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# Comparison of application methods to prolong the survival of potential biocontrol bacteria on stored sugar-beet seed

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## ABSTRACT

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**Aims:** To develop bacterial inoculation treatments on sugar-beet seed that will maintain a commercially acceptable degree of viability for a minimum of 4 months storage at ambient temperature.

**Methods and Results:** Single rifampicin-resistant ($Rif^+$) strains of both Gram-positive and negative bacterial isolates (mostly pseudomonads) were applied in turn to sugar-beet seed in a comparative study by seed soaking, encapsulation in alginate, pelleting using an inoculated peat carrier or seed priming. The treated seed was assessed for bacterial survival over a time course by plating out homogenized samples onto a selective medium. Priming inoculation offered a significant improvement over all the other application strategies tested. After pelleting with fungicides and drying at 40°C, *Pseudomonas marginalis/putida* P1W1 maintained populations of >6·6 $\log_{10}$ CFU $g^{-1}$ seed during 4 months storage at 15°C. Subsequent experiments verified a stabilized population under these storage conditions with commercial pellets at <7% moisture content.

**Conclusion:** An inoculation method was established which allowed the survival on seed of a Gram-negative bacterium at ambient temperature with little loss in viability.

**Significance and Impact of the Study:** This has promising implications for the delivery of beneficial bacteria, especially Gram-negative strains, on sugar beet.

**Keywords:** bacteria, inoculation, seed priming, shelf-life, sugar beet.

## INTRODUCTION

The key factor in the large-scale production of reliable bacterial seed treatments for the sugar-beet market is the ability of the inoculum to survive in storage at ambient temperature in a dried commercial pellet in such a way that seed viability and vigour is also retained. Within any given growing season, a minimum of 4 months is the typical storage period between commercial processing and packaging of sugar-beet seed and end-point use by the grower. While Gram-positive endospore-forming bacteria are ideal candidates for the production of such seed treatments (Paau 1988), the majority of the most promising plant growth-promoting rhizobacteria and rhizosphere competent bacteria are Gram-negative pseudomonads (Whipps 1997). The aim of this study was to examine the application strategies that would enable candidate bacteria, especially those that were rhizosphere-competent Gram-negative strains, to fulfil these survival criteria on sugar-beet seed.

A variety of strategies for the application of bacteria to sugar-beet seed have been explored in other studies. Application as an aqueous suspension or slurry is the most commonly employed laboratory-based approach and, despite its potential commercial limitations, such a strategy is particularly useful in aiding the selection and testing of candidate bacteria for biocontrol activity (Whipps 1997). In this study, the seed soaking method was used to establish survival profiles of the isolates applied to the pelleted seed type used commercially in the UK [EB3, produced by the Germain's Technology Group (GTG)] following a range of cultivation and storage conditions. Once these profiles had

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been established, other seed treatment strategies were examined in an attempt to optimize the survival of the bacteria on seed.

Encapsulation of microbial cells in alginate, a linear polymer extracted from kelp (*Macrocystis pyrifera*), has been widely reported as a strategy for maintaining their viability in storage (Bashan 1986; Bucke 1987; Daigle and Cotty 1997). The sodium salt of alginate, when mixed with a calcium chloride solution, forms the water-insoluble gel calcium alginate because of cation exchange. Microbial cells have been shown to remain physiologically active after being entrapped in the interstitial spaces of such gels (Bashan 1986). This strategy has been used for the application of *Pseudomonas fluorescens* F113 to sugar-beet seed in a laboratory to determine whether an alginate coating produced around the seed could successfully deliver the bacterium into the seedling rhizosphere (Russo *et al.* 1996).

Peat is a potential carrier material that is both cheap and already available commercially as a finely milled sterilized powder in bags, facilitating direct inoculation and colonization by bacteria. Peat has a proven track record of success as a *Rhizobium* inoculant which is incorporated directly into the furrow of leguminous crops or applied to the seed at sowing (Powell 1992). Existing commercial pelleting technology can be used to incorporate peat into a seed pellet (Walker 2002). Clearly, this product has potential for use as a substrate for inoculum production using bacteria as biological control agents (BCAs).

Filmcoating has been shown to be an effective way of delivering dormant bacterial and fungal spores as seed treatments but has limitations as an application strategy for Gram-negative bacteria because of the exposure of vegetative cells to the abrasive shear forces involved (Paau 1988). Oospores of *Pythium oligandrum* have been successfully delivered onto sugar-beet seed using a commercial filmcoating process (McQuilken *et al.* 1990). However, more than 90% of *Ps. putida* 40RNF inoculum applied to sugar-beet seed during filmcoating died in the process, probably as a result of cell damage and lysis (Shah-Smith and Burns 1997). Because of the emphasis placed on the survival of Gram-negative bacteria in this current study, the strategy of filmcoating was not pursued.

The conditions to which the seed are subjected during the various priming techniques (which include osmoconditioning, solid matrix priming and damp seed incubation) provide a potentially ideal environment for bacterial inoculation and colonization of the seed, as has been reviewed by McQuilken *et al.* (1998). Seed species which have been shown to respond to various priming inoculations include: tomato (Harman and Taylor 1988; Legro and Satter 1995; Warren and Bennett 1999), sweet corn (Callan *et al.* 1990, 1991) and carrot (Jensen *et al.* 2002).

The commercial use of a sugar-beet priming procedure (based on the method of Durrant and Jaggard 1988) has increased significantly in the USA and in the UK sugar-beet crop in the last 5 years. The microbial inoculation of sugar-beet seed during an existing commercial hydration process already known to produce physiological enhancement, would be both economically and agronomically attractive with increased beneficial value for the crop. Surprisingly there are only two reports, neither of which have been widely circulated as full papers, relating the use of priming inoculations for sugar-beet seed (Paternoster and Burns 1996; Paternoster 1997) and no published data with regard to shelf-life at ambient temperature in relation to commercial pellet moistures. Our work was therefore directed towards evaluating priming and other inoculation systems for a selection of bacterial species and also at investigating their survival for several months in typical seed storage conditions.

## MATERIALS AND METHODS

### Chemicals and microbiological media

Unless otherwise stated all chemicals were obtained from Sigma (Dorset, UK) and microbiological media from Oxoid (Basingstoke, UK).

### Isolation and maintenance of wild-type strains of bacteria

All of the bacterial isolates used throughout this study were obtained from roots of sugar-beet seedlings and their isolation, screening, selection and characterization have already been documented (Williams and Asher 1996). The isolates were identified by fatty acid methyl ester profiling (Dr David Stead, Central Science Laboratories, Sand Hutton, York, UK) as strains of; *Bacillus megaterium* (P8S105), *Arthrobacter histidinolovorans* (P2T9), *Ps. fluorescens* (P22P101), *Ps. syringae* (P22P104) and *Cytophaga johnsonae* (P1T139). An additional isolate, *Ps. marginalis/putida* strain P1W1 (Walker *et al.* 2002), was selected for application in the priming studies. Rifampicin-resistant ($Rif^+$-marked) strains of the isolates were used throughout the work. All wild-type and $Rif^+$-marked strains of the isolates were maintained on plates of nutrient agar (NA) (Oxoid CM3) at 30°C and stored on NA slopes in universal bottles at 5°C and in 1 ml aliquots of sterile distilled water (SDW) in cryovials (System 100$^{TM}$, Nalgene®; Nalge Nunc International, Rochester, NY, USA) at −20°C.

### Production of antibiotic-resistant marked strains of bacteria

Rifampicin-resistant strains of the isolates were produced by spread plating wild-type liquid cultures in log growth phase

in tryptone soya broth (TSB) (Oxoid CM0876) onto NA containing 10 $\mu$g ml$^{-1}$ rifampicin. The resulting rifampicin-resistant colonies were then streak plated onto NA containing 100 $\mu$g ml$^{-1}$ rifampicin (NAR) and the Rif$^{+}$-marked strains were subsequently maintained on this medium.

### Seed material

All 'raw' (processed, rubbed and graded) and pelleted seed used throughout this study was obtained from British Sugar (Peterborough, UK). Seed pelleted with EB3 blend material (GTG; GTG-UK, King's Lynn, Norfolk, UK) without other pretreatment or fungicides is referred to as 'pelleted seed'. Standard commercial seed (SCS) refers to seed steeped in 0·2% w/v aqueous suspension of thiram prior to pelleting followed by the addition of thiram and hymexazol to the EB3 blend at rates of 4·8 g active integredient (a.i.) and 10·5 g a.i. per 100 000 seed respectively.

### Application of bacterial strains to prepelleted seed (seed soaking)

The bacterial suspensions were prepared for application to seed as described by Walker *et al.* (2002). All liquid cultures were incubated at 30°C and 100 rpm in rotary culture (Economy Incubator, Gallenkamp, UK) unless otherwise stated. Conical flasks containing 100 ml TSB were inoculated with 100 $\mu$l bacterial suspension from 24 h cultures in nutrient broth (Oxoid CM1). Flasks were incubated until each isolate had reached the mid-log phase of growth (determined previously by growth curve studies). The bacterial cells were centrifuged at 8000 *g* and 22°C for 30 min (4K10 Centrifuge; Sigma). The supernatant was discarded and the pellet re-suspended in 100 ml SDW. This washing procedure was repeated. The concentration of each bacterial isolate was determined by total cell counts in an Improved Neubauer haemocytometer (Weber; Lancing, Sussex, UK) and was adjusted to 9 $\log_{10}$ cells ml$^{-1}$.

Bacterial strains were applied to prepelleted seed as described by Williams and Asher (1996). Standardization was achieved by applying 500 $\mu$l aliquots of the cell suspension to 10 seeds in a single compartment of a 100 mm 25-compartment repli-dish (Bibby Sterilin; Stone, Staffordshire, UK) to give a final application rate of $5 \times 10^7$ cells per seed. The treatments were allowed to soak into the seed pellet for 4 h at 22°C and then dried for 16 h in a laminar flow cabinet at *ca* 20°C.

### Re-isolation of Rif$^{+}$-marked strains from inoculated seed and carrier materials

Each replicate sample was homogenized separately in maximum recovery diluent (MRD) (Oxoid CM0733) (1 g tissue in 9 ml MRD) for 15–30 s at 32 200 *g* using Janke & Kunkel Ultra-Turrax T25 (IKA®-Labortechnik, GmbH & Co. KG, Staufen, Germany). Homogenized samples were serially diluted in MRD and selected dilutions were spiral-plated (Model D Spiral Plater; Don Whitley Scientific Limited, Shipley, West Yorkshire, UK) onto NAR containing the antifungal agent cycloheximide (NARC; 50 $\mu$g ml$^{-1}$). The spiral-plated samples were incubated at 30°C and the numbers of re-isolated CFU calculated.

An amendment was made to this protocol for assessing samples where preparations of alginate were involved. All replicate samples were soaked for 30 min in tri-sodium citrate buffer ($C_6H_5Na_3O_7{\cdot}2H_2O$) (pH 7·0) (1 g tissue in 9 ml buffer) to dissolve the alginate. The samples were then homogenized in MRD as described previously.

### Survival profiles of the Rif$^{+}$-marked isolates applied in aqueous suspension to pelleted seed

The following experiments were carried out to determine the effects of different cultivation temperatures, duration of cultivation and seed drying regimes on the subsequent survival of the Rif$^{+}$-marked isolates on pelleted seed stored at two different storage temperatures (5 and 22°C).

The Rif$^{+}$-marked strains of isolates *B. megaterium* P8S105, *Ps. fluorescens* P22P101, *Ps. syringae* P22P104 and *Cyt. johnsonae* P1T139 were grown in liquid shake culture (100 ml TSB in 250 ml conical flasks) for 5, 11 and 28 days at 20°C and for 6, 12 and 28 days at 8°C, and cell number in these cultures was determined microscopically just before the bacteria were applied to pelleted seed as described previously. Half of this inoculated seed was dried at 40°C for 4·5 h in a fan-assisted incubator (Economy Incubator) and the remainder was air-dried for 16 h at room temperature in a laminar flow cabinet. Samples of the treated seed were stored at both 5 and 22°C. The numbers of surviving CFUs were assessed at various sample times by the spiral plating of seed homogenates prepared as described previously. In addition, growth curves of the five Rif$^{+}$-marked isolates in liquid shake culture at both 20 and 8°C were determined over the 28 days inoculum preparation period. This experiment was repeated with all five Rif$^{+}$-marked isolates (including P2T9) but the treated seed was stored at 5°C only.

### Encapsulation of Rif$^{+}$-marked isolates in alginate polymer

The medium viscosity sodium salt of alginic acid (A-2033, Sigma) was mixed with SDW and the resulting aqueous solution was then autoclaved at 121°C for 15 min. The effects of alginate polymer on the survival of the Rif$^{+}$-marked isolates was assessed both in alginate beads and after coating pelleted seed with alginate.

Aqueous cell suspensions of the Rif$^+$-marked isolates were prepared as described previously. The concentration of each bacterial isolate was adjusted to $1 \times 10^9$ cells ml$^{-1}$ in 3% alginate/bacterial suspension. Beads of alginate/bacteria *ca* 3 mm in diameter were produced by extruding the suspension into a solution of 0·1 M $CaCl_2$ using a 1000 $\mu$l Gilson Pipetman (Gilson, Middleton, WI, USA). Encapsulation of seed was carried out either by dipping prepelleted seeds into the suspension of bacterial cells contained in 3% alginate or by soaking the seed in a bacterial suspension and then adding a layer of 3% alginate around the seed. Immediately after these treatments, the seeds were immersed in a 0·1 M $CaCl_2$ solution. After allowing the gel to harden for 30 min, treated seeds and beads were rinsed in SDW and air-dried in a laminar-flow cabinet at room temperature.

Using these procedures, the following treatments were prepared for the five Rif$^+$-marked isolates: alginate beads; seeds dipped in alginate/bacterial suspension (3% alginate final concentration); seeds soaked in aqueous bacterial suspension (seed soaking) for 3 h then dipped in 3% alginate, and seeds soaked in aqueous bacterial suspension (seed soaking) for 3 h (no alginate).

Samples of all the treatments were stored in screw-cap Sterilin containers (Bibby Sterilin) at both 5 and 22°C. Survival of the isolates was assessed as described previously.

## Survival of Rif$^+$-marked isolates in colonised peat (*Rhizobium* inoculant)

Experiments were undertaken to explore the possibility of using peat as a carrier material for incorporating bacteria in a commercial seed pellet. The peat product (formerly from Microbio Ltd, Littlehampton, West Sussex, UK and currently available from Legume Technology Ltd, Epperstone, Nottinghamshire, UK) is used commercially as a field inoculant for *Rhizobium*. This product consists of a finely milled, sedge-based peat that is sterilized and sealed in gas-permeable bags. Colonization of the peat with *Rhizobium* is achieved commercially by sterile injection of an aqueous bacterial suspension into the bag and 'massaging' the bag thoroughly to aid distribution of the introduced liquid, followed by a period of incubation known as 'curing'. The Rif$^+$-marked isolates were cultivated and harvested as described previously. The concentration of each bacterial isolate was determined microscopically and these cultures put through a 1/370 dilution step. Five peat bags per isolate were inoculated by aseptically injecting each bag with 180 ml of the diluted cultures. The bags were cured at 30°C for 11 days.

After curing, the number of bacteria colonizing the peat was assessed for each isolate. The peat was dried in a laminar flow cabinet and stored in 15 ml aliquots in sealed Sterilin tubes (Bibby Sterilin) at 5 and 22°C. The survival of the bacteria was then monitored against time at these two storage temperatures. In addition, four bags of each isolate were stored unopened at room temperature for long-term survival studies. At sample times of 6 and 27 months, two replicate bags were opened for each isolate and three replicate samples removed from each bag and assessed for bacterial survival.

*Survival of Rif$^+$-marked* Ps. syringae *P22P104 in a commercially produced EB3/peat pellet* The peat inoculant was tested for its suitability as a carrier material for introducing the best performing Gram-negative bacterium into the commercial EB3 seed pelleting process (conducted at GTG-UK). Peat inoculated with *Ps. syringae* was applied at three different rates in the seed pellet either by application to the seed followed by EB3 pelleting or by mixing with the EB3 blend prior to pelleting. The effects of two different drying regimes on the survival of the isolate were also compared. This yielded 12 different treatment combinations. The treatments were tested for inoculum survival on seed at room temperature against time with the first sample being assessed the day after pelleting ($T = 1$ day). Seed of the cultivar Madison (Danisco Seeds, Lincoln, UK) was used throughout.

## Survival of Rif$^+$-marked *Ps. marginalis/putida* P1W1 applied to seed during priming

Experiments were designed to explore the feasibility of applying a selected Gram-negative isolate to sugar-beet seed (cultivar Celt; Syngenta Seeds, Cambridge, UK) during the commercial priming process. The proprietary 'advantage' process used routinely for sugar-beet seed preparation (conducted by GTG-UK) involves a preliminary steeping stage (also including a thiram fungicide steep in the UK product to eradicate deep-seated *Phoma betae* infections), followed by incubation of the damp seed for several days under controlled temperature conditions and a moisture content selected for the seed lot in question (Thomas *et al.* 1993).

The survival of *Ps. marginalis/putida* P1W1 following various priming application strategies was monitored during seed storage at 15°C over a 4-month period, the parameters selected to represent typical conditions in the commercial storage and distribution environment of pelleted sugar-beet seed (B. Gummerson, personal communication).

*Comparison of the two seed steeping methods and of different inoculation rates of P1W1* The isolate was applied to seed during priming by addition of 20 ml of bacterial suspension in sterile water at rates of: $\log_5$ cells ml$^{-1}$, $\log_7$ cells ml$^{-1}$, neat suspension ($\log_{9-10}$ cells ml$^{-1}$) and untreated control. Each of these treatments was applied to sugar-beet seed during priming following

two different steeping strategies to give eight treatments in total. Although the exact conditions remain proprietary information, the methods involved in the commercial treatment used in the UK include thiram fungicide in the steep (to eradicate deep-seated *Phoma betae* infections) followed by incubation for several days under controlled moisture and temperature conditions. In these experiments, the thiram was replaced with an alternative proprietary biocidal steep with non-selective antimicrobial activity to explore whether this would improve seed colonization by P1W1 through displacement of some of the resident microbial community. This strategy was compared with steeping the seed in water alone. (In later experiments, the effect of the standard thiram steep on the subsequent survival of *Ps. marginalis/putida* P1W1 was also evaluated).

The surviving populations of P1W1 $Rif^+$ and the resident microflora on the stored seed were determined by spiral plating of selected dilutions of seed sample homogenates onto NAR, *Pseudomonas* selective medium (PSM) (Oxoid CM0559 supplemented with Oxoid SR0103) and NA. The first medium was selective for the growth of $Rif^+$-marked *Ps. marginalis/putida* P1W1, while the other two media determined the populations of resident *Pseudomonas* species and the total bacterial count respectively. All three media were supplemented with cycloheximide antibiotic (50 $\mu$g $ml^{-1}$) to prevent fungal colonization. Two replicate samples were analysed for each of the treatments at each sample time. The mean data were plotted against time to determine the survival profiles of P1W1 expressed as CFU $g^{-1}$ seed in relation to the resident microbial populations for each treatment.

*Effects of subsequent pelleting, drying temperature and the addition of fungicides on survival of P1W1* A range of treatments (Table 1) were prepared applying isolate P1W1 as 20 ml of neat suspension in SDW where indicated and using the standard seed steeping strategy throughout. Fungicides were applied to the pellet at the standard commercial rate where indicated. The populations of P1W1, resident Pseudomonads and the total bacterial count were monitored as described previously.

*Comparison of priming with other application techniques using P1W1* The survival of isolate P1W1 applied in the commercial priming process was compared with other application techniques (Table 2). $Rif^+$-marked P1W1 was cultured in TSB and the cells harvested. The treatments were prepared, dried at room temperature and then stored at 15°C followed by testing for survival at the sampling times described previously.

*Effect of drying to different pellet moistures on shelf-life of P1W1 primed seed* Thiram-steeped seed primed with isolate P1W1 was pelleted with fungicides applied at the standard rate and then dried at 30°C to produce four separate subsamples each with a different pellet moisture. The samples were stored at 15°C followed by testing for survival at the sampling times described previously.

## Statistical analysis

Bacterial numbers were expressed as CFU following logarithmic transformation. Significant differences between sample means (Fisher's protected LSD, $P = 0{\cdot}05$) were determined by ANOVA performed with Genstat V.

**Table 2** List of treatment combinations used to compare priming of $Rif^+$-marked *Pseudomonas marginalis/putida* P1W1 with other application techniques

| Treatment | Description |
|---|---|
| 1 | Alginate beads (no seeds) |
| 2 | Alginate/bacterial mix applied to pelleted seed |
| 3 | Soaked pelleted seed + alginate coating |
| 4 | Soaked pelleted seed |
| 5 | Peat stored in sealed tubes (no seed) |
| 6 | Primed raw seed |
| 7 | Primed raw seed (stored at 22°C) |
| 8 | Primed seed + pellet |

**Table 1** List of treatment combinations used to apply $Rif^+$-marked *Pseudomonas marginalis/putida* P1W1 to seed during priming

| Treatment | Description | EB3 pellet | Thiram steep | Fungicides in pellet | Drying regime |
|---|---|---|---|---|---|
| 1 | P1W1 raw seed (RT) | − | − | − | RT |
| 2 | P1W1 raw + thiram (RT) | − | + | − | RT |
| 3 | P1W1 EB3 pellet (RT) | + | − | − | RT |
| 4 | P1W1 SCS pellet (RT) | + | + | + | RT |
| 5 | P1W1 raw seed (40°C) | − | − | − | 40°C |
| 6 | P1W1 raw + thiram (40°C) | − | + | − | 40°C |
| 7 | P1W1 EB3 pellet (40°C) | + | − | − | 40°C |
| 8 | P1W1 SCS pellet (40°C) | + | + | + | 40°C |

RT, room temperature SCS, standard commercial seed (rubbed, polished and graded 3·25–4·25 mm).

## RESULTS

### Survival profiles of the Rif$^+$-marked isolates applied by soaking onto pelleted seed

For the four isolates tested, inoculum survival was poor when seed was stored at the higher temperature, with loss in viability of all treatments within 28 days of storage (data not shown). When treated seed was stored at 5°C, each isolate produced distinct survival profiles that shared common characteristics (Table 3).

Firstly, the survival profiles of the treatments occurred in pairs according to the temperature and length of incubation of the liquid culture, irrespective of the seed drying temperature. In general, drying the seed at 40°C to conform to commercial seed-pelleting practices had no significant effect on bacterial survival compared with drying at ambient temperature. In two treatment pairs (of the 24 treatments tested) the numbers of bacteria were significantly greater after drying at 40°C compared with drying at ambient temperature. There were no other significant differences between the two drying procedures.

Secondly, cells from fresh, active cultures displayed better subsequent survival on seed than cells from older cultures. In almost all cases, cells harvested from late log-phase or early stationary-phase cultures survived significantly better on seed than cells from late stationary and decline-phase cultures, irrespective of the incubation temperature or bacterial isolate.

Finally, although bacteria were shown to survive the application and drying procedures in high numbers (cf. samples taken at $T = 0$), a large population decline during the first 24 h of seed storage (cf. samples taken on $T = 1$ day) was typical for all treatments of each isolate (data not shown). This decline varied between 2 and 5 $\log_{10}$ CFU depending on the isolate and the culture formulation.

**Table 3** Effect of cultivation temperature, incubation time and drying of seed on subsequent survival of Rif$^+$-marked isolates on seed stored for 160 days at 5°C

| | Survival of Rif$^+$-marked isolates (log CFU g$^{-1}$)* | | | |
|---|---|---|---|---|
| Single treatments (LSD = 2·12†) | *Bacillus megaterium* | *Pseudomonas fluorescens* | *Pseudomonas syringae* | *Cytophaga johnsonae* |
| 20°C (5 days) RT | 7·20$^{a}$‡ | 1·95$^{b}$ | 3·61 | 0·00$^{b}$ |
| 20°C (5 days) D | 7·24$^{a}$ | 5·16$^{a}$ | 5·39$^{a}$ | 0·00$^{b}$ |
| 20°C (11 days) RT | 2·05$^{c}$ | 4·58$^{a}$ | 0·82$^{b}$ | 0·00$^{b}$ |
| 20°C (11 days) D | 1·17$^{c}$ | 4·81$^{a}$ | 0·98$^{b}$ | 0·00$^{b}$ |
| 20°C (28 days) RT | 0·00$^{c}$ | 0·00$^{b}$ | 0·00$^{c}$ | 0·00$^{b}$ |
| 20°C (28 days) D | 0·00$^{c}$ | 0·00$^{b}$ | 0·00$^{c}$ | 0·00$^{b}$ |
| 8°C (6 days) RT | 0·00$^{c}$ | 5·53$^{a}$ | 6·02$^{a}$ | 0·80$^{b}$ |
| 8°C (6 days) D | 0·72$^{c}$ | 5·22$^{a}$ | 5·97$^{a}$ | 0·72$^{b}$ |
| 8°C (12 days) RT | 4·10 | 3·66 | 2·79$^{b}$ | 1·60$^{b}$ |
| 8°C (12 days) D | 2·07$^{c}$ | 4·17$^{a}$ | 2·89$^{b}$ | 4·07$^{a}$ |
| 8°C (28 days) RT | 5·19 | 0·00$^{b}$ | 0·00$^{c}$ | 0·00$^{b}$ |
| 8°C (28 days) D | 4·21$^{b}$ | 0·00$^{b}$ | 0·00$^{c}$ | 0·00$^{b}$ |
| Total (LSD = 0·91†) | 2·83$^{a}$ | 2·92$^{a}$ | 2·37$^{a}$ | 0·54$^{b}$ |

*Data shown are means from all sampling times pooled for each isolate.
†Significance between isolates tested using Fisher's protected LSD ($P = 0·05$).
‡Significant differences between treatments for each Rif$^+$-marked isolate denoted by 'a–c'.
RT, dried at room temperature D, dried at 40°C.

### Encapsulation of Rif$^+$-marked isolates in alginate polymer

Calculation of the survival rates in the beads showed a stabilization of the populations of all five isolates (Table 4). A population decline of only <1 $\log_{10}$ CFU after 24 h storage and <3 $\log_{10}$ CFU after 300 days was recorded for *B. megaterium*, *Ps. syringae* and *A. histidinolovorans*. The remaining two isolates showed a population decline of only <3 $\log_{10}$ CFU after 24 h storage and <3–5 $\log_{10}$ CFU after 300 days. For all five isolates, survival was significantly greater following encapsulation in alginate beads compared with soaking pelleted seed in the absence of alginate. Results showed that the populations of all isolates on seed were greater in the presence of alginate and this was observed at all sampling times.

### Survival of Rif$^+$-marked isolates in colonized peat (*Rhizobium* inoculant)

All isolates showed excellent survival on peat in sealed gas-permeable bags and all populations stabilized at >6 $\log_{10}$ CFU g$^{-1}$ after 27 months storage. The levels of the pseudomonads and *B. megaterium*, at 8 $\log_{10}$ CFU g$^{-1}$, were particularly high (Table 5).

Colonized peat stored in sealed tubes at 5°C produced stable population numbers of the Rif$^+$-marked isolates (Fig. 1a) over the 4-month period studied, with the exception of *Ps. fluorescens* which suffered a decline of *ca* 3 $\log_{10}$ CFU g$^{-1}$ during the first week of storage. The populations of *B. megaterium* and *Ps. syringae* were particularly well sustained, with a decline of <1 $\log_{10}$ CFU g$^{-1}$ after storage for 300 days. This survival was significantly better than that achieved previously with the seed soaking method (Table 6). When the preparations were stored at 22°C, the performance of all isolates was reduced. Despite this, at the critical 4-month storage limit, the populations of both *Ps. syringae* and *B. megaterium* were at *ca* 6·5 $\log_{10}$ CFU g$^{-1}$ (Fig. 1b). When the peat was compared with alginate it was found to be as effective for four of the isolates and was only significantly poorer for *Ps. fluorescens*

**Table 4** Effect of alginate on survival of Rif$^+$-marked isolates on preparations stored for 300 days at 5°C

| Treatment (LSD = 1·59†) | Survival of Rif$^+$-marked isolates (log CFU g$^{-1}$)* | | | | |
|---|---|---|---|---|---|
| | *Bacillus megaterium* | *Arthrobacter histidinolovorans* | *Pseudomonas fluorescens* | *Pseudomonas syringae* | *Cytophaga johnsonae* |
| Alginate beads (no seed) | 8·47$^a$‡ | 7·00$^a$ | 6·52$^a$ | 9·08$^a$ | 6·01$^a$ |
| Alginate mix onto pelleted seed | 6·81$^b$ | 4·96$^b$ | 0·81$^c$ | 8·64 | 1·08$^b$ |
| Soaked pelleted seed + alginate | 4·08$^c$ | 5·45 | 2·59$^b$ | 7·80 | 1·55$^b$ |
| Soaked pelleted seed | 2·61$^c$ | 4·83$^b$ | 0·00$^c$ | 6·11$^b$ | 0·41$^b$ |

*Data shown are means at all sample times pooled for each isolate.
†Significance between isolates tested using Fisher's protected LSD ($P = 0·05$).
‡Significant differences between treatments for each Rif$^+$-marked isolate denoted by 'a–c'.

**Table 5** Comparison of survival of Rif$^+$-marked isolates on peat in gas-permeable bags stored at room temperature

| LSD = 2·11† | Survival of Rif$^+$-marked isolates (log CFU g$^{-1}$)* | | | | |
|---|---|---|---|---|---|
| | *Bacillus megaterium* | *Arthrobacter histidinolovorans* | *Pseudomonas fluorescens* | *Pseudomonas syringae* | *Cytophaga johnsonae* |
| 6 months | 7·67$^a$‡ | 7·41$^a$ | 7·83$^a$ | 7·50$^a$ | 4·15$^b$ |
| 27 months | 7·56$^a$ | 5·95 | 7·57$^a$ | 7·81$^a$ | 4·21$^b$ |
| Total (LSD = 1·49†) | 7·61$^a$ | 6·68$^a$ | 7·70$^a$ | 7·65$^a$ | 4·18$^b$ |

*Data shown are means from triplicate samples at all sample times pooled for each isolate.
†Significance between isolates tested using Fisher's protected LSD ($P = 0·05$).
‡Significant differences at each sampling time between Rif$^+$-marked isolates denoted by 'a–b'.

(Table 6). The best-performing Gram-negative isolate (*Ps. syringae*) was selected for incorporation into a commercially produced peat-EB3 pellet.

*Survival of Rif$^+$-marked* Ps. syringae *P22P104 in a commercially produced EB3/peat pellet* All of the pelleted preparations using the peat-based formulations performed poorly in terms of survival of the isolate when stored at room temperature. The pseudomonad could be recovered from only one of the 12 treatments after 21-day storage and in numbers just above the detection limit (data not shown). This survival behaviour was markedly poorer than the survival of the isolate on peat stored at 22°C in sealed tubes. Incorporating colonized peat into the commercial pelleting procedure offered no advantage in terms of survival of the isolate on seed stored at ambient temperature compared with the seed soaking method.

## Survival of Rif$^+$-marked *Ps. marginalis/putida* P1W1 applied to seed during priming

*Comparison of the two seed steeping methods and of different inoculation rates of P1W1* For each application rate, the population of P1W1 was significantly greater in the priming application following the standard water-steeping treatment compared with the corresponding biocidal treatment (Table 7a). The highest rate of application in both treatment strategies produced the best survival in storage, with the most concentrated cell suspension producing significantly greater populations than the lower application rates. An exception was the 5 $\log_{10}$ rate which was not significantly different to the 7 $\log_{10}$ rate after the biocidal treatment. However, both of these treatments performed poorly when compared with the highest application rate.

The biocidal treatment had a noticeable impact on the resident pseudomonads, causing detectable fluctuations in the population levels detected (Table 7b). This effect was also observed for the biocidal control treatment that was not subsequently treated with P1W1. By comparison, the water-steeped seed supported significantly greater populations of resident pseudomonads, with only a slow decline of *ca* 1 log unit over the course of the experiment. The addition of isolate P1W1 did not affect the resident pseudomonad population in either water steeped or biocidal treated seed.

All the biocidal treatments, except the one followed by the highest rate of P1W1 application, yielded lower total bacterial counts than the water steeped treatments (data

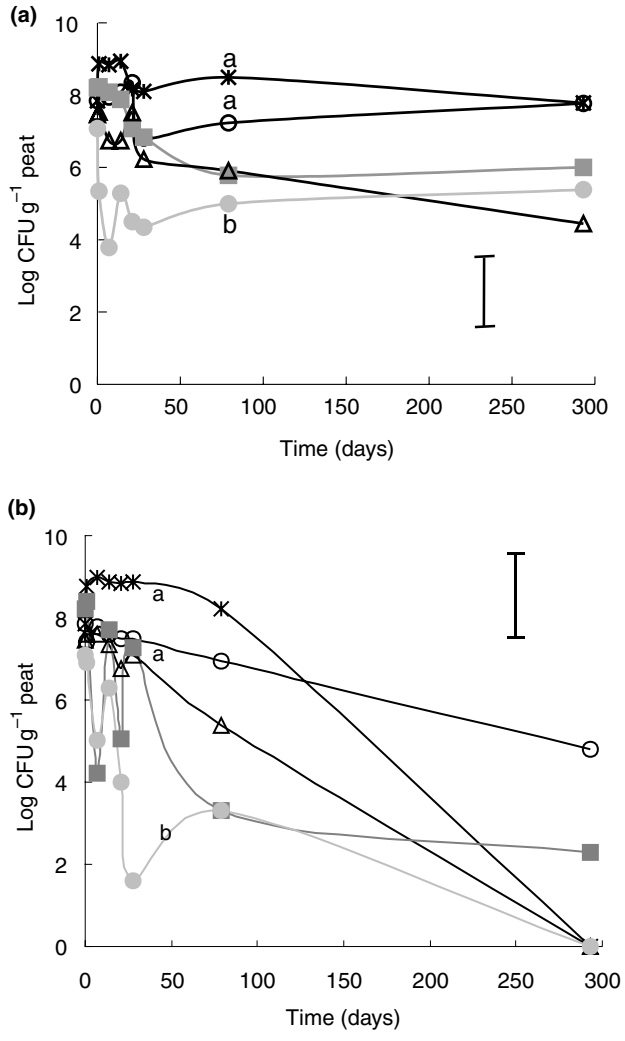


**Fig. 1** Comparison of survival of Rif$^{+}$-marked isolates on peat stored in sealed tubes at (a) 5°C and (b) 22°C. Treatments: *Bacillus megaterium* P8S105 (○), *Pseudomonas syringae* P22P104 (✱) *Arthrobacter histidinolovorans* P2T9 (Δ), *Cytophaga johnsonae* P1T139 (■) and *Pseudomonas fluorescens* P22P101 (●). Significant differences between treatment means denoted by (a–b). Bars represent least significant differences between treatment means (Fisher's protected LSD, $P = 0{\cdot}05$)).

not shown). The general effect of the biocidal treatments was therefore to slightly reduce the cell numbers in the total bacterial community, although there were no significant differences between treatments in terms of their total bacterial counts. There was no interaction between the introduced P1W1 isolate and the total bacterial community in either water steeped or biocidal treatments.

*Effects of subsequent pelleting, drying temperatures and the addition of fungicides on survival of P1W1* The most stable populations of P1W1 were produced when raw seed was dried at room temperature following priming inoculation (Table 8). Thiram-steeping prior to priming produced a reduction in population levels of P1W1 but this was only significant when the seed was dried at 40°C. Compared with nonpelleted seed, subsequent pelleting of the P1W1 primed seed resulted in a significant reduction in the P1W1 population. Addition of fungicides to the pellet had no detrimental effect on the population levels of P1W1 compared with the EB3-pelleted seed without fungicides. There were no significant differences between the two drying procedures. As with the previous experiment, there was no interaction between the introduced P1W1 isolate and either the resident pseudomonad population or the total bacterial community (data not shown).

*Comparison of priming with other application techniques using P1W1* The priming treatment promoted survival at *ca* 7·5 $\log_{10}$ CFU g$^{-1}$ seed after 4 months in storage at 15°C (Fig. 2). This survival rate was significantly greater than that following the other preparations. In addition, the survival of the isolate applied during priming was not significantly affected when the seed was stored at 22°C.

*Effect of drying to different pellet moistures on survival of P1W1 primed seed* The percentage moisture content recorded in the pellet of the four seed samples was 12·30, 10·08, 9·15 and 6·74. The rate of decline in P1W1 population numbers appeared to be directly related to the final moisture content of the seed pellet, with the fastest rate of population decline occurring in the seed sample with the highest pellet moisture and vice versa (Fig. 3). The survival of the P1W1 populations in the two samples with the higher pellet moistures showed marked fluctuations in the early storage period and suffered a rapid decline during later storage. However, the stabilization of P1W1 population levels in the two samples with the lower pellet moistures was comparable with the results obtained from earlier priming inoculation experiments where moisture content was not measured.

## DISCUSSION

In general, the isolates survived their initial application onto seed in high numbers irrespective of the strategy and drying procedure used. Subsequent survival in storage following the non-priming strategies was commonly characterized by a rapid initial decline in viable populations especially at ambient temperature. With the exception of prior biocidal steeping, all the priming strategies tested displayed a significant improvement with regard to the long-term survival of a selected Gram-negative bacterium at ambient temperature when compared with other application techniques.

**Table 6** Comparison of peat with alginate on survival of Rif$^+$-marked isolates for 300 days at 5°C

| | Survival of Rif$^+$-marked isolates (log CFU g$^{-1}$)* | | | | |
|---|---|---|---|---|---|
| LSD = 1·68† | *Bacillus megaterium* | *Arthrobacter histidinolovorans* | *Pseudomonas fluorescens* | *Pseudomonas syringae* | *Cytophaga johnsonae* |
| Peat | 7·93 | 6·48 | 4·13$^{b}$‡ | 8·52$^{a}$ | 7·18$^{a}$ |
| Alginate beads (no seed) | 8·45$^{a}$ | 6·98$^{a}$ | 6·59$^{a}$ | 9·01$^{a}$ | 6·09$^{a}$ |
| Alginate mix onto seed | 6·36$^{b}$ | 4·84$^{b}$ | 0·63$^{c}$ | 8·52$^{a}$ | 0·84$^{b}$ |
| Soaked seed | 2·63$^{c}$ | 4·69$^{b}$ | 0·00$^{c}$ | 6·04$^{b}$ | 0·55$^{b}$ |

*Data shown are means at all sample times pooled for each isolate.
†Significance between isolates tested using Fisher's protected LSD ($P = 0·05$).
‡Significant differences between treatments for each Rif$^+$-marked isolate denoted by 'a–c'.

**Table 7** Comparison of two priming techniques and three application rates of introduced Rif$^+$-marked P1W1 on (a) its subsequent survival in storage at 15°C and (b) its effect on the population of resident pseudomonads

| | Population levels on stored seed (log CFU g$^{-1}$)* (LSD = 2·14†) | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample time (days) | 0 | 1 | 7 | 14 | 28 | 59 | 84 | 137 | 188 | (LSD = 1·08†) |
| (a) P1W1 | | | | | | | | | | |
| Log 5 biocidal prime | 3·83$^{b}$‡ | 0·00$^{b}$ | 3·70$^{b}$ | 2·00$^{b}$ | 5·57$^{b}$ | 0·00$^{b}$ | 1·65$^{c}$ | 0·00$^{c}$ | 2·00$^{b}$ | 2·13$^{c}$ |
| Log 5 standard prime | 7·39$^{a}$ | 7·00$^{a}$ | 6·64$^{a}$ | 6·49 | 7·03 | 7·26$^{a}$ | 6·51 | 3·23$^{b}$ | 4·18$^{a}$ | 5·92$^{b}$ |
| Log 7 biocidal prime | 3·31$^{b}$ | 1·81$^{b}$ | 2·77$^{b}$ | 0·00$^{b}$ | 5·46$^{b}$ | 0·00$^{b}$ | 0·00$^{c}$ | 0·00$^{c}$ | 0·00$^{b}$ | 1·18$^{c}$ |
| Log 7 standard prime | 7·79$^{a}$ | 7·73$^{a}$ | 8·05$^{a}$ | 7·31$^{a}$ | 8·18$^{a}$ | 7·66$^{a}$ | 5·55$^{b}$ | 6·06$^{a}$ | 5·53$^{a}$ | 6·91$^{b}$ |
| Neat biocidal prime | 9·20$^{a}$ | 9·07$^{a}$ | 8·48$^{a}$ | 8·05$^{a}$ | 8·35$^{a}$ | 8·44$^{a}$ | 8·92$^{a}$ | 6·50$^{a}$ | 4·48$^{a}$ | 7·60$^{a}$ |
| Neat standard prime | 9·32$^{a}$ | 8·96$^{a}$ | 8·54$^{a}$ | 8·63$^{a}$ | 8·95$^{a}$ | 8·74$^{a}$ | 8·36$^{a}$ | 7·55$^{a}$ | 5·93$^{a}$ | 8·10$^{a}$ |
| (b) Resident pseudomonads | | | | | | | | | | |
| Log 5 biocidal prime | 4·27$^{b}$ | 2·87$^{b}$ | 0·00$^{b}$ | 2·41$^{b}$ | 4·75$^{b}$ | 0·00$^{c}$ | 1·89$^{b}$ | 1·65$^{b}$ | 4·63 | 2·19$^{b}$ |
| Log 5 standard prime | 7·02$^{a}$ | 6·61$^{a}$ | 6·71$^{a}$ | 6·61$^{a}$ | 6·41 | 7·09$^{a}$ | 8·05$^{a}$ | 6·76$^{a}$ | 5·49 | 6·73$^{a}$ |
| Log 7 biocidal prime | 0·00$^{c}$ | 1·61$^{b}$ | 0·00$^{b}$ | 0·00$^{c}$ | 5·03 | 2·49$^{b}$ | 4·92 | 0·00$^{b}$ | 3·75$^{b}$ | 2·31$^{b}$ |
| Log 7 standard prime | 7·33$^{a}$ | 5·76$^{a}$ | 6·95$^{a}$ | 5·77$^{a}$ | 7·09$^{a}$ | 6·27$^{a}$ | 6·55$^{a}$ | 6·39$^{a}$ | 6·02$^{a}$ | 6·43$^{a}$ |
| Neat biocidal prime | 6·02 | 5·29$^{a}$ | 6·79$^{a}$ | 5·85$^{a}$ | 5·61 | 2·49$^{b}$ | 4·92 | 0·00$^{b}$ | 2·40$^{b}$ | 4·01$^{b}$ |
| Neat standard prime | 6·63$^{a}$ | 6·26$^{a}$ | 7·03$^{a}$ | 6·27$^{a}$ | 7·28$^{a}$ | 5·63$^{a}$ | 5·04 | 6·09$^{a}$ | 6·48$^{a}$ | 6·26$^{a}$ |
| Control biocidal prime | 0·00$^{c}$ | 5·33$^{a}$ | 1·89$^{b}$ | 1·65$^{b}$ | 4·69$^{b}$ | 3·31 | 3·31$^{b}$ | 0·00$^{b}$ | 4·25 | 2·73$^{b}$ |
| Control standard prime | 7·73$^{a}$ | 3·97$^{a}$ | 5·77$^{a}$ | 5·74$^{a}$ | 5·61 | 5·38$^{a}$ | 6·44$^{a}$ | 5·80$^{a}$ | 4·72 | 5·64$^{a}$ |

*Data are means from duplicate samples.
†Significance tested using Fisher's protected LSD ($P = 0·05$).
‡Significant differences between treatments at each sample time denoted by 'a–c'.

With the seed soaking strategy, all the isolates survived the initial application stage in high numbers. Surprisingly, drying the seed at 40°C had no detrimental effect on bacterial survival. Shah-Smith and Burns (1997) reported the promising survival of cells of *Ps. putida* 40RNF applied to EB3-pelleted sugar-beet seed using a very similar laboratory-based seed-soaking technique and this has also been reported for a range of bacterial species (Paternoster 1997). In both of these earlier studies, drying the seed at raised temperature had no impact on bacterial survival, producing similar populations in the pellet irrespective of whether a drying temperature of 22, 30 or 45°C was used. Although the drying air is at the raised temperature, a damp, pelleted seed mass actually remains at a lower temperature during drying and only increases towards the maximal value when the process is almost complete (B. Gummerson, personal communication). Therefore the drying temperature would be expected to have less impact on bacterial viability than anticipated.

It has been suggested that bacterial cells from dormant cultures survive better than actively growing cells. Slininger *et al.* (1996) reported that, although young cells (from 24–48 h liquid culture) of *Ps. fluorescens* 2–79 survived drying processes significantly better than cells from older cultures (72–96 h), the older cells displayed a significantly higher survival rate on encapsulated wheat. In the experiments reported here, however, actively growing cells from log-phase cultures survived significantly better than cells

**Table 8** Effects of subsequent pelleting, drying procedures and addition of fungicides on subsequent survival of Rif$^{+}$ P1W1 in storage at 15°C

| Sample time (days) | Population levels of P1W1 on stored seed (log CFU g$^{-1}$)* (LSD = 0·66†) | | | | | | | | Total (LSD = 0·23†) |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 7 | 14 | 28 | 59 | 84 | 137 | |
| P1W1 raw seed (RT) | 8·61 | 9·00 | 8·85 | 8·88 | 8·97 | 7·77 | 8·67 | 8·57 | 8·66[a]‡ |
| P1W1 raw + thiram (RT) | 8·58 | 8·76 | 8·66 | 8·86 | 8·58 | 7·36 | 8·46 | 8·29 | 8·44 |
| P1W1 EB3 pellet (RT) | 7·67 | 7·98 | 7·55 | 7·71 | 7·64 | 5·46 | 5·40 | 6·43 | 6·98[d] |
| P1W1 SCS pellet (RT) | 7·70 | 7·55 | 7·67 | 7·56 | 7·79 | 6·83 | 6·74 | 6·76 | 7·32[c] |
| P1W1 raw seed (40°C) | 8·82 | 8·74 | 8·77 | 8·80 | 8·76 | 7·74 | 8·51 | 8·20 | 8·54[a] |
| P1W1 raw + thiram (40°C) | 8·55 | 8·48 | 8·44 | 8·32 | 8·64 | 7·13 | 8·22 | 7·67 | 8·18[b] |
| P1W1 EB3 pellet (40°C) | 7·17 | 7·31 | 7·22 | 7·31 | 6·57 | 6·40 | 6·70 | 6·77 | 7·93[d] |
| P1W1 SCS pellet (40°C) | 7·88 | 8·00 | 7·95 | 7·75 | 7·43 | 6·95 | 6·84 | 6·60 | 7·34[c] |

*Data are means from duplicate samples.
†Significance tested using Fisher's protected LSD ($P = 0·05$).
‡Significant differences between treatment means at each sample time denoted by 'a–d'.
RT, seeds dried at room temperature.

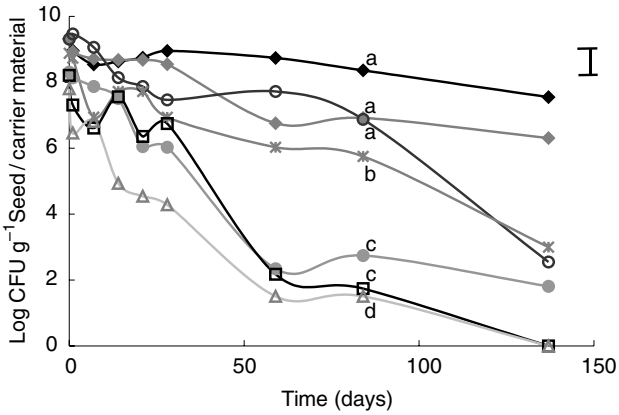


**Fig. 2** Comparison of priming inoculation with other application techniques in terms of survival of Rif$^{+}$-marked *Pseudomonas marginalis/putida* P1W1 on seed stored at 15°C. Inoculations: primed (◆), Primed (stored at 22°C) (◆), Alginate beads (no seed) (○), peat (no seed) (✱), soaked seed + alginate (●), alginate mix + seed (□), soaked seed (no alginate) (△). Significant differences between treatment means denoted by (a–d). Bars represent least significant differences between treatment means (Fisher's protected LSD, $P = 0·05$).

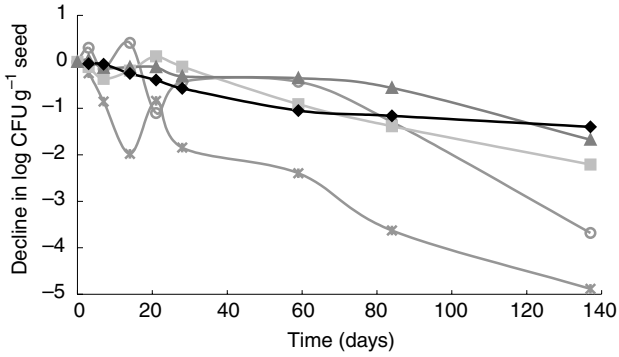


**Fig. 3** Effect of pellet moisture on shelf-life of Rif$^{+}$-marked *Pseudomonas marginalis/putida* P1W1 following priming inoculation. All samples were pelleted in EB3 with fungicides at the standard rate followed by raised temperature drying. Pellet moistures: 12·30 (✱), 10·08 (○), 9·15 (■) and 6·74 (▲) (dried at 30°C), and no pellet moisture recorded (dried at 40°C) (◆).

from older cultures. The harvesting of cells in mid-log-phase was accordingly adopted as a standard procedure throughout the remainder of this study.

Despite the promising bacterial survival after seed soaking and drying of the pellets, their numbers suffered a significant decline during initial seed storage. In addition, the seed soaking method only produced sufficient survival of bacteria on seed when a storage temperature of 5°C was used. Ambient storage temperatures of both 15 and 22°C resulted in a rapid decline in viability with this application technique. This phenomenon, which has been attributed to a decrease in metabolic activity of the cells at refrigerated temperatures (Wessendorf and Lingens 1989), has been widely reported for the storage of Gram-negative bacteria (Somasegaran 1985; Russo *et al.* 1996).

It was concluded that the low moisture environment offered by the EB3-pellet alone was insufficient for long-term bacterial survival when bacteria were applied directly to this pellet material. Results from other experiments carried out *in vitro* showed that the presence of EB3-pelleting material had no detrimental effect on the viability of the isolates (data not shown).

Attempts to stabilize the populations of the isolates with preparations of alginate both in the presence and the absence of EB3-pelleted seed produced mixed results. The survival of the isolates at 5°C was radically improved by encapsulation in alginate beads and alginate coating significantly increased bacterial survival on EB3-pelleted seed for some of the isolates tested. This stabilization maintained adequate

populations of isolates of *B. megaterium*, *A. histidonolovorans* and *Ps. syringae* for the critical 4-month storage period. However, a later study showed that all alginate preparations stored at 15°C performed poorly (data not shown) and that the presence of alginate around the seed significantly reduced seedling emergence (Walker 2002).

The isolates survived in high numbers in colonized peat in sealed gas-permeable bags, with the pseudomonads and *B. megaterium* showing a decline of $<1$ $\log_{10}$ CFU $g^{-1}$ after incubation for 27 months, well within the expected survival limits (P. Fiddaman, personal communication) and comparable data published by other workers using a similar approach supports this (Sparrow and Ham 1983; Somasegaran 1985; Rabindran and Vidhyasekaran 1996; Gasoni *et al.* 1998). On dried peat stored at 22°C, the populations of both *Ps. syringae* and *B. megaterium* remained within commercially acceptable limits for the critical 4-month storage period. This suggested that peat had potential as a carrier material for incorporating the isolates into the commercial pelleting process. However, although all of the peat-EB3 pellet treatments survived the commercial pelleting and drying processes with no significant loss in viability, subsequent survival at room temperature was very poor (Walker 2002), suggesting that the moisture content of the EB3-peat pellet was insufficient for survival of the bacteria in this carrier system.

A similar study, in which peat colonized with either *Ps. fluorescens*, *B. pumilis* or *B. cereus* was applied to radish seeds, reported that the *Bacillus* species produced stable populations of *ca* 5–6 $\log_{10}$ CFU $g^{-1}$ which survived for 150 days at 20–30°C whereas the pseudomonad had declined to $<4$ $\log_{10}$ CFU $g^{-1}$ after only 20-day storage (Gasoni *et al.* 1998). Similar shelf-life inadequacies have previously resulted in the biocontrol product Dagger G (a peat-based granular formulation of *Ps. fluorescens*) being withdrawn from the market (Lisansky and Coombs 1993).

Another study using a vermiculite carrier system to incorporate *Ps. fluorescens* F113 into the EB3 sugar-beet seed pellet reported a direct correlation between bacterial survival and pellet moisture (Moënne-Loccoz *et al.* 1999). Although a stable population of 7 $\log_{10}$ CFU $g^{-1}$ seed was achieved during storage at 12°C over a 21-day sampling period with a pellet moisture of *ca* 10%, the survival of the bacterium was compromised when a pellet with a moisture of 8% was stored under the same conditions. In contrast, Shah-Smith and Burns (1997) maintained populations of *ca* $2 \times 10^5$ CFU per pellet for 24 weeks with *Ps. putida* 40RNF applied in a commercial EB3 pellet stored at 18–20°C. This performance is within the bounds of commercial acceptability (Paau 1988) although the moisture content of the pellets was not reported.

A major focus of this study was to overcome the contradictory technical constraints of producing a seed pellet with a moisture content that was sufficiently low to maintain seed viability in long-term storage while sustaining an adequate population of a rhizosphere competent, non-spore-forming bacterium. For the long-term storage of sugar-beet seed, a pellet with *ca* 7% m.c. was recommended (B. Gummerson, personal communication). P1W1 applied as a priming treatment maintained populations of *ca* 6·6 $\log_{10}$ CFU $g^{-1}$ seed after 137 days storage at 15°C following subsequent pelleting, addition of fungicides and drying at 40°C, all standard commercial practices. Subsequent experiments verified a population decline of $<1{\cdot}7$ log CFU $g^{-1}$ seed over this storage period when commercial pellets were dried to $<7$% m.c. and stored in accordance with the standard commercial practice. An inoculation method, which allows the survival on seed of a Gram-negative bacterium at ambient temperature with little loss in viability, has thus been established for sugar beet.

It has been reported that the application of bacteria to seed during priming results in prior colonization (Callan *et al.* 1990, 1991). This may allow a certain degree of acclimatization to the seed coat environment and thus improve subsequent survival. Bacteria capable of producing protective exopolysaccharides *in situ*, such as pseudomonads, may do so during priming (Callan *et al.* 1997). Indeed, in our study, the performance of *Ps. marginalis/putida* P1W1 applied during priming by far surpassed survival of this isolate over a 4-month period at 15°C when applied to the seed by any other technique. In addition, bacterial survival was not significantly affected when the storage temperature was raised to 22°C or the final pellet moistures dried to $<7$%. It was also noted that higher moisture content in the stored pellets could actually be detrimental to bacterial survival and resulted in population fluctuations. This may be the result of the excess moisture encouraging the bacterial cells to proliferate rather than conserve energy for survival. Alternatively, the resident microbial population may increase during storage in this moist pellet environment to out-compete the introduced strain.

It had been anticipated in our work that the biocidal steep treatment prior to the introduction of the BCA on sugar-beet seed would facilitate more extensive colonization of the seed by P1W1 because of the removal of some of the resident microflora. It may be, therefore, that the biocidal treatment reduces P1W1 proliferation at low cell concentrations via a physical effect, either by removal of seed exudates utilized as nutritional substrates by colonizing bacteria or through the release of toxic substances from lysed microbial cells or the seed coat. In the water-steeped treatments, where P1W1 demonstrated better survival, colonization did not occur at the expense of the resident pseudomonad population or the total culturable bacterial community. There were no detectable interactions between P1W1 and any of the resident bacterial populations in any of the priming inoculation experiments.

Scanning electron microscopy (Fukui *et al.* 1994) has revealed that the colonization patterns of sugar-beet seed by introduced Gram-negative bacteria. Experiments in which surface-sterilized sugar-beet seed were subsequently inoculated with strains of *Ps. putida* and/or *Ps. fluorescens-putida* described bacterial colonies randomly distributed over the entire surface of the pericarp. Despite this distribution, no more than 10–40% of the total pericarp surface was colonized, even with very high-density inocula. Nutrients are not evenly distributed in this environment, suggesting that colonization is a function of nutrient availability rather than spatial accessibility.

Strain compatibility during colonization has been partly attributed to the utilization of differing carbon substrates (Wilson and Lindow 1994) suggesting that displacement of the resident microbial community by an introduced bacterium would only occur through direct competition for the same nutrient source. This may explain the apparent lack of interaction between the introduced *Ps. marginalis/putida* P1W1 and the resident microbial population in the present study.

When comparing the various components of the commercial seed-pelleting process on priming-inoculated seed, the addition of the pellet resulted in a significant reduction in the survival of P1W1, whereas addition of the standard rate of fungicides to the pellet did not produce any further significant reduction in survival. Steeping seed in thiram only had a significant effect on survival of the isolate when the seed was dried at 40°C.

The use of priming as a strategy to apply a BCA to sugar-beet seed has not been widely reported previously. Paternoster and Burns (1996) demonstrated that application of *Ps. fluorescens* (*Pf* 54/96) to sugar-beet seed during a prepelleting priming step resulted in significantly greater viable populations on pelleted seed than application during pelleting. Paternoster (1997) has demonstrated the successful application of a range of bacterial genera including *Flavobacterium*, *Arthrobacter*, *Bacillus* and *Pseudomonas* to sugar-beet seed during commercial priming inoculations with shelf-life responses at ambient temperature comparable with the data presented in this study.

Clearly, priming inoculation offers enormous potential for the delivery of Gram-negative bacteria as biological seed treatments on sugar beet. Further studies of the nutritional and spatial aspects of seed colonization and the processes involved during acclimatization are required in order to optimize this potential.

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