ESTABLISHMENT OF *SERRATIA ENTOMOPHILA* IN SOIL FROM A GRANULAR FORMULATION

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ABSTRACT

A granular formulation of *Serratia entomophila* has been developed to improve shelf-life and storage characteristics of this bacterium, which is used as a microbial control agent of the New Zealand grass grub. Bacterial establishment and survival of bacteria released from the granular and liquid formulations were assessed in a laboratory experiment. Bacteria were enumerated by dilution plating onto *Serratia* selective agar. *Serratia entomophila* populations in soil inoculated with granules remained stable in soil for up to five months at a range of soil moisture levels. Bacterial numbers declined more rapidly when soil was inoculated with the liquid formulation. High numbers of bacteria remained viable in the granules throughout the experiment demonstrating the potential for sustained release of inoculum after application of the biopesticide granules.

Keywords: *Serratia entomophila*, biopesticide, formulation, soil inoculation, soil moisture.

INTRODUCTION

The New Zealand grass grub (*Costelytra zealandica* (White)) (Coleoptera: Scarabaeidae) feeds on the roots of grasses and clovers, causing significant economic pasture damage. The bacterial pathogen *Serratia entomophila* causes amber disease of New Zealand grass grub, and has been developed as a commercial microbial control agent. For many years, the bacterium was applied to pasture soil as a liquid drench known as Invade® (Jackson et al. 1992). However, the need to store the bacterial product under refrigeration and the large volume of water required to apply the suspensions limited the use of this product. To overcome these limitations, new formulations that have more favourable storage, distribution and application properties have been developed. A clay-based prill formulation of *S. entomophila* gave improved shelf-life at ambient temperatures and application properties (Johnson et al. 2001).

In addition to improving the shelf-life of microbial inoculum, a biopesticide must be formulated to allow efficient release of pathogen from the site of inoculation, ensuring its subsequent availability to the target pest. Laboratory studies on the clay-based prill formulation demonstrated the successful release of *S. entomophila* into soil at several soil moisture levels (O'Callaghan et al. 2002). More recently, there has been further developmental work on the formulation of *S. entomophila*, and a new granular formulation, known as Bioshield™, has been developed.

This paper reports experiments on the prototype granular formulation, to examine release of *S. entomophila* from granules at different levels of soil moisture. Persistence of *S. entomophila* in soil treated with granules is compared with soil treated with the liquid drench application of *S. entomophila*.

MATERIALS AND METHODS

Growth and formulation of *Serratia entomophila*

Serratia entomophila strain 626 was originally isolated from pasture soil in Canterbury and is pathogenic to the New Zealand grass grub. This strain is held in the Microbial Control/Insect Pathogen Culture Collection, AgResearch, Lincoln. *Serratia entomophila*

was cultured by inoculating 100 ml of broth (4 g raw sugar, 1 g yeast extract, 0.2 g urea and 0.2 g NPK) with 1 ml of an overnight culture and incubating with shaking (180 rpm) at 30°C for 36 h. Cell counts were determined by serial dilution plating on Luria Bertani (LB) agar (Sambrook et al. 1989). Broth used in experiments contained $\sim 1 \times 10^9$ colony forming units (cfu)/ml. *Serratia entomophila* was formulated into granules using methods developed at AgResearch, Lincoln, for the stabilisation of non-sporeforming bacteria (NZ Patent 50687). Granules were approximately 5 mm in diameter, with a moisture content of 35–40% and a bacterial loading of 1.23×10^{10} cfu/g fresh weight of granule.

Quantitative recovery of formulated *S. entomophila* from granules and soil

Serratia entomophila populations in soil were enumerated by soil dilution plating onto a selective medium, caprylate thallos agar (Starr et al. 1976), and identity of colonies was verified as described previously (O'Callaghan & Jackson 1993). Granules recovered from soil microcosms were weighed and suspended in 9.9 ml extraction medium, containing 0.1% (w/v) tetra-sodium pyrophosphate and Tween 80 to aid dispersal of the cells. The tubes containing samples were vortexed for 30 sec and placed horizontally in an orbital shaker for 1 h at speed 6. Suspensions were dilution plated onto CTA as above.

Effect of soil moisture on release of *S. entomophila* from granules

Field soil (Wakanui silt loam, pH = 6.3) was collected at Lincoln, Canterbury. The soil was sieved and stored at 4°C until used. Soil moisture content was determined by drying 20 g soil at 60°C for 48 h. Samples of the soil were either air-dried or moistened with tap water to give the required soil moisture contents of 15% (equates to wilting point), 20%, 25% and 30% (w/w) (equates to field capacity). The equivalent of 20 g dry soil was added to 30 ml sterile plastic tubes. Granules (approximately 0.05 g) were buried in the soil microcosms to give a rate of $\sim 10^7$ *Serratia* cfu/g air dried soil. Addition of 0.1 ml of liquid InvaDe® gave a rate of $\sim 5 \times 10^6$ cfu/g soil. The tubes were randomly placed on one shelf of a constant temperature cabinet ($15 \pm 1^\circ\text{C}$). At each sampling date, tubes (4 replicates for granule treatments; 2 replicates for liquid InvaDe®) were randomly selected and destructively sampled. Granules were extracted from soil and *Serratia entomophila* in soil and in the granules were enumerated separately as described above.

Statistical analysis

Counts on *S. entomophila* cfu/g soil were logarithm transformed and subjected to an analysis of variance for each of the liquid and granule treatments separately because bacterial counts from soil treated with granules were more variable than when liquid treatment was applied. Four of the 96 data values were zero and were replaced by a value of 27 (half the minimum detectable level) to enable logarithm transformation of the data. Each analysis involved polynomial components for each of two factors, soil moisture and sampling date, and assumed a completely randomised design. Counts of *S. entomophila* (cfu/g) in the granules were also logarithm transformed and subjected to an identical analysis.

RESULTS

There was a significant difference in the rate of decline between *S. entomophila* populations from the liquid and granule formulations ($P < 0.001$) (Figure 1a versus 1b). At day 16, soil treated with the liquid formulation contained on average 2.3×10^6 cfu/g soil. The populations subsequently declined at all soil moisture levels, so that at day 145, an average of 4.6×10^1 cfu/g soil were recovered from 15% and 30% soil moisture treatments, while at 20 and 25% soil moisture, 3.4×10^2 cfu/g soil and 6.2×10^2 cfu/g were recovered, respectively (Figure 1a). The rate of decline differed significantly between soil moisture treatments ($P < 0.05$). Higher numbers were recovered at day 145 from soils maintained at moderate soil moistures (20–25%) than in soil at wilting point (15% soil moisture) and field capacity (30%).

FIGURE 1: Mean numbers (log₁₀) of *Serratia entomophila* colony forming units/g oven dry soil in soils held at soil moisture contents of 15, 20, 25 or 30% (w/w) following inoculation of soil with (a) *S. entomophila* broth and (b) granules containing *S. entomophila*. Error bars are LSD ($P < 0.05$) and apply to sampling dates up to and including day 145.

Lower numbers of *S. entomophila* were recovered from granule-treated soil at 16 days than following treatment with the liquid formulation as bacteria were still being released from granules into surrounding soil (Figure 1b). Numbers of *S. entomophila* established in soil 16 days after addition of granules were higher in the more moist soils (9.5×10^2 cfu/g at 15%, 1.68×10^3 at 20%, 5.1×10^3 at 25% and 5.5×10^3 cfu/g soil at 30% soil moisture) ($P < 0.05$). By 145 days, the populations had decreased gradually, with the rate of decline not differing significantly between the four moisture levels. However, the amount of bacteria remaining in the soil after 145 days differed, with significantly higher populations maintained at 20%, than at 15% and 30% soil moistures ($P < 0.01$).

Granules had a bacterial loading of 1.23×10^{10} cfu/g granule before they were added to soil. During the first 16 days after inoculation into soil, numbers remaining on the granules had dropped approximately 5-fold in all soil moistures except 30%, where numbers of *S. entomophila* were significantly lower at 1.3×10^8 cfu/g granule, approximately a 100-fold decrease ($P < 0.001$) (Fig. 2). From day 16 to 145, the rate of decline was roughly similar between the four soil moistures. Overall, soil moisture had a significant effect on the numbers of *S. entomophila* maintained on granules: highest numbers were maintained at 15 and 20% soil moisture, with lowest numbers recovered from more moist soils ($P < 0.01$).

DISCUSSION

Formulation continues to be a challenging and often success-limiting step in development of biocontrol products (Paau 1998), in particular where the active micro-organism is an environmentally sensitive non-sporeformer such as *S. entomophila*. In addition to enhancing shelf life, the formulation can enhance field efficacy by improving establishment and maintenance of microbial inocula in soil. In this experiment, bacterial counts at day 16 in liquid-treated soils were higher than those expected following application of the liquid formulation in the field and were equivalent to the "on-row" rate, immediately after application (M. O'Callaghan, unpubl. data). Typically, average counts of 10^4 cfu/g soil would be expected following treatment of pasture soil with the liquid

FIGURE 2: Mean numbers ($\log_{10}$) of *Serratia entomophila* colony forming units/g (wet weight) of granule recovered from soil held at soil moisture contents of 15, 20, 25 or 30% (w/w). Error bar is LSD ($P < 0.05$).

formulation, thus the laboratory experiment has overestimated the extent of persistence of *S. entomophila* applied as an unformulated liquid. However, *S. entomophila* populations in granule-treated soil declined more slowly than where unformulated cells were applied to soil, indicating that sustained persistence of the inocula is possible. Bacteria in granular formulations were less affected by soil moisture conditions in soil, which is the major environmental factor affecting persistence of this non-sporeforming bacterium.

High numbers of *S. entomophila* remained viable within the granules for up to five months, demonstrating the potential to extend the period when the biopesticide can be applied for grass grub control. Release of bacteria from the granules present in the field will be dependent on soil moisture conditions and influenced by the frequency of watering, either through rainfall or irrigation. The granule formulations have potential to be used in the development of other beneficial micro-organisms that are environmentally sensitive.

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