POTENTIAL FOR BIOLOGICAL CONTROL OF PORINA (*WISEANA* SPP.) WITH A NOVEL INSECTICIDAL BACTERIUM, *YERSINIA* N. SP. (MH96) EN65 STRAIN

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ABSTRACT

Porina (*Wiseana* spp. larvae) are endemic pests of pasture. If detected early, young larvae can be controlled with diflubenzuron, but generally damage is not predicted and organophosphate insecticides (e.g. diazinon, chlorpyrifos, chlorfon) are required. *Yersinia* n. sp. (MH96) cf. *entomophaga* (EN65 strain) – a novel bacterium with insecticidal properties – is pathogenic to late instar *Wiseana* spp. larvae in laboratory assays and has potential as a biopesticide. This trial measured the effect of two formulations of EN65 and a commercial formulation of *Bacillus thuringiensis* var. *kurstaki* (*Btk*) against late instar porina. EN65 was formulated in a sprayable biopolymer and on to a kibbled wheat bait. Both EN65 formulations caused significant mortality of porina larvae and reduced feeding damage on white clover; efficacy was superior to *Btk*. Survival of the bacterium in the sprayable biopolymer formulation was enhanced compared to a non-formulated broth culture. Bacterial survival on the kibbled wheat bait was better when stored at 4°C compared to 20°C over 3 months.
Keywords: porina, *Wiseana* spp., *Yersinia* n. sp., biopesticide, microbial control.

INTRODUCTION

Current high commodity prices for dairy products are driving the intensification of pastoral production systems (Clark et al. 2007). As a consequence, external inputs (e.g. improved grass varieties, fertilisers, irrigation) are increasingly used to sustain high milk production levels and to achieve the genetic potential of improved livestock. The need to maximise dry matter yield from pastures has reduced the tolerance for insect feeding damage, which can significantly reduce pasture productivity, and has increased the likelihood that pest control measures will be implemented. Porina, *Wiseana* spp. (Lepidoptera: Hepialidae), are endemic to New Zealand. They are polyphagous, feeding on both legumes (clover) and grasses, and are considered primary pasture pests, particularly in the South Island (Barratt et al. 1991; Dugdale 1994).

Traditionally, insecticides have been used to control porina. Feeding damage can be largely mitigated by monitoring adult flights and larval development, which enables targeted applications of the insect growth regulator diflubenzuron (Dimilin®) against young susceptible larvae. However, many farmers do not, or are unable to, do this. Instead, broad spectrum organophosphate insecticides (e.g. diazinon, chlorpyrifos, chlorfon)

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with wide ranging environmental effects are applied when large larvae are observed and pasture damage is clearly visible. Both methods have varying levels of success, which may be associated with timing of application particularly when more than one species of porina are present (King et al. 1985; Stewart & Ferguson 1989; Ferguson et al. 1996; Ferguson 2000). Availability of bioprotection methods that would be effective against all larval stages and against all species in the porina complex would be a significant benefit to farmers as they seek more sustainable pasture protection techniques.

Several biological control agents have been investigated for porina control, including entomopathogenic nematodes and fungi (van der Mespel et al. 1986; Nelson et al. 1996). In addition, ryegrass endophytes, *Neotyphodium* spp., can reduce survival and growth of porina larvae, although effects of different strains appear to be variable (Jensen & Popay 2004). More recently, the insecticidal activity of a novel non-spore forming bacterium, *Yersinia* n. sp. (MH96) cf. *entomophaga*, against porina and other lepidopteran pests has been demonstrated (Hurst et al. 2007). This paper reports on trials to evaluate *Yersinia* MH96, applied as a sprayable biopolymer formulation or coated onto a kibbled wheat bait, for control of porina larvae on white clover.

METHODS

Production of EN65

Stock cultures of *Yersinia* n. sp. are maintained at AgResearch, Lincoln, New Zealand (type strain MH96 in the AgResearch Bacteria Culture Collection; the commercial patent to the strain used in the current trial is owned by EnCoate Ltd, Hamilton, and the strain is designated as EN65; this designation is used hereafter). The bacterium was produced in Luria-Bertani (LB) broth using standardised techniques. Briefly, a starter culture was prepared on LB agar, from inoculum stored at -85°C in the AgResearch bacteria culture collection. Plates were incubated at 25°C for 24 h. Five colonies were removed from the surface of the medium and used to inoculate an Erlenmeyer flask (250 ml) containing 50 ml LB broth; duplicate flasks were prepared for the production run. Flasks were incubated on a rotary shaker at 25°C, 200 rpm for 20 h. After 20 h, production flasks (500 ml containing 100 ml LB broth) were each inoculated with 5 ml of the seed culture and incubated at 25°C, 200 rpm for 20 h. Cultures were immediately formulated after incubation. Samples were also taken at this time to estimate live cell density (according to colony forming units – CFU) using the drop plate technique on LB agar (Herigstad et al. 2001). Target cell densities of about 1×10^{10}/ml medium were achieved in each of the shake-flask cultures.

Formulation of EN65

Bacterial cells were incorporated into a biomatrix gel formulation for spray application, or were coated onto kibbled wheat for a bait treatment using patented biopolymer technology (NZ Patent Nos 506484, 550494). Viable cell concentrations in the respective formulations were determined using the drop plate method. A 1% biopolymer gel concentrate containing 2.6×10^9 CFU/ml was prepared for spraying, and was diluted in water at a 1:1 ratio immediately prior to application. The bacterial loading on the kibbled wheat was approximately 7×10^8 CFU/g.

The suitability of the two formulation methods for preservation of EN65 was assessed. For the sprayable gel preparation, 1.5 ml samples were pipetted into sterile 1.8 ml cryovials. As a control, samples of the LB broth cultures were distributed into sterile cryovials. Cryovials from each treatment were then randomly divided into two groups that were held at 4 or 20°C. For the kibbled wheat formulation, ~2 g samples were transferred to a series of sterile plastic pottles (50 ml capacity), which were randomly assigned for storage at 4 or 20°C. Formulations were destructively sampled after 1, 2 and 7 days and 2, 4, 8, 12, 20 (all formulations) and 40 (gel, LB only) weeks, and viable cell densities determined by CFU counting. Duplicate vials or pottles were sampled at each time point.

Porina larvae

Late instar (7th–9th) *Wiseana* spp. (most likely a mixture of *W. cervinata* and *W. copularis*) were collected from a 3-year old ryegrass/white clover pasture on Barewood

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Station, Hindon, between 1 and 13 June 2007. After collection the larvae were held individually in a cool laboratory in 60 ml specimen containers filled to approximately 40 mm with sphagnum moss peat until required for the trial. White clover leaves were provided as food and were replaced every 3–4 days.

Trial containers, treatments and experimental protocols

Forty plastic containers, measuring at least 42 x 34 x 30 cm, were filled to 15 cm with peat. Nine white clover plants with between five and nine trifoliate leaves were transplanted into each container on 5 June 2007. These plants had previously been grown in "Yates Thrive" potting mix in 20 x 20 x 40 mm deep cells. No fertiliser other than that contained in the potting mix was supplied to the plants.

Eighteen larvae were introduced into each container 24 h before treatment by inverting the 60 ml specimen containers, complete with one larva each, on to the peat surface and inserting the containers approximately 1 cm into the peat. These were left in position overnight. This technique ensured that the larvae burrowed into the peat, that an even distribution of larvae was obtained and that larval combat was avoided.

All treatments were applied on 14 June. Ten containers were allocated to each of the following:

(1) 1% EN65 biopolymer gel diluted 1:1 with water and sprayed on plants until runoff (approx 35 ml/container)

(2) EN65 on kibbled wheat applied at 50 kg/ha (0.7 g/container) but with two grains placed within 20 mm of each plant and the remainder sprinkled as evenly as possible throughout the container

(3) *Bacillus thuringensis* var. *kurstaki* (Dipel®) (0.1 g/100 ml; equivalent to the high label rate recommended for control of diamondback moth on brassicas) was prepared in 0.05% Triton X-100 as a surfactant and sprayed onto plants until runoff (approx 35 ml/container)

(4) Untreated (control).

Both spray treatments were applied using hand-pumped pressurised sprayers (Results™ 1 Litre Pressurised Sprayer, Mitre 10, Mosgiel).

Containers were then arranged in ten randomised blocks, each block incorporating one of each treatment. The experiment was conducted in an unheated glasshouse at Invermay Agricultural Centre, Mosgiel. Plants were watered as required and the surface of the containers checked for dead larvae on a daily basis. Larval feeding on the clover plants was recorded on 18 and 25 June and plants scored as: untouched (0%), less than 50% of leaves and shoots removed, more than 50% of leaves and shoots removed, or all above ground plant destroyed (100%). On 27 June all live plants were harvested from the containers and dried at 80°C overnight before weighing to determine their dry weight. The peat within the containers was searched on 29 June to recover surviving larvae.

Statistical analyses

Data were statistically analysed using an analysis of variance for a randomised complete block design with 10 blocks and 4 treatments. Individual treatment means were then compared using an unrestricted least significant difference (LSD) procedure (Saville 1990).

RESULTS

Porina trials

Damage to the plants in all treatments was evident after 24 h and increased in severity over time. The mean number of plants per treatment in each damage category is presented in Table 1. The early appearance of damage indicated the larvae had adapted well to the conditions within the containers. When assessed for damage 4 days after treatment (18 June), significantly ($P<0.05$) more plants in the control and *Btk*-treated containers had been destroyed or severely damaged than in either of the EN65 treatments (Table 1). One week later (25 June), surviving larvae were exerting pressure on plants regardless of treatment and some feeding damage was observed across all treatments, although again, significantly fewer plants were destroyed in the EN65 treatments.

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TABLE 1: Damage to white clover plants 4 and 11 days after treatment (DAT). Values presented are the proportion (%) of plants (n=9) in each damage category.

	18 June (4 DAT)				25 June (11 DAT)			
	0%	<50%	>50%	100%	0%	<50%	>50%	100%
Control	8	50	26	17	0	28	24	48
Btk	8	51	20	21	0	24	27	51
EN65 Gel	13	62	20	4	0	72	22	6
EN65 Bait	19	59	12	9	0	68	19	13
LSD (P<0.05)	17	16	12	8	-	13	11	14

Survival of the porina larvae within the control treatment was high (mean 88%) (Table 2) indicating that the experimental set up used was satisfactory. Although *Btk* significantly reduced survival of the larvae compared to the control (P<0.01), overall efficacy against porina was poor and there was substantial feeding damage to clover foliage. In contrast, both EN65 treatments significantly reduced larval survival compared to the control and *Btk* treatments (P<0.001) (Table 2), but the difference between the gel and bait treatments was not statistically significant.

Effects of the treatments on feeding damage were also reflected in the final dry weights of the white clover plants (Table 2). Clover dry weight was lowest in the control and *Btk* containers where porina survival was greatest. Dry weights in both EN65 treatments were significantly higher than the control and *Btk* treatments (P<0.001). Dry weight was approximately 3.5x greater than the control in the EN65 gel treatment and 2.5x greater in the EN65 bait treatment. Clover dry weight was greatest in the gel treatment, but was not significantly higher than the bait treatment.

TABLE 2: Mean survival (%) of *Wiseana* spp. larvae and mean dry weight (g/ treatment) of white clover leaves, 15 (29 June) and 13 (27 June) days after treatment, respectively.

Treatment	Surviving larvae (%)	Log_{10} (dry weight (g) of clover leaves)	(Back-transformed means)
Control	87.8	-0.623	(0.24)
Btk	67.8	-0.651	(0.22)
EN65 Gel	25.6	-0.080	(0.83)
EN65 Bait	31.1	-0.205	(0.62)
LSD (P<0.05)	11.3	0.221	-

Survival of EN65 in gel and bait formulations

Survival of *Yersinia* EN65 cells in the gel formulation and LB broth is presented in Figure 1. Cell survival declined in the gel and LB broth over time. The rate of decline was strongly influenced by temperature. After 7 days, fewer viable cells were recovered from samples stored at 20°C compared to those stored at 4°C. At 4°C stability was maintained for 14 days. Thereafter, cell viability began to drop by orders of magnitude below the starting concentrations of ca $2.59x10^9$ CFU/ml in the gel and $4.19x10^9$ CFU/ml in the

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broth. Overall, though, cell survival was better in the gel formulation than the broth. For example, in samples stored at 20°C there were 2.10 $x10^8$ viable cells/ml in the gel formulation compared to $0.80x10^8$ viable cells/ml LB broth by day 56; after 280 days, there were $0.33x10^8$ viable cells/ml in the gel and $0.02x10^8$ viable cells/ml LB broth. Trends were similar for both gel and broth suspensions at 4°C up to day 56. Survival in the gel was superior thereafter such that after 280 days there were still $5.50x10^8$ viable cells/ml in the gel, over 1000 times greater than in the LB broth.

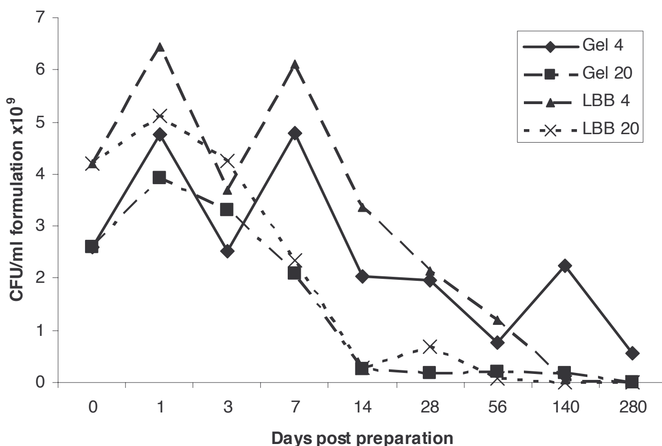

FIGURE 1. Number of viable cells (CFU/ml formulation x 10^3) of *Yersinia* n. sp. EN65 in a biopolymer gel and LB broth at 4 and 20°C.

When coated onto kibbled wheat, cell survival was greatest on samples held at 4°C, with minimal decline in cell viability over 3 months (Table 3). When held at 20°C, viable cell concentration declined rapidly after 1 week, and no viable cells were recovered after 2 months.

TABLE 3: Number of viable cells (CFU/g) of *Yersinia* n. sp. EN65 on biopolymer-coated kibbled wheat at 4 and 20°C.

Time of storage	Storage temperature 4°C	Storage temperature 20°C
0	7.0 $x10^8$	7.0 $x10^8$
24 h	9.1 $x10^8$	5.5 $x10^8$
48 h	6.7 $x10^8$	2.9 $x10^8$
1 week	7.7 $x10^8$	1.9 $x10^8$
2 weeks	5.2 $x10^8$	4.9 $x10^7$
1 month	2.2 $x10^8$	<1 $x10^4$
2 months	2.4 $x10^8$	0
3 months	1.1 $x10^8$	not tested

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DISCUSSION

The sprayers used were of a standard design, providing satisfactory foliage coverage with *Btk*. However, the EN65 gel formulation was too viscous to produce a fine mist, and large droplets were deposited on clover leaves. While this was adequate for the trial, a better quality (higher pressure) sprayer may have provided more homogenous coverage. Alternatively, a lower concentration of gel in the final spray mix would reduce the viscosity for application. The air temperature in the glasshouse ranged between 6 and 30°C throughout the trial, but seldom dropped below 10°C. Porina larvae are known to be active over this temperature range so conditions were suitable for larval feeding on contaminated foliage or bait.

Results from the trials clearly demonstrated the potential utility of *Yersinia* n. sp. EN65 for porina control. Both sprayable and bait treatments not only caused high levels of larval mortality, but also prevented significant damage to the clover plants in the trial containers. The relatively poor performance of the Btk formulation was not unexpected. The product is not registered for use against porina and efficacy against this pest has not previously been demonstrated.

Although the two *Yersinia* n. sp. EN65 formulations caused similar levels of larval mortality, in a working situation, a bait formulation is more likely to be used for porina control. Availability of a robust biopesticide that could be easily applied without specialised equipment would significantly enhance the uptake of a new pest management technology. Development of a granular bait formulation of EN65 for application by a broadcast spreader could be readily utilised by farmers in outbreak areas.

Bacteria showed reasonable persistence on the prototype kibbled wheat bait formulation but this would have to be enhanced for a commercial preparation. Biopolymer technologies have been successfully used to stabilise other non-spore forming bacteria, such as *Serratia entomophila*, which is formulated in the product Bioshield (Ballance Agri-Nutrients, Mt Maunganui) that has excellent shelf life under ambient conditions (Johnson et al. 2001; Brownbridge et al. 2008). Work to refine the formulation, validate field efficacy and optimise application strategies will be priorities in the future development of EN65.

There is a recognised need for effective bioprotection products in agriculture. This is being driven by changes in regulations in New Zealand and overseas governing the use of pesticides and their residues in agricultural commodities. Market- and environment-driven demands also mean that more sustainable production practices have to be devised to ensure access for export products. In addition, insect resistance to Bt and Bt-derived toxins expressed in transgenic crops creates a new niche and opportunities for insecticidal bacteria with alternative modes of action (Moar 2003; ffrench-Constant et al. 2007). *Yersinia* n. sp. EN65 toxins have a wide host range and unique mode of action (Hurst et al. 2007) and represent an exciting prospect for future bio-based crop protection strategies.

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REFERENCES

Barratt BIP, van Toor RF, Ferguson CM, Stewart KM 1990. Grass grub and porina in Otago and Southland. A guide to management and control. MAF, 104 pp.

Brownbridge M, Townsend RJ, O'Callaghan M, Bell NL, Mander C, Jackson TA 2008. Developing opportunities for entomopathogenic microbes and nematodes in crop protection. In: Butcher MR, Walker JTS, Zydenbos SM ed. Future Challenges in Crop Protection: Repositioning New Zealand's Primary Industries for the Future. New Zealand Plant Protection Society Incorporated, Hastings, New Zealand. Pp. 129-142.

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Clark DA, Caradus JR, Monaghan RM, Sharp P, Thorrald BS 2007. Issues and options for future dairy farming in New Zealand. New Zealand Journal of Agricultural Research 50: 203-221.

Dugdale JS 1994. Fauna of New Zealand No. 30, Hepialidae (Insecta: Lepidoptera). Manaaki Whenua Press. 164 pp.

Ferguson CM 2000. Susceptibility of *Wiseana* species to diflubenzuron and implications for field applications. New Zealand Plant Protection 53: 430-435.

Ferguson CM, Kerse GW, Evans AA 1996. Evaluation of diflubenzuron for porina control in south Otago. Proceedings of the 49th New Zealand Plant Protection Conference. Pp. 249-252.

ffrench-Constant RH, Dowling A, Waterfield NR 2007. Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. Toxicon 49: 436-451.

Herigstad B, Hamilton M, Heersink J 2001. How to optimize the drop plate method for enumerating bacteria. Journal of Microbiological Methods 44: 121-129.

Hurst MRH, Young S, Nelson T, Jackson TA, Becker A, Glare TR 2007. Species identification and host range testing of a new entomopathogenic member of the Enterobacteriaceae. Fortieth Annual Meeting of Society of Invertebrate Pathology, Quebec City, Canada, 12-16 August. p. 105.

Johnson VW, Pearson JF, Jackson TA 2001. Formulation of *Serratia entomophila* for biological control of grass grub. New Zealand Plant Protection 54: 125-127.

Nelson TL, Low A, Glare TR 1996. Large scale production of New Zealand strains of *Beauveria* and *Metarhizium*. Proceedings of the 49th New Zealand Plant Protection Conference. Pp. 257-261.

Jensen JG, Popay AJ 2004. Perennial ryegrass infected with AR37 endophyte reduces survival of porina larvae. New Zealand Plant Protection 57: 323-328.

King PD, Meekings JS, Phillip KC 1985. Porina control in Waikato dairy pastures. Proceedings of the 38th New Zealand Plant Protection Conference. Pp. 15-18.

Moar WJ 2003. Breathing new life into insect-resistant plants. Nature Biotechnology 21: 1152-1154.

Saville DJ 1990. Multiple comparison procedures: The practical solution. American Statistician 44: 174-180.

van der Mespel GJ, Kain WM, Wouts WM 1986. Evaluation of an entomophagous nematode (*Steinernema bibionis*) for the control of porina larvae (*Wiseana* spp.) in the Otaki district. Proceedings of the 39th New Zealand Weed and Pest Conference. Pp. 114-116.

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