacterium* sp. (Stormo and Crawford 1992). From an agricultural standpoint, these ultra-small beads have two main disadvantages, namely insufficient bacterial capacity and their wet preparation. Theoretically, a 50-pm bead cannot contain more than 10^4 CFU bead-t of *Azospirillum*-like bacteria. It is well established that for successful inoculation with *A. brasilense,* for example, 10^6 CFU seed-t are required (Bashan 1986b). Even higher populations are required of some biocontrol agents. Beads of 200 µm diameter may theoretically hold $>10^6$ CFU $bead^{-1}$, so even one bead of this diameter will be sufficient to inoculate the seed if there is insufficient attachment to accommodate the desirable larger number of microbeads. Wet beads are impractical for agricultural use since very few farmers have the technical capacity to make appropriate seed coatings under field conditions, but future developments may make possible the use of these wet preparations like fertilizer in a drip irrigation system.

In the industry, a large concern about the activity of the entrapped bacteria in macrobeads is the restriction of O_2 diffusion into the bead (Chang and Mo 1988; Sun et al. 1989). Active aerobic cells may be found to a depth of only 50-200 µm into the bead, so about 80% of the volume of a 2-mm bead may contain inactive or even dead cells (Chen and Humphrey 1988). Although microaerophylic bacteria such as *A. brasilense* or *Azospirillum*-related microorganisms that have no difficulty with low O_2 tensions are easily encapsulated, aerobic PGPBs will be stressed in a macrobead formulation. In the case of the microbead formulation proposed here, this is not a problem since the diameter is smaller than the critical depth. A minor disadvantage of microbead production, however, is that the entrapping procedure kills a large number of the bacteria because of cross-linking of the alginate-calcium complex with the bacterial cell wall. This phenomenon was observed earlier with *A. brasilense* (Bashan 1986a) and with *Burkhuldaria cepacia* in an alginate-clay matrix (Fravel et al. 1985). However, it is easy to avoid this problem by secondary incubation of the microbeads in a new growth medium. The surviving bacteria will multiply and restore the concentration to that in the original growth medium. Furthermore, another advantage of the microbeads is the possibility to produce them dry. In the dry agricultural preparation the bacteria are inactive but alive, as is preferable since their activities are needed only after seed germination and the decomposition of the beads.

In conclusion, a simple method is being proposed in this study to produce microbeads useful for the application of PGPB to agricultural plants. Field application of this novel application is still pending.

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