Alginate Beads as Synthetic Inoculant Carriers for Slow Release of Bacteria That Affect Plant Growth†‡

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Uniform synthetic beads were developed as carriers for the bacterial inoculation of plants. The beads are made of sodium alginate and skim milk and contain a large reservoir of bacterial culture which releases the bacteria at a slow and constant rate. The beads are biodegradable and produce no environmental pollution. The strength of the beads, the rate of bacterial release, and the time of their survival in the soil can be controlled by several hardening treatments. The final product, lyophilized beads, is simple to use and is applied to the seeds concomitantly with sowing. The released bacteria are available for root colonization immediately at seed germination. Dry beads containing bacteria can be stored at ambient temperature over a long period without loss of bacterial content; storage requires a limited space, and the quality control of a number of bacteria in the bead is simple. The level of plant inoculation with beads was similar to that with previously used peat inoculants, but the former method yielded more consistent results, as the frequency of inoculated plants was much higher. The former method provides a different approach for inoculation of plants with beneficial rhizosphere bacteria.

Inoculation of plants with nonsymbiotic rhizosphere bacteria for crop improvement is a complicated task. In most studies reported to date, variable and inconsistent results were obtained (1, 15, 17, 18, 23–25). Since the modes of action of these bacteria are as yet unknown (22), any application method with uncertain efficiency may be a crucial factor determining such inconsistent results.

Inoculation of legumes with their symbiotic rhizobia (27), a worldwide-common practice for the last several decades, is carried out by two main methods: (i) direct inoculation with bacterial culture, e.g., dipping seeds in a bacterial suspension, aerial spraying of bacteria, or spreading the bacteria in the sowing furrow by various drip systems mounted on the sowing drill and (ii) use of various solid-phase bacterial inoculants based on organic materials (mainly peat variants), including organic granular particles. Both methods are inexpensive, easy to use, and have simple formulation preparations, both for the farmer and the bacteria industry.

During the last decade there has been an increased interest in synthetic beads of various materials and dimensions for immobilization of microorganisms and cells and enzymes, antibodies, and other proteins. The main purpose of these beads was to immobilize or entrap the target organism or proteins for a long period to collect products produced by the entrapped living material. Release of the immobilized living material was considered a disadvantage of the system (4, 5, 7, 8, 12, 13, 16, 20, 21, 26). A few reports suggested the use of several polymer gels for entrapping fungi and bacteria for use as agricultural inoculants (9, 11, 14, 19).

The purpose of this study was to develop beads as a bacterial slow-release synthetic inoculant carrier which can substitute for the organic inoculants.

MATERIALS AND METHODS

Chemicals. Sodium alginate was obtained from BDH, Poole, England, iota-carrageenan was from Sigma Chemical Co., St. Louis, Mo., and skim milk was from Difco Laboratories, Detroit, Mich. All other chemicals were of analytical grade and were obtained from various commercial sources.

Bacteria. *Azospirillum brasilense* Cd (ATCC 29710) was used as a model organism throughout the study. *Pseudomonas* sp. strain 84313 was used in some experiments.

Plants and growth conditions. Plants of common wheat (*Triticum aestivum* cv. Deganit) were used as the model plant organisms. Seeds were obtained from Zeraim Gedera Co., Israel. Seeds were surface disinfected by immersing them in 1% NaOCl for 3 min at room temperature, thoroughly washed in tap water, and imbibed for 3 h in tap water. Sowing was either on wet filter paper (Whatman no. 1) in petri dishes or in pots containing soil. Planting in pots was at a depth of 0.5 cm with two to four plants per 5-liter pot.

Culture conditions, bacterial entrapment within solidified beads, and seed coating. *A. brasilense* Cd was cultured in nutrient broth (Difco) in a rotary shaker (200 rpm) at 30 ± 2°C for 24 to 48 h to a final concentration of 10^9 CFU/ml. Entrapment of bacteria within beads was carried out under sterile conditions in a laminar flow hood with organic polymers which were gamma irradiated with 2.5 megarads by using a cobalt-60 source at the Weizmann Institute of Science, Rehovot, Israel. The bacterial culture was aseptically mixed with 2% (wt/vol) sodium alginate powder and stirred gently for 1 h. In alginate-skim milk bead preparations, 10% (wt/vol) sterilized skim milk was applied prior to the alginate treatment. The obtained mixture was stirred for 1/2 to 2 h to ensure complete dissolution of all ingredients. The mixture was added dropwise with the aid of a 10-ml sterile syringe into gently stirred, sterilized 0.1 M $CaCl_2$ at room temperature, and beads immediately formed in the $CaCl_2$ solution. The resulting alginate beads (mean diameter, 2 mm) entrapped the bacterial cells. The beads were maintained in the solution at room temperature for an additional 1 to 3 h to obtain regular solid beads. The $CaCl_2$ solution was then pumped out, and the beads were washed twice with sterilized tap water. After washings, the beads were incubated in fresh nutrient broth medium for an additional 24 h for *P.*
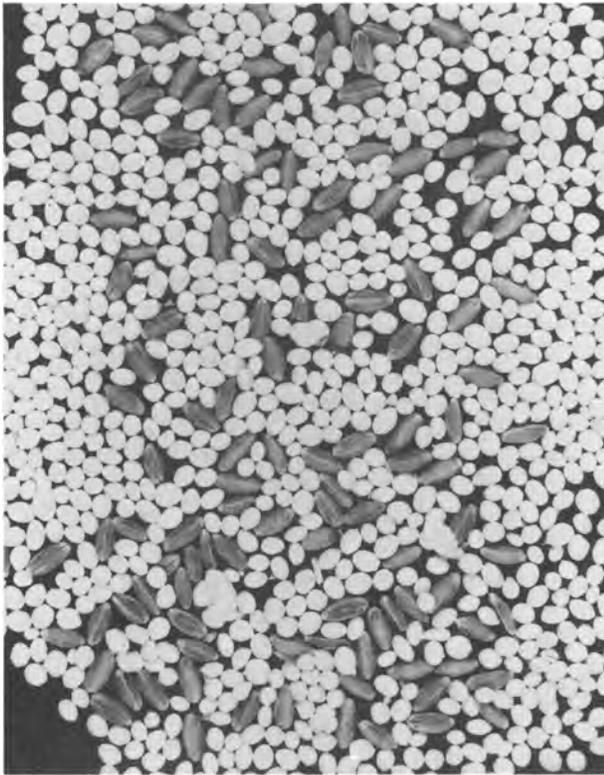
FIG. 1. Lyophilized alginate-skim milk beads mixed with wheat seed before sowing.

fluorescens and 48 h for *A. brasilense* in the rotary shaker at 30 ± 2°C to allow bacteria to multiply inside the beads. Then the beads were washed twice and collected. The wet beads were kept at 4 ± 1°C in hermetically sealed flasks under moist conditions for several days.

Thin layers of washed beads (after multiplication) soaked in a small amount of tap water in flasks were immediately frozen at −70°C by dipping the flasks in a bath containing dry ice immersed in technical-grade ethanol. After freezing, the beads were lyophilized to complete dryness and kept until usage in hermetically sealed flasks containing silica gel (Fig. 1).

Surface-disinfected wheat seeds were immersed in a solution containing 2% (wt/vol) alginate dissolved in 10^9 CFU of *A. brasilense* Cd per ml in sterile saline (0.85% NaCl) for 5 s, pulled out, immediately immersed in 0.1 M $CaCl_2$ for 30 s, and washed three times in sterile tap water. The alginate-coated seeds were air dried at 30 ± 2°C in a forced-draft oven, lyophilized as previously described for dry beads, or maintained under approximately 100% relative humidity in sealed petri dishes at 4 ± 1°C.

Hardening of beads containing immobilized bacteria. The rate of bacterial release and bead degradation in soil was controlled by hardening the beads by using the following methods.

(i) A portion of 100 beads containing immobilized bacteria was added to 20 ml of 0.1 M glutaraldehyde (aqueous solution) and gently stirred for 2.5 h at 30 ± 2°C. Excess glutaraldehyde was removed by washing with tap water at 10°C. To this preparation, 100 mg of gelatin (300 bloom, Sigma) was added with an additional 20 ml of 0.2 M glutaraldehyde (aqueous solution), and the mixture was

gently stirred for 1 h at 30 ± 2°C. The excess gelatin and glutaraldehyde were removed as previously described (26).

(ii) Sodium metaperiodate (5 mg) was added to 5 g of sodium alginate dissolved in 125 ml of distilled water and stirred at room temperature for 1 h. Another 5 g of sodium alginate and 10 ml of culture suspension (containing approximately 10^9 CFU/ml) were mixed with the sodium metaperiodate-alginate suspension. The obtained slurry was added dropwise into 0.1 M $CaCl_2$, and the beads formed were incubated for 1 h at 4 ± 1°C. The beads were then washed in distilled water and incubated in 400 ml of 0.5% (vol/vol) polyethyleneimine hydrochloride in 0.1 M $CaCl_2$ (pH 7.0 ± 0.2) for 24 h at 4 ± 1°C. Finally, the beads were washed again with tap water and stored wet at 4 ± 1°C (4).

(iii) Beads (5 g) containing immobilized bacteria were suspended in 100 ml of 0.5% (vol/vol) polyethyleneimine-hydrochloride in 50 mM $CaCl_2$ (pH 7.0 ± 0.2) with gentle stirring and kept for 24 h at room temperature. The beads were rinsed in water and incubated in 100 ml of 0.05% (vol/vol) glutaraldehyde dissolved in 10 mM sodium phosphate (pH 7.0 ± 0.2) at room temperature for 10 min with stirring. The beads were then washed with tap water and stored at 4 ± 1°C in a minimum amount of water (4).

Solubility of beads. To dissolve the alginate beads for bacterial counts, beads were immersed in various concentrations of potassium phosphate buffer (0.06, 0.2, and 0.4 M; pH 6.8 ± 0.1), known for its ability to dissolve alginate gels (7) (one bead per 1 ml of buffer in a test tube), and incubated for 30 to 60 min (depending on gel type) at 30 ± 2°C. To facilitate the solubility, the beads were vigorously shaken on a Vortex mixer (Vortex-Genie Scientific Industries). The released bacteria were counted by the plate count method on a nutrient agar plate (Difco).

Slow release of entrapped bacteria. A portion of 20 washed beads containing immobilized bacteria was transferred into 75 ml of sterile saline solution (0.85% [wt/vol] NaCl) and gently shaken at 30 ± 2°C for 24 h. Then triplicate samples of 0.5 ml of saline solution were collected, and the number of released bacteria was determined by the plate count method on nutrient agar plates. Then the beads were rinsed twice with sterile tap water and transferred into a fresh saline solution, and the procedure was repeated after an additional 24 h. Afterwards, the beads were kept at 4 ± 1°C under a thin layer of water, and the procedure was repeated after 30 days of this incubation.

Peat inoculant preparation. Peat inoculant was prepared by the method of Thompson (27). For preparation of 100 g of inoculant, 45 g of ground peat (40 mesh, Nitragin; Nitragin Mil.) was thoroughly mixed with 5 g of $CaCO_3$ and 20 ml of tap water (final pH, 6.8) in polyethylene bags sealed with cotton plug. The bags were gamma irradiated (2.5 megarads), and 30 ml of a 24-h-old bacterial culture (approximately 5×10^9 CFU/ml) was aseptically added to each bag, mixed, and incubated for an additional 7 days at 30 ± 2°C. Every 2 days the peat was mixed by shaking the bags. The final number of bacteria in the inoculant ranged from 5×10^7 to 5×10^8 CFU/g of inoculant. The bags were stored for up to an additional 7 days at 4 ± 1°C. At 1 day before plant inoculation, the bags were transferred to 30 ± 2°C.

Immobilization of bacteria in iota-carrageenan. A solution of 1.5% sterile iota-carrageenan was aseptically dissolved in bacterial suspension with gentle stirring, and the mixture was added, as described in the formation of alginate beads, to 0.1 M $MgCl_2$ solution and left in the solution for a further 30 min (26).

Scanning electron microscopy. For each observation with

TABLE 1. Effect of various treatments of seeds coated with alginate on the survival of *A. brasilense* in the coating matrix and on seed germination

Treatment of seeds	No. of bacteria surviving in the seed coating matrix			% Seed germination		
	A. brasilense[a]	*A. brasilense* after secondary bacterial multiplication[b]	Other bacteria	100 h after treatment	100 h after secondary multiplication[b]	After treatment, at normal moisture conditions[c]
Air dried	$(2.1 \pm 0.11) \times 10^1$	$(9.7 \pm 0.11) \times 10^1$	$(2.8 \pm 0.2) \times 10^3$	0	80 ± 2	89 ± 2
Lyophilized	$(4.8 \pm 0.21) \times 10^1$	$(7.2 \pm 0.36) \times 10^6$	$(3.1 \pm 0.4) \times 10^3$	0	0	7 ± 3
Wet	$(4.3 \pm 0.76) \times 10^4$	$(8.5 \pm 0.19) \times 10^8$	$(4.6 \pm 0.4) \times 10^5$	92 ± 4	92 ± 3	92 ± 3
Air dried without *A. brasilense*	ND[d]	ND	$(2.6 \pm 0.3) \times 10^3$	0	83 ± 2	88 ± 3
Lyophilized without *A. brasilense*	ND	ND	$(3.4 \pm 0.4) \times 10^3$	0	0	8 ± 3
Not coated or treated	ND	ND	$(3.9 \pm 0.6) \times 10^4$	0	ND	91 ± 2

[a] Inoculum level of 10^9 CFU/ml; 20 seeds were coated with 1 ml of solution.

[b] Seeds were shaken for 24 h at $25 \pm 2°C$ in nutrient broth.

[c] Seeds were placed on wet filter paper in Parafilm-sealed petri dishes.

[d] ND, Not determined.

scanning electron microscopy, 10 beads were fixed for 5 h in 5% glutaraldehyde solution (in distilled water), washed three times in 0.2 M cacodylate buffer (pH 7.2), fixed again for 1 h in 1% OsO_4, washed three times in cacodylate buffer, and then dehydrated with ethanol at the following concentrations: 50% for 30 min, 70% for 30 min, 70% for 10 h, 100% for 30 min, and 100% for 60 min. The whole procedure was carried out at $4 \pm 1°C$. The samples were dried in a critical point dryer (Tousimis Research Corp.) in Freon and later under a CO_2 atmosphere. The dried samples were stuck to stubs, covered with gold, and examined under a Philips SEM 505 scanning electron microscope at 10 to 15 kV. Lyophilized beads were not fixed and were treated as dry beads after critical point dryer treatment.

Inoculation of plants by beads. Inoculation by beads was carried out in petri dishes and pots. For petri dish inoculation, disinfected wheat seeds germinated on wet filter paper (Whatman no. 1) were inoculated by aseptically placing three beads per seedling near the growing roots. For pot inoculation, 10 beads per pot (4 to 5 beads per seed) were mixed with seeds before sowing or beads alone were placed in a sealed delicate nylon-cloth bag and buried near the seeds, approximately 5-cm deep in the following soils or substrate: vermiculite, brown-red degrading sand soil (Rehovot, Central Israel), loess raw soil (Kibbutz Nir-Am, Northern Negev, Israel), and alluvial soil (Kibbutz Negba, Northern Negev, Israel). Soils either in their natural state or gamma sterilized (2.5 megarads) were maintained near field capacity. Wheat plants (two per pot) were grown and underground drip irrigated with regular water daily. After inoculation, the surface of each pot was covered with a layer of 2 to 3 cm of a dry sterile vermiculite above the driplet position, which resulted in a dry sterile vermiculite layer maintained for several weeks to avoid the quick contamination of pots. A group of five pots was sampled weekly. The bags were opened, and the beads were examined under a stereoscopic light microscope for degradation with the following scale: 0, no degradation; 1, slight visible degradation on bead edges; 2, one-half to three-fourths of the beads were degraded; 3, the beads were fully degraded to the extent that they were not found in the nylon bag. In addition, bacterial inoculation of roots was estimated as explained in the following section.

Estimation of root bacterization and soil bacterial population. Root bacterization was estimated by several methods: (i) direct observations of roots with a scanning electron microscope; (ii) qualitative estimation by crushing roots in a fine glass homogenizer (Kontes Co., Vineland, N.J.) in 0.06

M potassium phosphate buffer (pH 6.8) and counting the CFUs on nutrient agar medium. This method is not quantitative because the bacteria tend to form massive aggregates on roots, and those cannot be dissolved by the homogenizer; (iii) count by the improved selection technique (3); (iv) count by the recently developed enzyme-linked immunosorbent assay technique (H. Levanony, Y. Bashan, and Z. E. Kahana, FEMS Symp. Microb. Communities Soil, p. 77–77a, 1985).

Soil bacteria were estimated by vigorously shaking 1.0 g of soil (in triplicate) in 100 ml of 0.06 M potassium phosphate buffer (pH 6.8) in Erlenmeyer flasks for 1 h at $25 \pm 2°C$ (200 strokes per min). The homogenate was immediately serially diluted by 10-fold dilutions, plated on nutrient broth medium, and incubated for 72 h at $25 \pm 2°C$. Bacterial CFUs were then determined.

Experimental design and statistical analysis. All experiments were repeated two to eight times and, unless otherwise noted, with 10 replicates in each. The replicates, arranged in a randomized design, consisted of pot, bead, Erlenmeyer flask, tube, or microwell. The results given are from a representative experiment. The significance is given with the designated standard error.

RESULTS

Effect of alginate seed coating on seed germination and viability of immobilized bacteria. Seeds coated with alginate were tested for germination and survival of the bacteria in the solidified matrix. Germination of air-dried coated seeds was similar to that of the untreated control seeds, but the survival of bacteria in the dried matrix was very poor; less than 100 bacteria of an initial population of 10^8 bacteria per seed survived (Table 1). Lyophilization of coated seeds improved the survival of the bacteria but prevented seed germination. Coated seeds with the immobilized bacteria under conditions of high humidity (without any drying procedure) maintained the highest population of bacteria, but the seeds began to germinate and thus were useless for mechanical sowing even if the germination itself was unaffected (Table 1).

Direct immobilization of bacteria in the beads during bead formation and after secondary multiplication in relation to alginate concentration. As bacteria were entrapped in the process of bead formation, most of the initial population was lost regardless of the alginate concentration or the addition of skim milk into the formed beads (less than 10^2 CFU/g of beads was detected). However, these few entrapped bacte-
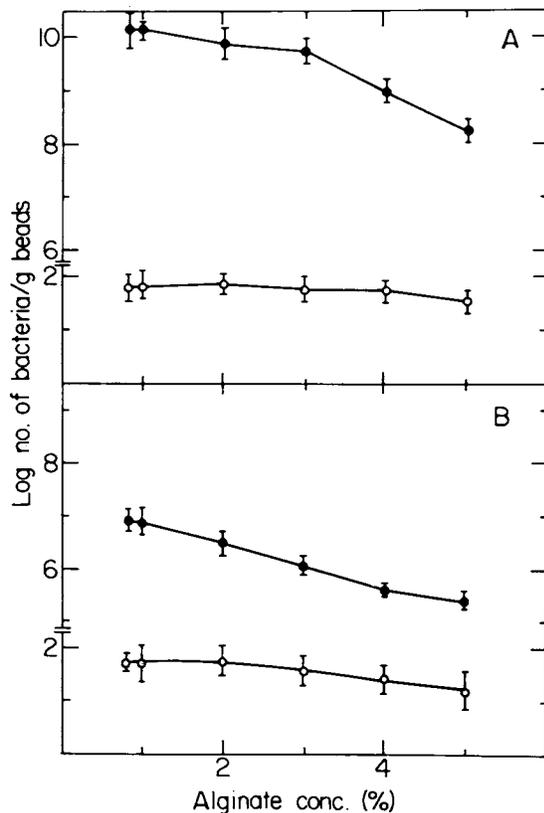
FIG. 2. Immobilization and multiplication of bacteria in beads with (A) and without (B) skim milk in relation to the alginate concentration. Symbols: O, number of immobilized bacteria; ●, number of bacteria after multiplication. Bars represent the standard error.

rial cells could multiply inside the beads to form a massive bacterial population, and in beads containing skim milk in their basic structure, the population increased even higher. An increase in the alginate concentration of the beads resulted in a decrease in the number of the bacterial population that multiplied in the beads (Fig. 2). However, even at the highest alginate concentration which allowed minimum secondary multiplication, the final number of bacteria was several orders of magnitude higher than the number of bacteria directly immobilized in the beads. However, beads of high alginate concentration were rarely produced because of the high viscosity of the initial alginate solution. Similar results were obtained with *Pseudomonas* sp.

Scanning electron microscopy of beads. Examination of beads at various stages of production revealed that the alginate bead is a rounded structure with shallow grooves every 10 to 15 μm and a few cavities on its rough surface (Fig. 3a, b, and c). Multiplication of the bacteria inside the bead resulted in the formation of numerous bulges all over the bead surface area (Fig. 3d, e, and f) inside which the bacteria population multiplied (arrows). Further examination of an exploded bulge revealed that it was formed of a bacterial microcolony covered with a solidified matrix (Fig. 3g), while the whole external surface was covered by bacteria (Fig. 3h). This pattern of multiplication was found in alginate beads with or without skim milk. When the bead was cut into a thick section, these bulges appeared also inside the beads (Fig. 4b). After lyophilization, alginate

beads without skim milk showed concentric layers of dried solidified matrix (Fig. 4a) on which dried bacterial microcolonies appeared (Fig. 4b). When alginate beads formed with skim milk were cut, the revealed inner structure was not organized by layers but contained various-shaped cross-linkages also containing numerous microcolonies (Fig. 4c and e).

Drying and storage effects on the size of the bacterial population in the beads. In general, drying of the beads reduced the number of the entrapped bacteria by several orders of magnitude. However, drying the beads by lyophilization caused a smaller reduction than drying in a forced-draft oven (Fig. 5A). Lyophilization of the *A. brasilense* culture yielded 0.05 to 0.1% recovery.

The lyophilized and wet beads stored for 3 months at 4 ± 2°C showed no decline in the bacterial population. In the wet beads there was even a slight increase during the first 2 months, but later the population decreased. Although there was a difference in the initial bacterial numbers between beads containing and those not containing skim milk, the same trend in the size of the bacterial population was observed (Fig. 5B).

Solubilization of beads. Beads were solubilized by immersing them in potassium phosphate buffer of various concentrations. In wet beads without skim milk, rapid solubilization occurred within 30 min. When skim milk was incorporated into the polymer the time required for solubilization was doubled, although it still remained relatively short (less than 1 h). The time required for solubilization of lyophilized beads was much longer; 18 h of incubation in the phosphate buffer did not result in total solubilization even when the beads were vigorously shaken several times in a Vortex mixer. Under these conditions, the beads were degraded into small particles but not solubilized. Increasing the molar concentration of the phosphate buffer up to 0.4 M did not enhance solubilization, and a decrease in the molar concentration from 0.2 to 0.06 M decreased the rate of solubilization. The immobilized bacteria in the beads in all bead types had no effect on the solubilization. Identical results were obtained with *Pseudomonas* sp.

Hardening of alginate beads. Three different hardening treatments were used on skim milk alginate beads. As compared with the total solubilization of untreated beads, the hardening procedure decreased solubilization. There was no significant difference between the three hardening treatments. Hardening of the beads had only a marginal effect on bacterial multiplication inside the beads. Hardening of the beads during bead formation slightly decreased the number of immobilized bacteria inside the bead, but after multiplication no significant difference in the bacterial number was found between hardened and nonhardened beads. Hardening of beads also decreased their biodegradation in soil (Table 2).

Effect of matrix solidification on bacterial colony morphology. A typical colony of *A. brasilense* Cd was immobilized and multiplied inside the beads. After bead dissolution and growth of the bacteria on a rich nutrient broth medium, polymorphic colonies were formed. In addition to the typical, dark pink, dry colonies of *A. brasilense* Cd, there were also white or pale pink, smooth-surfaced colonies with or without dry ridges or with an undefined border. Such polymorphic appearance of *A. brasilense* Cd was reported previously (10). These colonies were positively identified as *A. brasilense* Cd by the enzyme-linked immunosorbent assay (Levanony et al., FEMS Symp. Microb. Communities Soil, 1985).
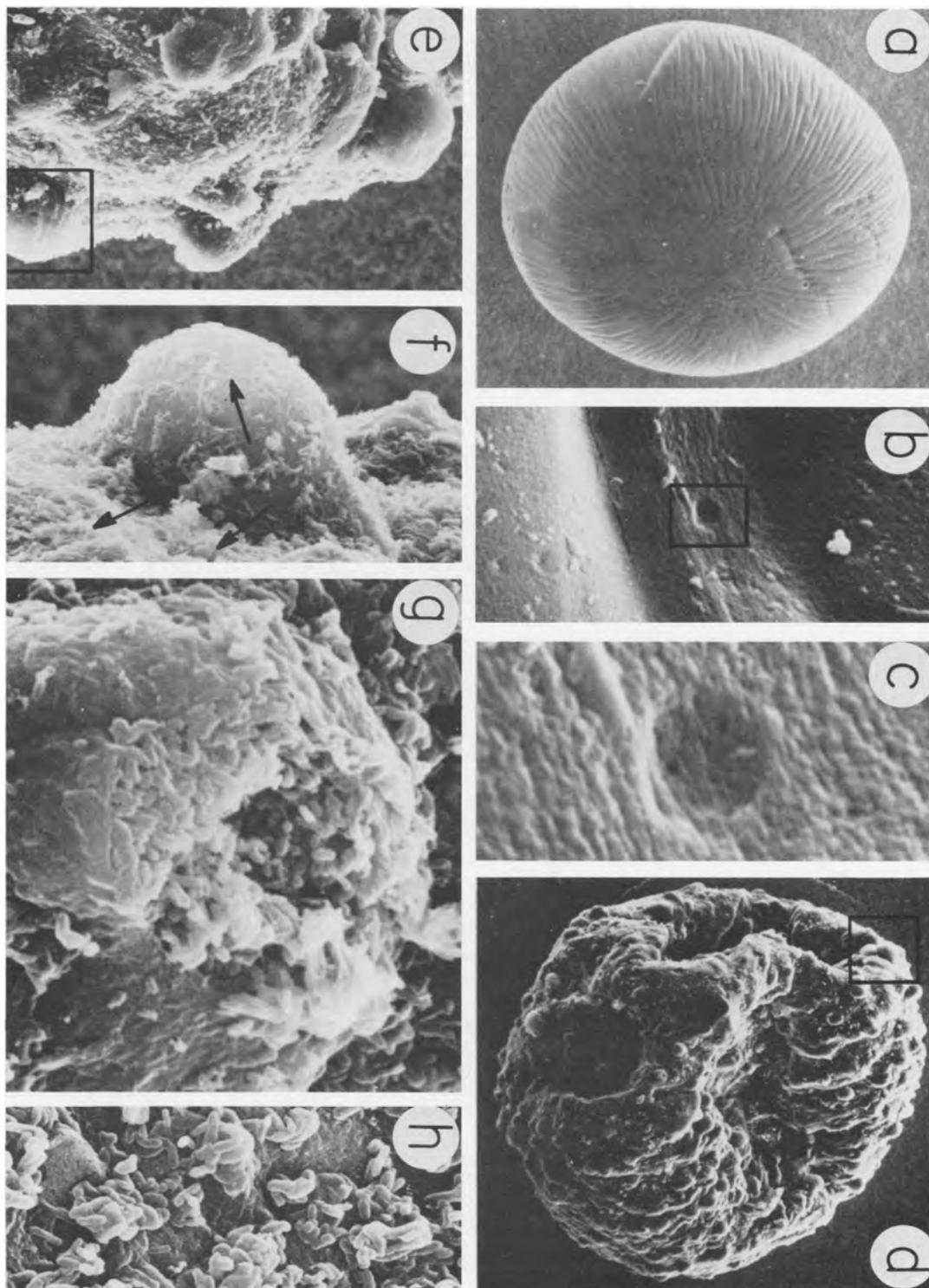
FIG. 3. Scanning electron micrographs of (a) wet alginate bead without bacteria (magnification, ×60), (b) grooves in bead surface (magnification, ×1,700), (c) insert of Fig. 2b, showing shallow cavity on the bead surface (magnification, ×7,700), (d) beads after bacterial multiplication (magnification, ×70), (e) insert of Fig. 2d, showing bulges (magnification, ×350), (f) insert of Fig. 2e, showing a single bulge (magnification, ×900), arrows indicating bacteria on bead surface, (g) exploded bulge, showing its internal content of bacterial colony (magnification, ×2,700), and (h) the area surrounding the exploding bulge (magnification, ×2,700), indicating a mass of released bacteria.
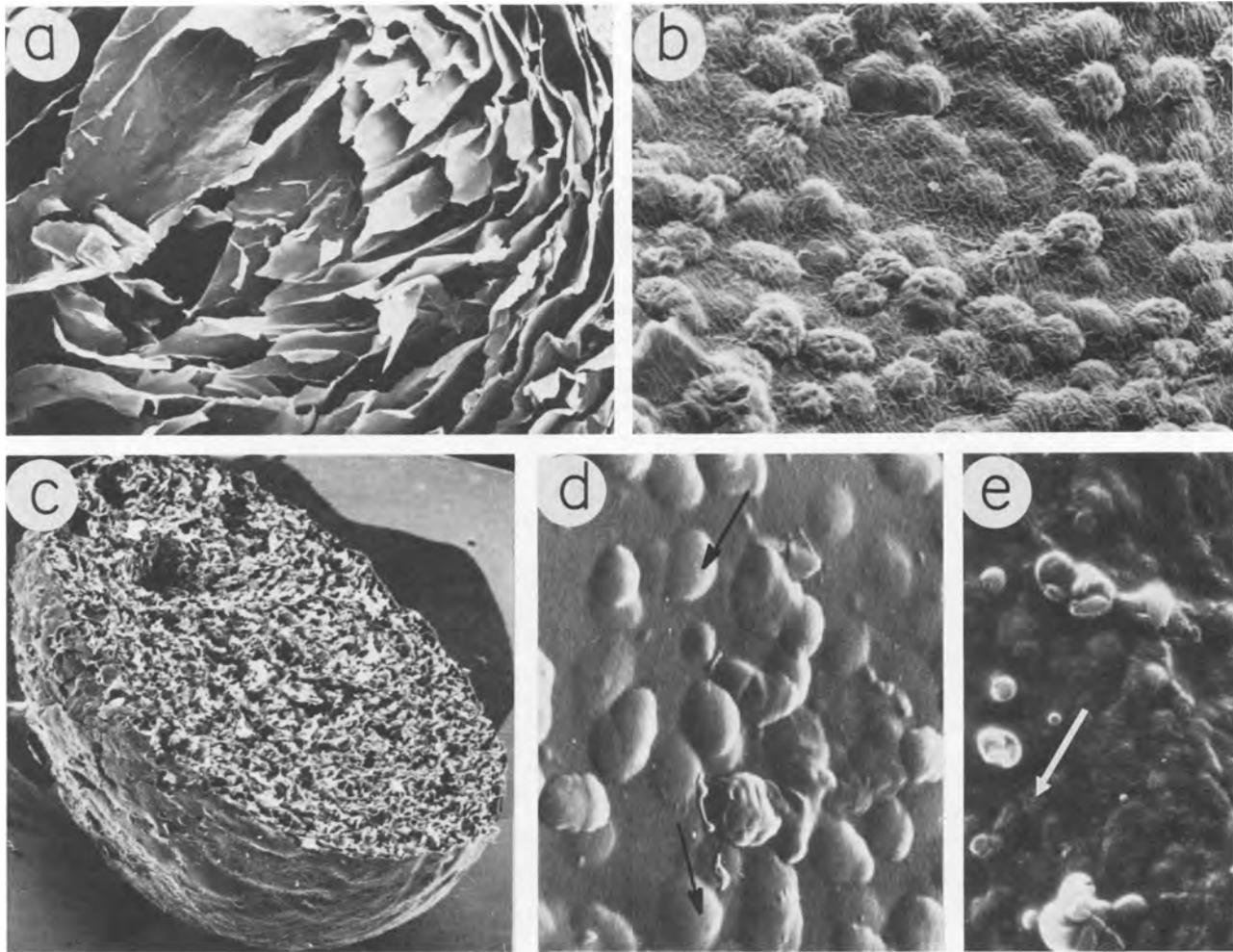
FIG. 4. Scanning electron micrographs of thick-section cut of (a) lyophilized alginate bead without skim milk, showing layers of the dried polymer (magnification, ×50), (b) wet bead indicating formation of similar internal bulges containing bacteria (magnification, ×2,000), (c) lyophilized alginate bead with skim milk (magnification, ×25), (d) lyophilized bulges in beads without skim milk containing bacteria (arrows) on the dried polymer layers (magnification, ×2,500), and (e) lyophilized bulges in beads with skim milk containing bacteria (arrow) (magnification, ×1,000).

Factors affecting bead size. With a 21-gauge syringe (dropwise, 2 to 3 beads per s), we formed beads of various sizes, ranging from 1.5 to 2.0 mm in diameter. Reducing the syringe size to 25 or 27 gauge decreased the diameter of the beads formed to a minimum of 0.7 to 0.8 mm. Further decrease was impossible because of the high viscosity of the initial alginate solution. The speed of bead production was limited to five beads per s, but the bead size remained constant. During the formation of approximately 500,000 beads, their size remained uniform as mentioned.

Bacterial slow release from beads. Beads containing bacteria after bacterial secondary multiplication were tested for their ability to release the bacteria. Figure 6 showed that beads either with or without skim milk released about 10^5 to 10^6 CFU/g of beads per 24 h from initial populations in the beads of 1.2×10^{10} and 5×10^7 CFU/g of beads, with and without skim milk, respectively. The samplings were stopped after 48 h because the bacteria were starved, and the beads were stored. After storage at 4 ± 2°C for an additional 30 days, the beads released bacteria when transferred to 30 ± 2°C. However, the number of released bacteria was smaller than after 48 h. Similar results were obtained with *Pseudomonas* sp.

Biodegradation of beads containing immobilized bacteria in soil. Bacteria-free beads as well as those containing bacteria either with or without skim milk were buried in nylon nets in two soil types (light-texture and heavy-texture soils) and examined weekly for their biodegradation. In general, beads containing skim milk were degraded at a faster rate in both soil types (Fig. 7A and B). Degradation was more complete in the heavy soil (Fig. 7B). Presence of bacteria inside the beads did not affect their degradation. In initially sterilized soils, beads were not degraded for the first 3 weeks and began to degrade only after 4 weeks (Fig. 7C and D). At that time, the total bacterial soil population increased in this sterilized soil, perhaps as a result of soil contamination. In nonsterilized soil, total bacterial population remained constant and was greater in heavy soil (Fig. 7E). The possibility of bead degradation by root development was eliminated, since roots did not penetrate the nylon net bags in which the beads were maintained. In addition, when roots were inoculated in petri dishes they did penetrate the beads but did not

TABLE 2. CFUs of *Azospirillum brasilense* Cd, degree of bead solubility, and degree of bead degradation of alginate beads hardened by several procedures[a]

Hardening procedure	No. of CFU[b]:		Degree of bead solubility[c]	Degree of degradation in soil 6 weeks after inoculation[d]
	Immediately after immobilization	After secondary bacterial multiplication		
Not treated	$(1.05 \pm 0.05) \times 10^2$	$(1.04 \pm 0.17) \times 10^{10}$	3	3
Glutaraldehyde + gelatin	$(7.4 \pm 1.4) \times 10^1$	$(9.4 \pm 0.11) \times 10^9$	1.8 ± 0.2	0.9 ± 0.1
Sodium metaperiodate + polyethyleneimine hydrochloride	$(6.9 \pm 1.7) \times 10^1$	$(8.8 \pm 0.19) \times 10^9$	1.4 ± 0.3	1.2 ± 0.2
Polyethyleneimine hydrochloride + glutaraldehyde	$(9.1 \pm 0.04) \times 10^1$	$(7.4 \pm 0.21) \times 10^9$	1.6 ± 0.3	1.3 ± 0.2

[a] For each experiment, 25 beads and three replicates were used.

[b] The number of CFUs was determined in bead fragments; CFUs should be considered the minimal bacterial number.

[c] Solubility scale: 0, no solubility; 1, visual deterioration of bead surface; 2, deterioration into fragments; 3, total solubility. For each treatment, 100 beads were tested.

[d] Biodegradation scale: 0, no degradation; 1, slight visible degradation on bead edges; 2, one-half to three-fourths of the bead degraded; 3, full degradation, to the extent that the beads were not found in the nylon bag. A sample of 100 beads was tested.

cause any additional visible damage to them during 4 weeks after inoculation.

Inoculation of plants by beads under controlled environments. Experiments were conducted in petri dishes containing filter paper and in pots containing various types of soils with a mixture of beads with and without skim milk and by using wet beads and lyophilized beads. The experiments indicated that plant roots were colonized when beads containing bacteria were used. Inoculation was better in a petri dish than in soils. Colonization of plant roots by bead inoculation was similar to that obtained by peat inoculation. However, the variation of colonization level by beads was smaller than that by inoculation with peat; i.e., all the plants
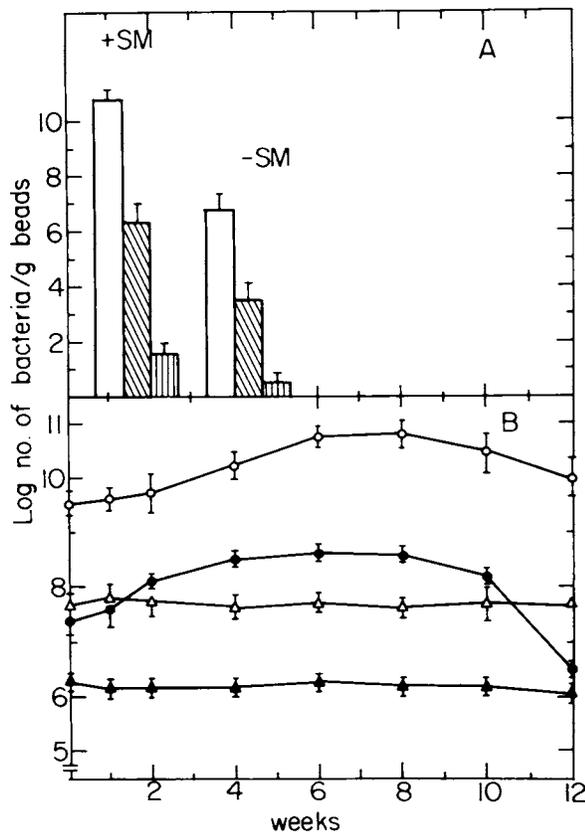

FIG. 5. Effect of bead drying on survival of bacteria immediately after drying (A) and after longer periods of time (B). Symbols: ☐, number of bacteria in wet beads; ▨, number of bacteria after lyophilization; ▥, number of bacteria after air drying; ○, survival in wet beads with skim milk; ●, survival in wet beads without skim milk; △, survival in lyophilized beads with skim milk; ▲, survival in lyophilized beads without skim milk. Bars represent the standard error.
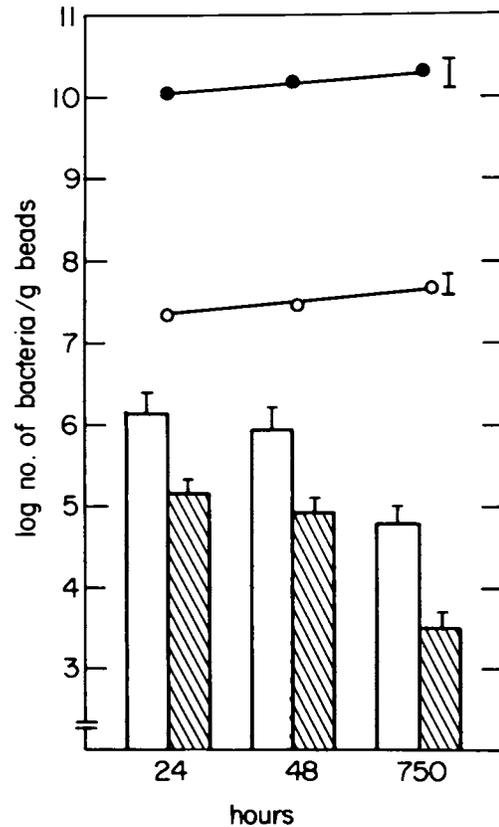

FIG. 6. Bacterial slow release from beads with time. Symbols: ☐, Release from beads containing skim milk; ▨, release from beads without skim milk; ○, number of bacteria in beads without skim milk; ●, number of bacteria in beads with skim milk. Bars represent the standard error.
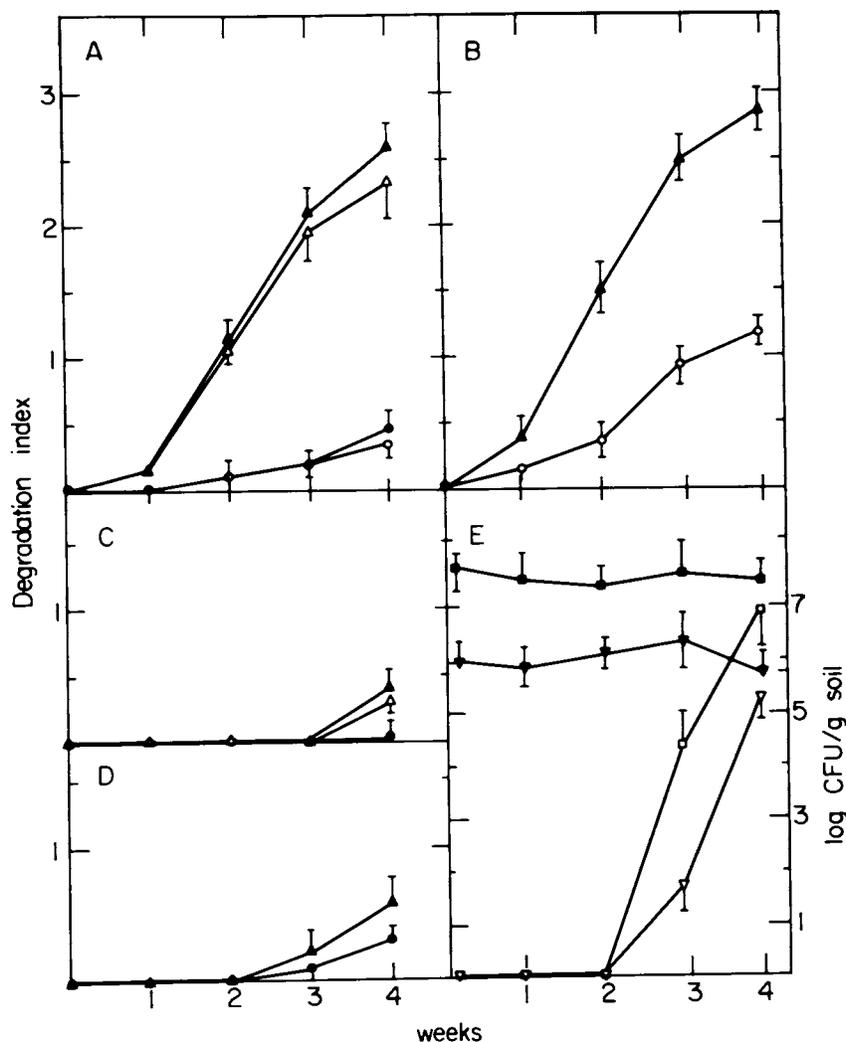
FIG. 7. Biodegradation of immobilized beads in natural light-texture (A) and heavy-texture (B) soils and in sterile light-texture (C) and heavy-texture (D) soils and the bacterial population in the soil (E) during the course of experiment. Symbols: ▲, beads containing bacteria and skim milk; △, beads with skim milk and without bacteria; ●, beads containing bacteria without skim milk; ○, alginate beads only; □, sterilized heavy-texture soil; ■, natural heavy-texture soil; ▼, natural light-texture soil; ▽, sterilized light-texture soil. Bars represent the standard error. Biodegradation scale: 0, no degradation; 1, slight visible degradation on bead edges; 2, one-half to three-fourths of the bead degraded; 3, full degradation to the extent that the beads were not found in the nylon bag.

were inoculated at about the same level, a phenomenon that did not occur with peat inoculant. Slight differences were found between the types of soils. Wet beads inoculated plants better than dry ones, but the difference was not significant (Fig. 8A and B).

Bead formation with iota-carrageenan. Iota-carrageenan beads were formed by the same method as alginate beads and had a similar appearance. However, their internal structure was weak. After bacterial multiplication inside the beads, 44 ± 8% of the total number of these beads was fragmented, and the remaining beads were very fragile and deteriorated after lyophilization.

DISCUSSION

Bacterial inoculation of plant roots with beneficial rhizosphere bacteria, such as Azospirillum sp. and Pseudomonas sp., presents many difficult problems. Bacteria should reach the root even if the root system is widely dispersed; soil particles adsorbed the bacteria in great num-

bers, and bacterial application should be at the precise time needed by the plants (6; Y. Bashan, Soil Biol. Biochem., in press). In addition, inoculation techniques should be practical for the farmer and simple to apply. Inoculation of nonirrigated plants, such as wheat sown in dry soil, is further complicated; bacteria should remain viable in the dry soil for a long time, until it rains, and should proliferate rapidly and immediately to colonize the root system of the seedling when the seed germinates (2). All these difficulties may explain the limited commercial use of bacterial inoculation, except for the legume-Rhizobium system (27). To date, only a few different methods of inoculation are used; the simplest and primitive inoculation method is the application of bacteria in liquid broth (1, 18, 23, 25) or dried bacterial cultures on seeds (28). More reliable procedures use various organic inoculants, most of which are based on peat (15, 24). These inoculants cannot solve most of the problems raised above, thus resulting in inconsistent results.

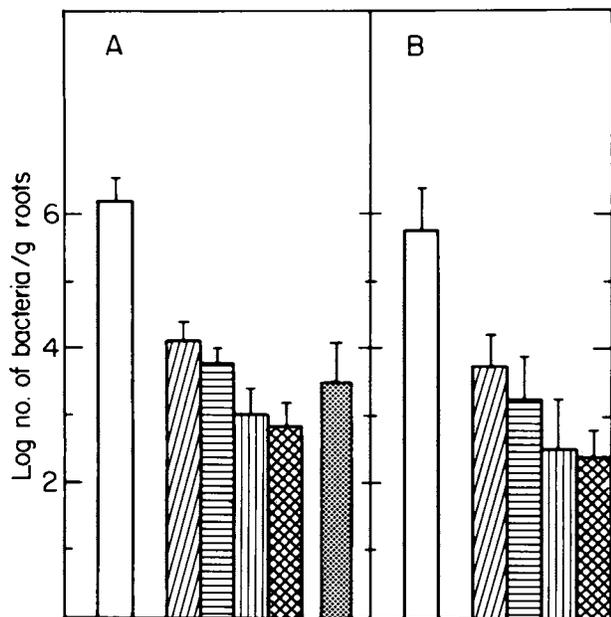The type of bacterial inoculant described in this study may

FIG. 8. Level of inoculated bacteria in wheat root after inoculation with wet beads (A) and lyophilized beads (B) in petri dishes containing filter paper (☐) and in pots containing vermiculite (▨), brown-red degrading sand soil (▤), alluvial soil (▥), and loess raw soil (▩). ▨, Level of plant inoculation with peat inoculant. Bars represent the standard error.

fulfill many of the requirements for a good practical inoculant. It is dry, synthetic, simple to use, uniform, biodegradable by soil microorganisms, and nontoxic in nature; it contains a large uniform bacterial population, provides for the slow release of the bacteria for long periods, causes no ecological pollution, may be produced on a large scale by the proper industry; and its biological characteristics can be effectively controlled by manipulating its chemical features. The beads can be stored for long periods in a relatively small volume without any apparent effect on the size of the bacterial population. Quality control of bacterial number is simple, and the bacteria released from the beads can inoculate the plants efficiently.

Recently, by encapsulation of several biocontrol agents of plant diseases in an alginate-clay matrix, Fravel et al. (11) showed that various fungi species survived well in the dry matrix. However, cells of Pseudomonas cepacia died during the encapsulation process, similar to A. brasilense Cd in this study. Since no secondary bacterial multiplication of P. cepacia was induced, it was assumed that alginate-clay beads were not a suitable carrier for this Pseudomonas species. In addition, the viability of most organisms tested hitherto decreased appreciably with storage time. On the other hand, studies with entrapped Rhizobium in various nonsterile polymer gels by nonsterile procedures (9, 14) showed that the entrapping process did not significantly decrease either the number of bacteria or their viability as shown in the present study and in the work of Fravel et al. (11).

From biological and agricultural standpoints, the beads solve some of the main problems of inoculation of beneficial rhizosphere bacteria. Their application concomitantly with sowing and by the same devices will save additional treatment and requires no change in agrotechnical procedures. In addition, the beads remain undegraded in the dry field until

it rains (Y. Bashan, unpublished data). At this stage, the germination of the seeds will be accompanied by the "awakening" of the bacteria, which will thus reach the germinating seed at the right place and time. Since the optimal inoculation time is not always known (Bashan, Soil Biol. Biochem., in press), the slow release of the bacteria will ensure a constant supply of the bacteria over a relatively long period. Moreover, the number of released bacteria can be controlled by the hardening procedure. Additionally, since the young roots will be close to the bacterial source, this may reduce the problem of bacterial adsorption to soil particles (Y. Bashan, unpublished data).

The biodegradation of the beads depends on soil microflora. The higher the density of the microflora surrounding the bead, the faster the biodegradation. Biodegradation is carried out from the outside layer inward, thus sequentially exposing different layers of the bead to the surrounding soil until degradation of the bead is complete.

Encapsulation of seeds with alginate-containing A. brasilense Cd was found impractical; the relatively high moisture on the seeds required to keep the bacteria alive tended to cause germination, and thus seeds could not be mechanically sown. When the beads were air dried, most of the bacteria died, whereas lyophilization of coated seeds killed the seeds. Additionally, as mentioned, the immobilization procedure resulted in the death of most bacteria, and a second multiplication of bacteria inside the beads was needed. Such multiplication cannot be done in the presence of seeds without the beginning of seed germination.

Although his paper presents data on two rhizosphere bacteria, the system is capable of using many other beneficial rhizosphere bacteria of the genera Pseudomonas, Bacillus, Azotobacter, and Klebsiella (Y. Bashan, unpublished data), and it may be recommended for the inoculation of rhizobia and growth-promoting bacteria of the genus Pseudomonas as well.

To date, the bead production has been carried out on a relatively small scale, and a scaling-up procedure for full commercialization is needed. From the analysis of the available data on the present procedure, it is suggested that the concept of bacterial synthetic inoculants opens a new approach in this field of research.

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