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A NEW ENCAPSULATION TECHNOLOGY FOR BACTERIAL INOCULANTS AND SEED BACTERIZATION

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ABSTRACT

Inadequate storage and release of inocula frequently cause inconsistent results in bacterization. To avoid these problems, a new encapsulation process was perfected. The aim of this technology of double inclusion was to ensure good preservation and release of the beneficial inoculum in stable paraspherical granules. As many as 10^8 bacteria can be encapsulated in a 6 mm diameter granule. Dehydration to 15% residual moisture maintained 10^7 viable bacteria per granule that can be kept at $+4^\circ\text{C}$ for at least six months without any loss of bacteria. Furthermore, a derivative of the process, described as a multiple inclusion technique, was adapted to granules and seeds and allowed the encapsulation of seeds with 10^5 to 10^7 living bacteria per seed. Results and future prospects are discussed.

INTRODUCTION

Although some specific characters of bacterial strains are important (such as genetic stability, colonization and antagonistic ability, resistance to moisture and to temperature stress), in most cases, the type of chosen formulation determines the efficiency of the inoculum to bacterize a substrate. In bacterization experiments of soil, substrates and seeds with PGPR (*i.e.* *Pseudomonas fluorescens-putida*), it has been observed that populations drop during the first few days after inoculation.

Liquid formulations are the easiest to prepare, but they are not always satisfactory. In fact, in liquid media, bacteria are unprotected and dispersed at the time of inoculation in soil or substrates or on seeds. Under these conditions, the balance of forces is often in favor of the resident microflora, and results of inoculation may be more disappointing the more powerful the antagonism of indigenous microflora. Moreover, storage and preservation of liquid inocula are difficult. Solid formulations, dehydrated or lyophilized, such as powders, occasionally give better results, and the life of inoculum can be extended. The addition of an inert but hydrophilic protective carrier, such as clay, peat or cellulose is necessary to obtain good efficiency (11). However, one of the main problems is that microorganisms, before becoming active, have to be rehydrated, which might cause a loss in viability due to osmotic shock. Gelled formulations, using polymers such as polyacrylamide (7) polysaccharides such as xanthan (8) or al-

ternatively alginate (2) resolve, in part, the drawbacks previously described. In these cases, the bacteria are directly mixed in polymer gels.

The aim of this study is to describe a new process of inclusion (3, 5) which avoids these disadvantages allowing a true encapsulation of bacteria and seed and leading to a well-adapted delivery system for inoculation of soil and seed.

MATERIALS AND METHODS

1. Core of capsule and bacterial inoculum

In this procedure, the bacterial inoculum was concentrated in the core. Bacteria such as *Pseudomonas* or *Rhizobium* grew in liquid culture to the stationary phase (about 10^9 CFU/ml). This stationary phase was reached by *Pseudomonas fluorescens-putida* strains after 48 hr at 26 °C in Misaghi's medium (9) and by *Bradyrhizobium japonicum* strain G49 after 90 hr at 28 °C in YEM medium (12). Micronised Algae powder extracted from horsetail kelp (*Laminaria digitata* L., *Laminariaceae*) was sterilized by gamma irradiation. It was added to the bacterial suspensions at the rate of 9% (w/v) to increase the viscosity of the medium to about 80 centipoises (cps) with a viscosimeter (Contraves TV type). The pH was adjusted to neutrality.

2. Envelope of the capsule and coating

The envelope material was made with a sterile aqueous mixture of 1.5% sodium alginate and 10% kaolin (w/v) with a viscosity of about 350 cps. The reticulation agent of the envelope was a sterile saturated solution of calcium gluconate. The granule was finally surrounded with a thin pellicle of clay such as bentonite for dehydration and to avoid the packing of granules during the drying procedure.

3. Seeds

Several types of seeds were used. Shape, volume and weight of seeds affected encapsulation. The spheric or paraspheric shape and a satisfactory weight of seeds were prerequisite. Pre-pelleted seeds such as lettuce and sugar beet seeds could also be encapsulated. The tested species were: soya, pea, corn as non-treated seeds and lettuce, sugar beet and tomato as pre-pelleted seeds.

4. Equipment for encapsulation

4.1. Bacterial granules

The paste suspension, which was intended to form the core, arrived under pressure via the central channel of an injector, forming a viscous drop at the end of the needle. Simultaneously, the material intended to form the envelope flowed into the annular space of the injector surrounding the inoculum drop. Together

they emerged from the base of the injector in the form of drops which fell into a 0.1 M calcium gluconate solution (Fig. 1). The calcium gluconate induced an ion exchange at the surface: calcium ions of gluconate took the place of sodium ions of alginate, inducing the reticulation of alginate and making a resistant calcium alginate layer. The head of the disk-shaped equipment was composed of three elements (A, B and C) of 10.5 cm diameter, superposed and assembled in a leak-tight manner (Fig. 2). The capacity of equipment was determined by the number of impulses per minute of the pump. The external diameter and the volume of the core of granules depended on the diameter of the end nozzles of central and coaxial channels and might vary from 1 to 8 millimeters. For example, to obtain granules with a 6 millimeters external diameter and a 113 μ l core, the nozzle diameter of the central channel was 1.5 mm and the coaxial channel 3.0 mm.

4.2. Seed encapsulation

Bacterial inoculum and envelope used for seed encapsulation were made with the same material as bacterial granules. They were directed simultaneously under pressure toward a specific injector in such a way they formed a meniscus of which the upper part was intended to form the bacterial inoculum and the lower part the envelope. The seed fell on the meniscus and was surrounded by the two

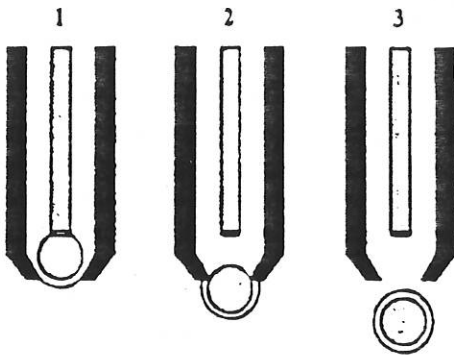


Fig. 1. Steps of encapsulation

1. Formation of the core by a viscous drop at the end of the central channel.
2. Formation of the envelope which flows into the annular space and surrounds the core granule.
3. Granules exit from injector.

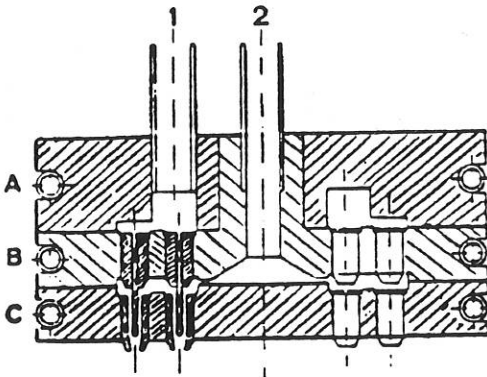


Fig. 2. Head of encapsulation equipment.

1. Core channel.
 2. Envelope channel.
- A, B, C, elements.

materials (Fig. 3). Together they emerged from the base of the injector and fell into a 0.1 M calcium gluconate solution which finally formed a resistant envelope. After permeating for 1 min, the encapsulated seeds were immediately dried. The diameter of the internal orifice of injection depended on the shape and volume of seed to be encapsulated. The greater the size of seeds, the higher the viscosity of material.

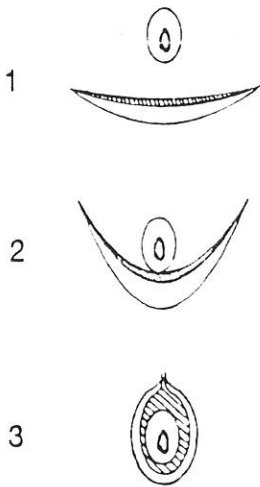


Fig. 3. Steps of seed encapsulation.

1. Formation of a double meniscus and free drop of the seed.
2. Bending of meniscus under the weight of the seed.
3. Seed encapsulation.

5. Dehydration, storage and assessment of survival of encapsulated bacteria

Survival of *Pseudomonas fluorescens-putida* strain M3 in granules v.s. residual hygrometry and dehydration time at 40 °C was studied. Strains of different bacterial species (*P. syringae* pv. *pisi*, *P. solanacearum*, *Bradyrhizobium japonicum*) were seed encapsulated and bacterial survival was assessed after dehydration for 1 hr at 40 °C. In pre-pelleted lettuce seeds, survival of *P. fluorescens* strain L26.1 v.s. residual hygrometry and dehydration time at 24 °C was determined. In addition dehydrated bacterial granules (15% residual hygrometry) were kept at +4 °C in sealed flasks for six months for bacterial survival assessment.

To release bacteria, samples of 100 granules and 100 seeds were immersed and vigorously shaken at 4 °C in 100 ml of a sterile phosphate buffer (pH 6.4, 0.02 M). After 1 hr, the supernatant was serially diluted and plated on King's medium B. Colony counts were made after 48 hr incubation at 26 °C. *Bradyrhizobium japonicum* was numerated by indirect immunofluorescence technique.

6. Induced degradation of the envelope of bacterial granules

Three chemical products capable of inducing degradation of the envelope were tested. Ammonium phosphate, ammonium carbonate and sodium carbonate

were solubilized in sterile water at 1 M concentration. For "in vitro" experiments, products came directly in contact with the granules for 24 hr. For "in soil" experiments, five granules were incorporated in 20 ml of soil at pH 7.3 containing 16% clay, 27.1% loam, 52.5% sand and 4.1% organic matter. Then the soil samples were drenched to saturation by degrading solution and the control by sterile water. The degradation of the envelopes was visually observed every day for 8 days.

RESULTS

1. Structure of bacterial granules and equipment capacity

Characteristics of hydrated granules are summarized in Table 1. The discrepancy in size was relatively small. Under our experimental conditions, granules made from a bacterial inoculum of 10^9 CFU/ml contained a mean of 10^8 bacteria. A slow rate of pump (1 impulse/second) and an equipment head with three injectors allowed more than one kilogram of granules per hr to be made, *i.e.* about 6,500 granules. But it was possible to increase the capacity 4-fold by increasing the number of injectors and the number of impulses.

2. Structure of encapsulated seeds and equipment capacity

Under our experimental conditions, the bacterial inoculum volume laid on each encapsulated seed was about 100 μ l which corresponded to about 10^8 bacteria, but there was some variation according to the seed type (Table 2). On the other hand, the equipment is a laboratory prototype, and the obtained encapsulations were not perfectly regular. With one injector, the encapsulation rate was one seed per second.

Table 1. Characteristics* of bacterial granules.

Diameter (mm)	6
Weight (mg)	168
Volume (μ l)	250
- envelope	140
- core	110
Residual hygrometry (%)	93
Bacteria (CFU)	10^8

*These data represent the average calculated from 1000 fresh granules.

3. Survival of bacteria in granules and encapsulated seeds

Dehydration in a ventilated atmosphere at 40 °C for 1 hr allowed survival of more than 10^7 bacteria per various types of encapsulated seeds with different bacterial species (Table 2). In granules, survival of *P. fluorescens-putida* M3 v.s. dehydration time was correlated with residual hygrometry. A positive correlation between the graphs of bacterial survival and of residual hygrometry was observed. After 50 minutes of dehydration at 40 °C, more than 10^6 bacteria were alive in the granules at a 15% residual hygrometry (Fig. 4). This significant dehydration resulted in a twofold decrease in granule diameter. These dehydrated granules were kept in sealed flasks at +4 °C for six months without any loss of bacteria. In encapsulated lettuce seeds, survival of *P. fluorescens* L26.1 v.s. dehydration time at 24 °C also related to residual hygrometry but less closely than in granules. After 120 minutes of dehydration at 24 °C, more than 10^7 bacteria were alive for a 10% residual hygrometry (Fig. 5).

4. Induced degradation of the envelope of bacterial granules

In vitro degradation induced by ammonium and sodium carbonates was extremely rapid. One hour after immersion of granules in 1 M carbonate solution, the envelope was entirely degraded, while 24 hours of immersion were needed to

Table 2. Survival of bacteria in encapsulated seeds.

SEED SPECIES	BACTERIAL SPECIES	VIABLE BACTERIA* PER SEED (cfu)		
		GROWTH MEDIUM (STATIONARY PHASE)	DEHYDRATION** BEFORE	AFTER
SOYA (naked)	<i>Pseudomonas fluorescens-putida</i> M3	4.67 x 10 ⁹	2.63 x 10 ⁸	2.35 x 10 ⁸
	<i>Pseudomonas fluorescens</i> L26-1	2.43 x 10 ⁹	2.82 x 10 ⁸	2.47 x 10 ⁸
	<i>Bradyrhizobium japonicum</i> (i 49)	6.97 x 10 ⁹	4.07 x 10 ⁸	3.58 x 10 ⁷
PEA (naked)	<i>Pseudomonas fluorescens-putida</i> M3	4.67 x 10 ⁹	6.00 x 10 ⁸	5.81 x 10 ⁷
	<i>Pseudomonas syringae</i> pv <i>pisi</i> (virulent strain)	5.20 x 10 ⁷	3.56 x 10 ⁶	1.98 x 10 ⁵
SUGAR BEET (pre-potted)	<i>Pseudomonas fluorescens-putida</i> M3	1.40 x 10 ⁹	1.11 x 10 ⁸	5.03 x 10 ⁷
		2.43 x 10 ⁹	2.04 x 10 ⁸	1.17 x 10 ⁷
TOMATO (pre-potted)	<i>Pseudomonas solanaceorum</i> 2515 (avirulent mutant)	3.21 x 10 ⁹	2.75 x 10 ⁸	1.63 x 10 ⁷
LETTUCE (pre-potted)	<i>Pseudomonas fluorescens</i> L26-1	4.36 x 10 ⁹	3.20 x 10 ⁸	2.15 x 10 ⁷

* Bacterial enumeration was achieved by serial dilution and plating on King's medium B and indirect immunofluorescence technique for *Bradyrhizobium* from samples of 100 encapsulated seeds. Numbers of bacteria are the average of 3 replications.

** Dehydration during 1 hr at 40 °C.

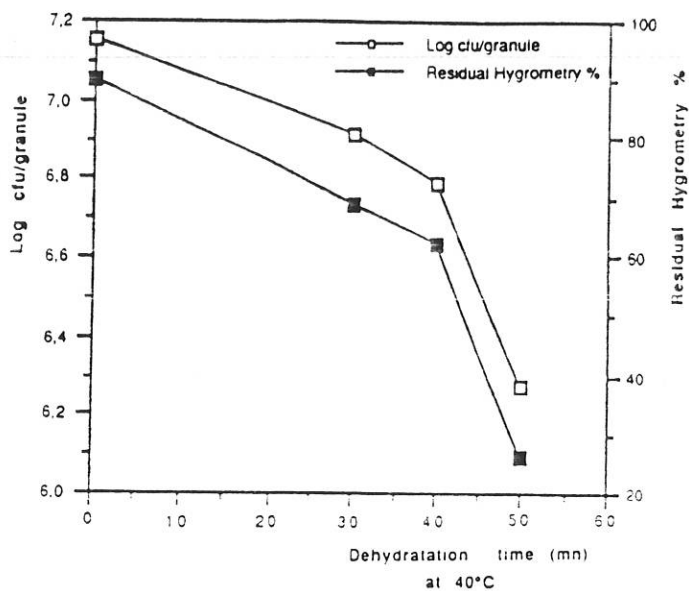


Fig. 4. Survival of bacteria in granules.

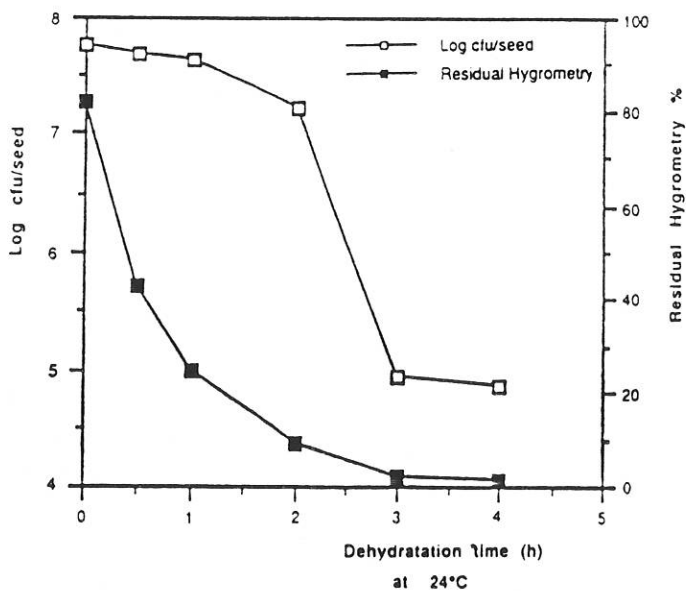


Fig. 5. Survival of bacteria in encapsulated seeds.

induce the degradation of the whole envelope in 1 M phosphate solution. On the other hand, in soil, 24 hours was necessary to induce the degradation of the whole envelope with each of the three substances tested and eight days for the control (Table 3).

Table 3. Envelope degradation of granules *in vitro* and in soil.

Degrading agent (1 M solution)	Degradation time (hours)	
	<i>in vitro</i>	in soil
Ammonium phosphate	24	24
Ammonium carbonate	1	24
Sodium carbonate	1	24

DISCUSSION AND FUTURE PROSPECTS

The size of inoculum, delivery systems, and the method of release directly affect the establishment and dynamics of the bacterial population on the target (1).

This new encapsulation process resulted in a high concentration of bacteria in a small volume. Furthermore the bioactive charge of the core may be increased ten-fold if the bacterial concentration of nutrient broth is increased up to 10^{10} CFU/ml. Because bacteria were suspended in their nutrient broth and then mixed with Algae extract, it is expected that nutritional stress will be less severe during the release into the environment. Algae extract seems to be a well-adapted medium for bacterial survival. One of the key factors in survival is the preservation of a residual hygrometry of about 15% in granules and 8% to 10% in encapsulated seeds.

In the specific case of *Pseudomonas*, strain resistance to temperature stress could also be a survival factor because of the detrimental effect of high temperatures which are frequently required for dehydration (6). Because speed of seed germination and root development are rarely synchronized with speed of inoculum release, it would be of interest to obtain a better assessment of timing of envelope degradation.

New applications of the process can also be adapted to embryo encapsulation in order to mimic teguments (envelope of granule) and albumen (core of granule) and to develop artificial seeds. The granules, being entirely biodegradable in soil, are environmentally compatible and would contribute to forming a microbial inoculum for plant-growth promotion and biocontrol of plant pathogens.

To optimize bacterization, it would be judicious to use both encapsulated seeds and bacterial granules because both are complementary (4). Finally, the system offers the possibility of mixing several bacterial strains, or different species of bacte-

ria, or bacteria and beneficial fungi in the core. Alternatively, bacteria can be included in the envelope and beneficial fungi in the core or vice-versa.

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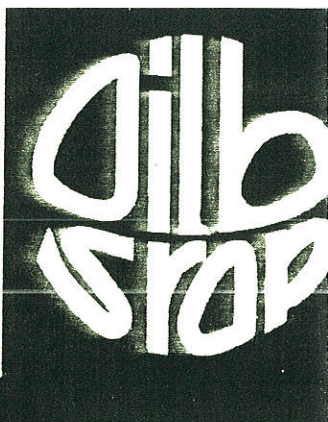
REFERENCES

1. Bahme, J. B., Schroth, M. N., Van Gundy, S. D., Weinhold, A. R., and Tolentino, D. M. 1988. Effect of inocula delivery systems on rhizobacterial colonization of underground organs of potato. *Phytopathology* 78(5):534-542.
2. Bashan, Y. 1986. Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. *Appl. Environ. Microbiol.* 51:1089-1098.
3. Digat, B. 1988. Structure for including active substances. International Patent no. WO 88/08876.
4. Digat, B. 1989. Strategies for seed bacterization. *Acta Horticulturae* 253:121-130.
5. Digat, B. 1990. Granules à multicouches contenant des substances actives enrobées: leur procédé de fabrication, dispositif pour la mise en oeuvre de ce procédé et utilisation des granules obtenus. International Patent PCT no. 90/00604.
6. Digat, B., and Mattar, J. 1990. Effects of temperature on growth and siderophore production of *Pseudomonas fluorescens-putida* strains. *Symbiosis* (9): 207-213).
7. Dommergues Y. R., Diem, H. G., and Divies, C. 1979. Polyacrylamide-entrapped *Rhizobium* as an inoculant for legumes. *Appl. Environ. Microbiol.* 37:779-781.
8. Jung, G., Mugnier, J., Diem, H. G., and Dommergues, Y. R. 1982. Polymer entrapped *Rhizobium* as an inoculant for legumes. *Plant and Soil* 65:219-231.
9. Misaghi, J., Stowell, L. J., Grogan, R. G., and Spearman, L. C. 1982. Fungistatic activity of water soluble fluorescent pigments of fluorescent pseudomonads. *Phytopathology* 78:33-36.
10. Okon, Y., and Hadar, Y. 1987. Microbial inoculants as crop yield enhancers. *CRC Critical Reviews in Biotechnology* 6:61-85.
11. Suslow, T. V., and Schroth, M. N. 1982. Rhizobacteria of sugarbeets: effects of seed application and root colonization on yield. *Phytopathology* 72:199-206.
12. Vincent, J. M. 1970. A manual for practical study of root-nodule bacteria. IBP Handbook 15. Blackwell Scientific Publications, Oxford.

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