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Survival, root colonisation and biocontrol capacities of *Pseudomonas fluorescens* F113 LacZY in dry alginate microbeads

A Russo[1], M Basaglia[2], E Tola[2] and S Casella[2]

[1]*Dipartimento di Chimica e Biotecnologie Agrarie, Via del Borghetto, 80, 56124 Pisa, Italy;* [2]*Dipartimento di Biotecnologie Agrarie, Agripolis — Università di Padova, Strada Romea, 16, 35020 Legnaro, Padova, Italy*

Cells of *Pseudomonas fluorescens* F113 LacZY were encapsulated in alginate and their survival and ability to colonise sugar beet were evaluated. To assess survival, the formulation, composed of dry alginate microbeads of 300- to 700-μm diameter, was stored 1 year at 28±2 and 4±2°C and then tested against pathogenic fungi *Pythium ultimum* and *Rhizoctonia solani* in *in vitro* inhibition experiments. The same material was also used as inoculant for protection of sugar beet against *Py. ultimum* in microcosm experiments. The results obtained indicated that, although drying alginate beads resulted in a significant reduction of bacterial viability, the use of microbeads enabled a satisfactory level of root colonisation and protection, at least under microcosm conditions. The capability of the encapsulated cells to produce the antifungal metabolite 2,4-diacetylphloroglucinol (Phl) was not significantly affected by 12 months storage. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 337–342.

Keywords: biopesticide; alginate; immobilised bacteria; inoculant; *Pseudomonas*; *Pythium*; *Rhizoctonia*

Introduction

The increased reliance on expensive fertilizers and chemical pesticides to ensure competitive yields and protection of crops has generated an interest in developing less expensive alternative procedures. The application of beneficial root colonizing bacteria as phytostimulators, biopesticides and biofertilizers is one possible approach.

When bacteria are released into the soil for biological control of plant diseases, they should be protected against several adverse factors such as chemicals, environmental fluctuations and biological aggression in terms of competition for nutrients and colonisation sites. Liquid formulations of bacteria rarely provide sufficient protection; moreover, they are susceptible to contamination during manufacturing and to uncontrolled off-site dispersion during application. In recent years several technologies have been proposed and developed to safely introduce microorganisms in soil by adsorbing bacteria to different carriers such as peat, charcoal, vermiculite and other organic materials [1,7,19,20]. Alternatively, immobilized cells can be used [2]. Attaching and/or entrapping cells to inert supports can optimize their efficacy, stability, safety, and flexibility of application. The immobilisation of cells in an alginate matrix has recently been proposed as an efficient method for delivering microbial inoculants such as *Pseudomonas fluorescens* [19,23].

Several strains of fluorescent pseudomonads, isolated from soil or the rhizosphere of various plants species, have been shown to promote plant growth through the control of plant pathogens [3,4,6,22]. *P. fluorescens* F113, used in this work, has been widely studied as biocontrol agent of damping off of sugar beet [9,14].

Correspondence: Prof S Casella, Dipartimento di Biotecnologie Agrarie, Agripolis — Università di Padova, Strada Romea, 16, 35020 Legnaro, Padova, Italy
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Previous results indicated a long survival of the genetically modified biocontrol strain F113 LacZY encapsulated in alginate polymers, both in the moist alginate and in the rhizosphere. Moreover, colonisation and mobility on the root, as well as the *in vitro* inhibitory activity of the cells was unaffected by immobilisation [15]. Nevertheless, the moist formulation is not easy to handle and is not compatible with existing farming equipment. These limitations could be overcome with a dry formulation.

The objective of this work was to evaluate the effects of drying on the survival and colonisation capability of *P. fluorescens* F113 LacZY immobilised in alginate microbeads, compared to those of unencapsulated cells (free cells). The bead size was also reduced in order to reduce nutrient diffusion barriers, to minimise oxygen limitation, and to improve carrier effectiveness.

Materials and methods

Bacterial strain

P. fluorescens F113 was originally isolated from the rhizosphere of sugar beet [17]. A derivative strain carrying *lacZY* genes was obtained as previously described [8]. This modified strain can utilise lactose and cleave the chromogenic molecule 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The strain was maintained on LB agar medium [11] and cultured in LB broth at 28°C.

Preparation of dried, ground alginate beads (microbeads)

Beads were manufactured using sodium alginate from *Macrocystis pyrifera* (Sigma, St. Louis, MO) as previously described [15] using a sterile solution of phosphate-buffered saline (PBS, 1/5 strength) to resuspend the cells. The moist beads were air dried for about 20 h in a laminar flow hood at 25°C and ground at 10,000

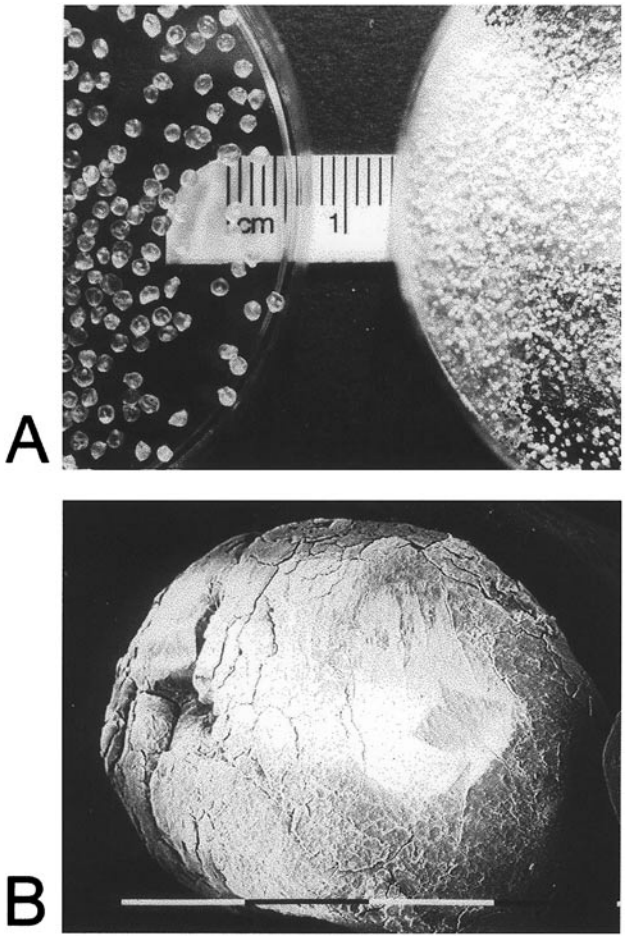

Figure 1 (A) Dried alginate beads containing *P. fluorescens* F113 LacZY before (left) and after (right) grinding. (B) Scanning electron micrograph of a dried alginate bead containing *P. fluorescens* F113 LacZY. Bar = 100 μm.

rpm using a Retsch Ultra-Centrifugal Mill ZM 1000 (ring sieve in stainless steel, holes=0.50 nm). The dimension of the microbeads obtained was evaluated using a scanning electron microscope (Philips SEM 505 at 10 kV). Moisture content of the microbeads was determined by the Karl Fisher titration method (using a Radiometer TTT80 automatic titrator).

Survival of P. fluorescens *F113 LacZY in microbeads*

Microbeads containing bacteria were stored at 4±2 and at 28±2°C in a desiccator, and the number of culturable cells was periodically monitored for 12 months. To determine the number of the culturable encapsulated cells, the microbeads were completely dissolved in sterile 1% sodium citrate solution, pH 6.5 for 30 min at room temperature. The suspensions were serially diluted in sterile physiological solution (0.9% NaCl) and plated on sucrose asparagine agar (SA) [16] supplemented with the chromogenic substrate X-gal (25 μg ml^{-1}). Blue colonies were counted after 4–5 days incubation at 28°C. All experiments were done in triplicate.

Microcosm experiments

A loamy sandy soil (pH 7.5) from Lecce, Italy, passed through a 4-mm sieve, was used for microcosm tests to study the root colonisation ability of encapsulated cells. Three plastic pots, each containing 200 g of soil, were used for each treatment. They were maintained at about 20% soil moisture using distilled water and sown with five sugar beet seeds (*Beta vulgaris* cv. Golf, Agra, Italy) inserted 2 cm into the soil. Each seed was inoculated directly with 0.012 g of microbeads that had been aged 12 months at 4°C and containing about 10^6 colony-forming units (CFU). Positive controls consisted of sugar beet seeds inoculated with the same number of bacteria from a fresh culture, washed and suspended in physiological solution (NaCl 0.9%). The pots were incubated in a humid growth chamber at 12°C operated at a 16/8 h light/dark cycle and watered every 3 days as necessary.

Twenty days after inoculation the full root system was placed on minimal medium M9 agar [11] containing ampicillin (50 μg ml^{-1}), chloramphenicol (13 μg ml^{-1}), cycloheximide (100 μg ml^{-1}), X-gal (25 μg ml^{-1}) and lactose (5 g l^{-1}) as the sole carbon source. The occurrence of root colonisation by the inoculated strain was indicated by the development of blue colour around roots after incubation at 28°C [15]. For soil analyses, 20 g of soil was resuspended in a sterile 0.9% NaCl solution and maintained on a rotary shaker for 45 min at the maximum speed. Soil serial dilutions (1:10) were prepared and aliquots inoculated on modified King's B medium [10] for determinations of total fluorescent pseudomonads. For determination of the rhizosphere population, one entire sugar beet root with closely adhering soil, was resuspended in 2 ml sterile 0.9% NaCl solution, vortexed for 5 min, serially diluted and aliquots were plated on modified King's B. Plates were incubated at 30°C for 3–5 days before recording colony numbers. To monitor *P. fluorescens* F113 LacZY, aliquots of soil and rhizosphere serial dilutions, were spread on SA agar [16] containing ampicillin (50 μg ml^{-1}), chloramphenicol (13 μg ml^{-1}), cycloheximide (100 μg ml^{-1}) and X-Gal (25 μg ml^{-1}). After 3–7 days incubation, blue colonies were recorded as *P. fluorescens* F113 LacZY. All determinations were conducted at least in triplicate. Detection limits for LacZY-tagged *P. fluorescens* in soil were around 10–100 CFU g^{-1} [5].

In vitro *inhibition test*

Strains of *Pythium ultimum* and *Rhizoctonia solani* were kindly supplied by the Department of Microbiology of U.C. Cork, Ireland and Eridania Béghin Say, Massalombarda, Italy, respectively. The strain of *R. solani* was isolated directly from the rhizosphere of infected sugar beet and identified by morphological characteristics and by its pathogenic properties.

Microbeads (0.01 g) that had been aged 12 months and contained about 8×10^5 CFU (from 4°C storage) or 1×10^4 CFU (from 28°C storage), or alternatively the same number of free cells as a control, were placed on SA plates containing 50 mM $FeCl_3$ to support fungal growth and incubated at 28°C. After 20 h of incubation, fungal plugs (*Py. ultimum* or *R. solani*) were placed in the middle of the SA agar plates. Following an additional incubation of 3 and 7 days at the same temperature, the inhibition of hyphal growth was recorded. The experiments were done in triplicate.

Biocontrol ability of immobilised P. fluorescens *F113 LacZY in soil artificially infested with* Pythium

The *Pythium*-based inoculum for microcosm experiments was grown on finely chopped potatoes combined with vermiculite in the ratio 4:1 (wt/wt). The mixture was sterilised by autoclaving it

Table 1 Recovery of culturable bacterial cells after manufacturing process

	Liquid starter	Wet beads	[a]Dry beads	Ground dry beads (microbeads)
Bead number (or g)		25000±586	[a]25000±421	[b]50 g
Detected CFU/bead		1.8 (±0.5)×10^7	1.0 (±0.3)×10^6	[c]9.4 (±1.7)×10^5
Total CFU	5.0 (±0.8)×10^{11}	4.5 (±0.7)×10^{11}	2.5 (±0.5)×10^{10}	2.3 (±0.6)×10^{10}
[d]Recovery (%)		90.0	5.6	4.7

Results are the mean of three independent determinations (±SD).
[a]The average weight of one dry bead is 2 mg.
[b]Equivalent to 25,000 dry, unground beads.
[c]Calculated for 2 mg of ground beads.
[d]Percent recoveries are related to liquid starter.

twice for 1 h on two consecutive days. *Py. ultimum* was grown on PDA agar for 2 days at 24°C. Six mycelial disks (12-mm diameter) of actively growing *Py. ultimum* were used to inoculate 1.0 l of the sterilised potato–medium mixture in 2-l flasks. After 2 weeks of incubation at 24°C, the inoculum was air-dried for 1–2 days and vigorously mixed with sieved field soil, at two infestation levels: 0.75 g pathogen inoculant kg^{-1} soil (low) and 3.0 g pathogen inoculant kg^{-1} soil (high). Two hundred grams of the infested soil was potted and sown with (a) untreated sugar beet seeds, (b) seeds inoculated with free cells or (c) seeds inoculated with ground immobilised cells with eight seeds/pot and five pots/treatment. Soil moisture was adjusted to 20% with distilled water. Uninoculated seeds were sown in noninfested soil as a control. Pots were watered every 3 days. Plants were grown for 21 days in a humidity growth chamber at 12°C and a 16/8 h light/dark cycle. Seed emergence was recorded as plant health index because *Pythium* infection of sugar beet seed surfaces causes pre-emergence damping off and devitalization of seedlings.

HPLC analysis for 2,4-diacetylphloroglucinol (Phl) evaluation

After 12 months storage at 4°C, microbeads were transferred to LB agar plates. Five *P. fluorescens* F113 LacZY colonies derived from cells released from the beads were cultured in LB broth. After 12 and 24 h production of Phl in the cultures was evaluated as previously described [18] and compared to that of free cells. The amount of Phl recovered was expressed as micromoles per milliliter of culture adjusted to the same OD_{600}±standard deviation.

Results and discussion

Root colonisation obtained by inoculation of seeds with dried beads of 1-mm diameter (about 10^6 cells per seed) fluctuated greatly giving consistently lower colonisation than that obtained using free cells. In several sets of experiments, for instance, only sporadic colonisation was achieved with 1-mm beads, resulting in an average of three to four barely-colonised roots on 10 inoculated plants. This indicates a heterogeneous distribution of the inoculant, probably due to the excessive size and the consequent limited number of beads.

The size of the beads was therefore reduced from the initial 1-mm diameter to 300–700 μm (Figure 1A) by grinding. The final moisture content of the beads was about 10–11% as determined by the Karl Fisher method. These resulting microbeads are shown in Figure 1B.

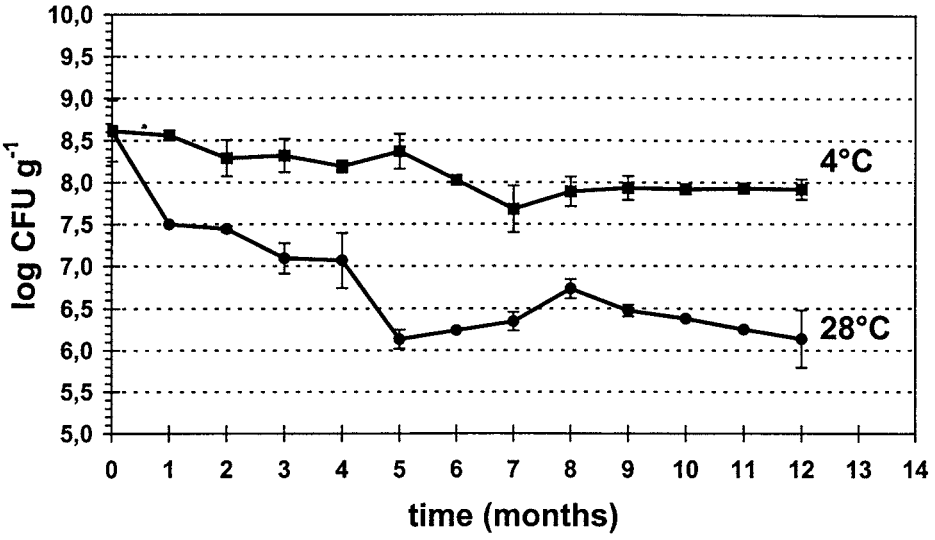

Figure 2 Viability of *P. fluorescens* F113 LacZY in microbeads during 12 months storage at 4 and 28°C. Results are the mean of three independent determinations with bars representing standard deviations.

Table 2 Differences in root colonisation, 20 days after sowing, by free and immobilised cells of *P. fluorescens* F113 LacZY, after 12 months storage at 4°C

	Fluorescent pseudomonads	*P. fluorescens* F113 LacZY	Fluorescent pseudomonads	*P. fluorescens* F113 LacZY
	CFU g^{-1} dry root		*CFU g^{-1} dry soil*	
Seed+microbeads	1.4 ($\pm$0.4)$\times10^8$	2.2 ($\pm$0.8)$\times10^7$	4.7 ($\pm$1.2)$\times10^5$	1.5 ($\pm$0.5)$\times10^5$
Seed+free cells	1.5 ($\pm$0.3)$\times10^8$	8.7 ($\pm$2.1)$\times10^7$	4.8 ($\pm$1.0)$\times10^5$	4.0 ($\pm$0.9)$\times10^5$
Uninoculated seeds	3.4 ($\pm$1.2)$\times10^7$	nd	3.2 ($\pm$0.9)$\times10^5$	nd

Results are the mean of three independent determinations ($\pm$SD).
nd: not detected.

The recovery of bacterial cells at different steps of manufacturing is shown in Table 1. Encapsulation into alginate made it possible to maintain 90% living cells, with 4.5×10^{11} of the 5.0×10^{11} inoculum surviving. Drying the alginate beads caused an additional reduction to 2.5×10^{10} cells, about 5–6% of the initial inoculum. A slight decrease in viable cells was also observed after grinding the dry beads to reduce their dimension. This gave a final total recovery (of 2.3×10^{10}) or 4.7% of the cell number in the liquid starter. Using this information we estimated the number of living cells present in 2 mg of microbeads (corresponding to the average weight of a single bead before grinding) to be 9.4×10^5. These microbeads were used for all the following investigations to evaluate survival of encapsulated cells and their biocontrol properties.

Storing microbeads at 28$\pm$2°C resulted in viability reduction of the entrapped dried bacteria by about 300-fold, mainly occurring within the first months. By storing the same formulation at 4°C the observed decline remained under 1 log during the entire 12-month storage period (Figure 2).

After 12 months of storage the ability of entrapped cells to colonise and protect plant roots was determined. At 20 days after seed inoculation, *P. fluorescens* F113 LacZY cells in microbeads colonised the sugar beet rhizosphere at a population density comparable to that obtained with free cells (Table 2).

Evaluating the number of cells introduced from the beads versus the number of total fluorescent pseudomonads indicated a good colonisation ability of *P. fluorescens* F113 LacZY especially in soil, where it seems to almost completely replace the resident population (Table 2). The whole root systems were colonised by both entrapped and free cells as confirmed by the blue colour developed extensively on the roots in the presence of X-Gal and lactose.

P. fluorescens F113 LacZY inhibited the growth of both *Py. ultimum* and *R. solani in vitro*. Nevertheless, the hyphal growth inhibition of *Py. ultimum* was higher with both encapsulated and free cells (Figure 3). Similar results were obtained with microbeads stored at 4°C and 28°C.

To verify the biological control ability of *P. fluorescens* F113 LacZY, *in vivo* experiments were carried out using soil with increasing levels of contamination. The results confirmed that immobilised cells of *P. fluorescens* F113 LacZY retained the ability to act as biocontrol agents toward *Py. ultimum* (Figure 4). In the soil infested by low numbers of *Pythium* no significant difference was observed in the emergence of sugar beet inoculated with free or immobilised cells. At high levels of infestation, free cells seemed to perform better when compared with the bead formulation. The level of plant protection observed with encapsulated cells remained significant, however.

The reduced effect on plant protection observed for the encapsulated inoculant could be due to (i) a reduced number of living cells still able to act as biocontrol agent or (ii) a reduced capability of the restored cells to produce the antifungal compounds. The data reported in Table 2 suggest the first hypothesis is unlikely. The second hypothesis can be tested by cultivating cells from alginate beads and comparing their Phl-producing activity with that of cells deriving from a stock. Production of the antifungal metabolite Phl, as evaluated by HPLC analysis, was 192.6$\pm$34 μmol ml^{-1} for bead-derived cultures, whereas stock-derived cultures, adjusted to the same OD_{600}, gave 168.6$\pm$16 μmol Phl ml^{-1}, both measured after 12 h growth. After

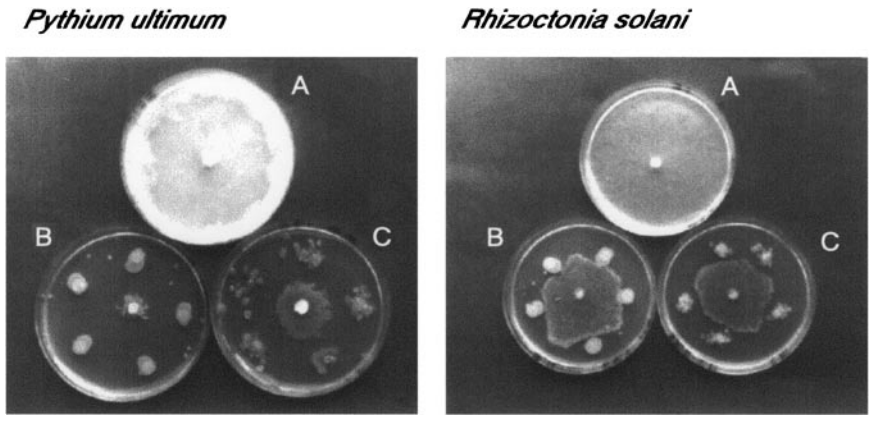

Figure 3 *In vitro* inhibition of *Py. ultimum* and *R. solani* by *P. fluorescens* F113 LacZY after 3 days of incubation. (A) Control plate; (B) unencapsulated cells; (C) microbeads.

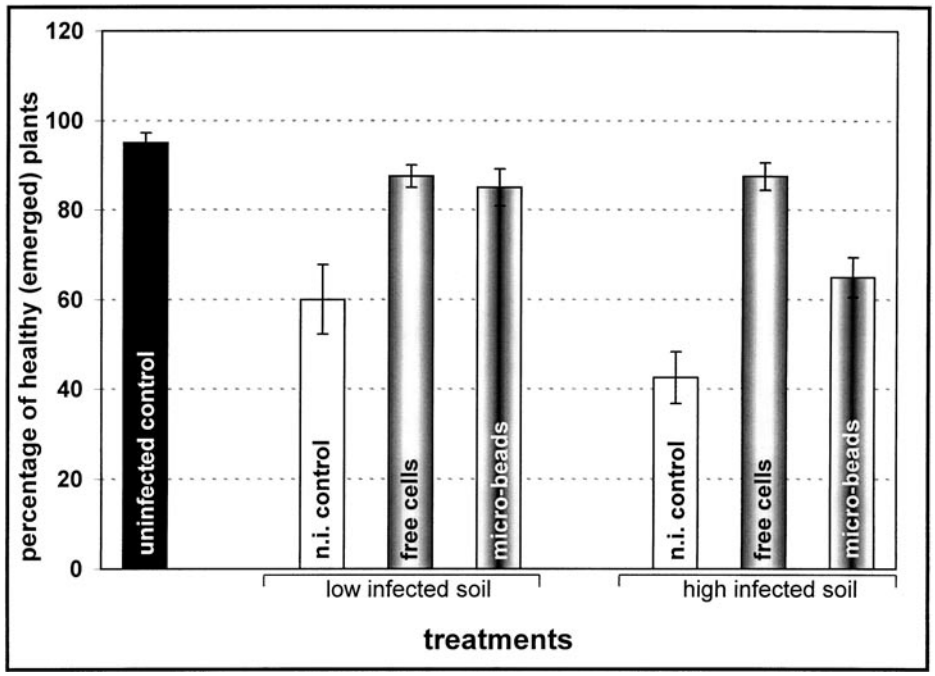

Figure 4 Effect of liquid suspension or beads, containing *P. fluorescens* F113 LacZY, on the emergence of sugar beet in soil infested with two different levels of *Py. ultimum*. The values are the mean of three independent determinations with bars representing standard deviations.

24 h, both values were consistently reduced although maintaining the same relative levels of production.

Pseudomonads have received increased attention for use as microbial inoculants in connection with their production of inhibitory compounds such as secondary metabolites. The generation of genetically modified derivatives of the wild-type biocontrol *P. fluorescens* strain F113 with increased levels of Phl biosynthesis represents a promising strategy to improve biocontrol activity [14]. Such a biosynthetic property may be maintained under soil conditions and, even before, in the manufactured formulation. The results obtained indicated that encapsulation within alginate, drying and grinding to obtain microbeads does not affect Phl production.

Although there are a number of advantages of the use of encapsulated cells for soil applications, some limitations can also occur. The most important is likely to be the diffusion of substrates such as gases, particularly oxygen, and solutes into beads, especially to cells located in the central section of the beads. Decreasing cell density, carrier concentration and gel particle size is thought to be helpful. Although some authors report that extreme dehydration does not significantly affect the survival of encapsulated cells [12,13] even if they are affected by rehydration, very little has been reported about the affect of water on encapsulated cells introduced into the soil. van Elsas *et al* [21] reported long-term colonisation by *P. fluorescens* when released as dry alginate beads, compared with early colonisation with a wet formulation. In the present study, this problem was overcome by reducing the bead size. The capability of *P. fluorescens* F113 to survive and colonise the soil and the beet root system suggested that the physiological properties of the bacteria used were not affected by encapsulation in alginate beads of such a reduced size, even after a long period of storage. This conclusion is supported by the activity of encapsulated bacteria in controlling *in vitro* hyphal growth and *in vivo* fungal infection as revealed by the emergence results reported above. This formulation is also probably useful for longer term storage since the living cells recovered after 18 months of storage at 4°C was 2.1×10^7 culturable cells per gram.

Conclusions

These promising results meet the commercial requirements for a suitable formulation of biopesticides in terms of stability, shelf life, easy application and user friendliness. The most promising data emerging from the literature, however, are still far from reasonable cost and applicability requirements. For instance, data reported by Trevors *et al* [19] indicated a favourable performance of alginate inoculants, but when a real situation is hypothesised (e.g., application of the inoculant to sugar beet seed at the same ratio reported in that study) the amount of inoculant needed would be 200 kg ha^{-1}. Obviously, this is not suitable for real agriculture situations. In the present study, the amount of inoculant per hectare to be applied to obtain 10^6 cells per seed remains within an acceptable limit of 0.425 kg. These data were calculated on 2×10^5 seeds, which are roughly used per hectare, and by assuming that 50 g of microbeads contain (2.3×10^{10}) living cells. This evaluation, based on a total recovery percentage of 4.7%, as reported in Table 1, can be improved. For instance, by increasing the initial cell number to 2×10^{12} the amount of inoculant needed to ensure 10^6 cells per seed becomes 0.125 kg ha^{-1}. This permits increasing the amount of beads to be applied to the same amount of seed, while reducing the concentration of living cell in the alginate formulation. The most suitable concentration can be determined once the amounts of microbeads applied to the seeds are optimised between 0.125 and 0.425 kg ha^{-1}. This will need further investigation and a careful economic evaluation of the substrate.

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