Effect of the bacterium *Yersinia entomophaga* on adult bronze beetle

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Abstract The entomopathogenic bacterium *Yersinia entomophaga* MH96 (Ye MH96) was produced by aerobic fermentation and applied in laboratory and field bioassays to control bronze beetle (*Eucolaspis* sp.). In the laboratory, bacteria were applied to apple leaves at a range of cell densities using a Potter Tower. After 6 days of exposure, the LC₅₀ of field-collected beetles was calculated as approximately 5.0×10^6 cells/cm² of leaf surface with high concentrations killing all treated adults. Time to death was inversely correlated with dose rate with the LT₅₀ at the highest dose being 3.6 days. Marked apple shoots were sprayed in the field, each shoot enclosed in a sleeve cage and healthy beetles added. After 7 days, beetle mortality averaged 42% following a single application and 66% after multiple treatments. Leaf damage was reduced by bacterial application. The results show that Ye MH96 has potential for development as a biopesticide against this intractable pest of organic apple production.

Keywords *Yersinia entomophaga*, Ye MH96, *Eucolaspis*, bronze beetle, biopesticide, apple.

INTRODUCTION

Bronze beetle (*Eucolaspis* sp., Coleoptera: Chrysomelidae) is a serious pest in organic apple orchards, with feeding by adult beetles on fruit resulting in up to 50% of apples at harvest being damaged in the worst affected orchards (Rogers et al. 2006, 2007). Furthermore, expansion of the organic sector and associated higher premiums are limited by this pest. Bronze beetle has an annual lifecycle. In apple orchards, larvae feed below ground on the roots of grasses (Miller 1971; Kay 1980), before emerging in late spring/early summer as adults to feed on apple leaves and fruit. There are currently no effective,

organically-acceptable insecticides to control this pest. Historically, soil cultivation has been used against bronze beetle (Lysaght 1930) and recently this measure has been widely adopted by organic apple growers, but unfortunately this is at best a short-term, partial solution (Rogers et al. 2009).

There is a recognised need for effective biological pesticides in agriculture. This is driven by changing regulations in New Zealand and overseas governing the use of pesticides and the resulting occurrence of their residues in agricultural commodities. In addition, market and environmental demands mean that more

sustainable production practices are required to ensure access for export products. The enterobacterium *Yersinia entomophaga* strain MH96 (Ye MH96) is a microbial biocontrol agent under development by AgResearch Limited (Hurst & Glare 2006). The bacterium has bio-insecticidal activity, and research under laboratory conditions has shown that it is effective against a number of insect pests that are important in New Zealand agriculture and horticulture (Hurst et al. 2011). Preliminary laboratory trials of Ye MH96 in a leaf bioassay caused high mortality of adult bronze beetle in 2009 (M.R.H. Hurst, unpublished data).

This study sought to determine the efficacy of Ye MH96 against adults of the bronze beetle (*Eucolapis* sp.) feeding on apple leaves and fruit. If sufficient efficacy can be demonstrated, it is thought that the organism will have potential for development into a bio-insecticide suitable for use in both organic and conventional production systems and could provide an environmentally-sustainable alternative to existing chemical insecticides.

MATERIALS AND METHODS

Bacterium and preparation

The bacterium *Yersinia entomophaga* (Ye MH96) was cultured by fermentation and concentrated by centrifugation, followed by suspension in a 1% biopolymer gel (Ye MH96) using patented biopolymer technology (Patent WO 02/15702A1) for transportation and laboratory bioassay. Viability of cells prior to bioassay was confirmed using serial dilutions in 0.1 M phosphate buffer (10 mM sodium phosphate buffer, pH 7.4; 0.65 mM K_2HPO_4 , 0.35 mM KH_2PO_4) spread plated onto Luria-Bertani agar plates incubated at 30°C for 48 h prior to enumeration of bacterial cell numbers. Cell concentrates were produced with cell densities ranging between 9.3 and 12.8×10^{10} cells/ml.

Laboratory bioassays

Three bioassays were carried out by AgResearch, at the Lincoln Research Centre in November and December 2010. Field-collected bronze beetle

adults and freshly picked 'Royal Gala' apple leaves from a Hawke's Bay organic orchard were used for each of the assays. For each assay, five concentrations of Ye MH96 were prepared in a 4:1 dilution series in water to produce cell densities ranging from 2.0×10^{10} to 3.3×10^7 cells/ml with the sixth treatment a water only control. Apple leaves were pre-sorted by size and age, trimmed to a standard length, and randomly allocated into treatment groups. For each treatment, single leaves were treated by spraying in a Potter Tower with 3 ml aliquots on both top and bottom leaf surfaces. When the spray had dried on the leaf surfaces the leaf stalk was inserted into a water soaked plug of florist's foam (Oasis™) to maintain the leaf in a fresh condition for the duration of the assay. Individual leaves were then placed into screw-topped 120 ml plastic tubes and ten live, active bronze beetle adults were added to each tube. Eight replicate tubes of the six treatments were arranged in a randomised block design and kept on a laboratory bench at 20°C under standard fluorescent light during the day with normal ambient lighting outside working hours. Assay 1 was assessed 4, 6 and 8 days post application, Assay 2 each day for 7 days after application and Assay 3 on 3, 4, 5, 6 and 7 days post application. Numbers of live and dead beetles were recorded and feeding damage to the leaf surface was noted. At the conclusion of each assay all the containers, leaves and beetles were bagged and autoclaved before disposal.

Field assays – single application

Yersinia entomophaga (Ye MH96) preparations were applied to run-off to tagged apple shoots with fruitlets (Plant & Food Research breeding selection, 'Sciros' and 'Pinkie' cross) using a 1 litre pressurised hand-held sprayer. The cell concentrate gel, containing approximately 1.0×10^{11} cells/ml, was diluted with an equal volume of water, allowed to dry and a second application was made to the tagged shoots to achieve the desired bacterial concentration. The adjuvants Du-Wett® (0.01%) and lignosulphonic acid (0.2%) w/v were added as a surfactant and UV protectant respectively. When dry, the shoots were covered with nylon mesh sleeve cages

(290 mm × 600 mm) and 20 field-collected bronze beetles were added before the cage was secured around the shoot with a plastic cable tie. Ye MH96 treatment was replicated three times (weekly) using fresh shoots with two shoots on each of five trees being treated on each occasion. Ye MH96 treated shoots were compared to control shoots that had the adjuvant products applied at the same rate in water. After 1 week the shoots were cut and the mortality status of the beetles was assessed in the laboratory. Beetles that failed to move after persistent prodding were classified as dead. Furthermore, the number of fruit, stems and leaves damaged by beetle feeding was recorded.

Field efficacy – multiple/cumulative applications

Applications of Ye MH96 and the control adjuvant products were applied to tagged shoots exactly as described for the single application above (Table 1). However, the same shoot was reused for three applications at weekly intervals with fresh beetles being added after each application. Mortality assessments were conducted as described above but damage was not recorded.

Field persistence

A single field/laboratory bioassay was carried out to determine the field persistence of Ye MH96. Two lower tier branches, one on either side of the tree, were treated with either Ye MH96 or the adjuvants alone (control) as described above. Once dry, 10 leaves, five from each side of the tree, were harvested and placed individually in Petri dishes (85 mm diameter) with 10 field-collected bronze beetles. After 7 days the vitality of the beetles was determined and the area of the leaf consumed by the beetles was calculated based upon leaf area difference measurements (LI-3100 area meter, Li-Cor Inc., Nebraska, USA). Leaves were collected from the treated trees on Day 0 (application day), 1, 2, 5 and 7 and bioassays established with 10 replicates (a Petri dish with leaf and 10 beetles) per sample day.

To quantify viable Ye MH96 bacteria on the leaves and their persistence, leaves were sampled approximately 2 h after application and again after

24 h. Three shoots were sampled from the north and south sides of apple trees after treatment with Ye MH96 as described above. Three leaves were removed randomly from each branch. Bacteria were enumerated by taking three 0.7 cm diameter discs from each leaf and dispersing bacteria from each leaf disc independently. Bacteria were separated from the leaf surface by shaking in 1 ml of 0.1% Triton-X-80, 2 mM tetrasodium pyrophosphate dispersed in water in a 1.5 ml Eppendorf tube on a Labnet VX100 vortex at maximum speed for 15 min. A 10-fold serial dilution series to 4 dilution factors was prepared and 100 µl aliquots plated onto a selective agar for Ye MH96 (M.R.H. Hurst, unpublished data) and the plates incubated overnight at 30°C. This allowed mean bacterial deposition rates to be estimated and expressed as cells/cm² of leaf.

Statistics

In the laboratory assays, the average beetle mortality (and standard error) after 6 days following each treatment were calculated from the observed mortalities separately for each trial, and for the three trials combined. The LC₅₀ was calculated from the combined data. The LT₅₀ (time to 50% mortality) for each treatment of the laboratory bioassay was obtained from the day-to-day mortality data for the combined trials by applying a set of % survival (or cumulative % mortality) functions and selecting the best-fit function to the data.

Field trial data were assessed using Minitab (Minitab® 15.1.0.0. ©Minitab 2006 Inc.) to calculate means and standard errors and to undertake an analysis of variance on angular transformed percentage data (arcsine ($\sqrt{x}$)). Model adequacy checks were carried out by examining plots (scatter, histograms and normal probability) of the residuals.

RESULTS

Laboratory assays

Beetles fed normally on the untreated apple leaves, producing characteristic small holes through the lamina. The amount of feeding was reduced, the degree of mortality increased and

time to death decreased with increasing bacterial cell density (Figure 1, Table 1). Ye MH96 applied at the highest rate ($8.00 \times 10^7/\text{cm}^2$) produced 100% mortality of beetles within 6 days. The LC_{50} dose rate was calculated as 5.0×10^6 cells/ cm^2 of leaf area. At the highest application rate, insects ceased feeding and died rapidly with an LT_{50} of 3.6 days. At the lowest dose rate used ($1.28 \times 10^5/\text{cm}^2$), there was still significantly less survival than in the control treatment and the life of treated beetles was reduced by 43% with an LT_{50} of 8.78 days (Table 1).

Field efficacy – single application

Beetle mortality after a single application of Ye

MH96 to apple shoots in the field reached an average of 52.4% compared with 18.7% in the control. Beetle mortality for weeks 2 and 3 was highly significant ($P < 0.01$, Table 2) but high control mortality and variability in the first week following application meant that at this time the difference in mortality approached but did not reach significance ($P = 0.07$). Damage to apple leaves was significantly reduced ($P < 0.01$) by the bacterial treatment; $28.1 \pm 1.9\%$ of leaves from the treated shoots had some damage compared to $44.3 \pm 2.5\%$ (mean $\pm$ SEM) from untreated control shoots. Most shoots had some fruitlet damage from bronze beetle feeding whether treated or not.

Table 1 Time to death (LT_{50} , days) for bronze beetle adults treated with different concentrations (cells/ cm^2) of *Yersinia entomophaga* MH96 applied to leaves using a Potter’s tower.

Concentration	LT_{50}	Standard error	95% Confidence interval
8.00×10^7	3.60	0.07	3.47, 3.74
1.60×10^7	4.44	0.15	4.15, 4.75
3.20×10^6	7.71	0.47	6.85, 8.69
6.40×10^5	8.74	0.73	7.42, 10.29
1.28×10^5	8.78	0.61	7.66, 10.06
0	15.14	2.92	10.37, 22.11

Figure 1 Mortality (%) of bronze beetle at 6 days after treatment with increasing rates of *Yersinia entomophaga* MH96 in laboratory trials. Values are the average of three trials and data were corrected for control mortality using Abbott’s formula (Abbott 1925).

Field efficacy – multiple/cumulative applications

Beetle mortality after multiple applications of Ye MH96 at 73% was also significantly greater than in the adjuvant-only controls (22.8%) on all occasions ($P < 0.01$, Table 2), despite a high control mortality in the Week 1 assay. Damage to fruitlets (feeding on the stems and surface) was lower on treated shoots than control ones, although not significantly so, and by week 3 cumulative damage on the treated shoots caused by the continued addition of beetles was only slightly lower than on the controls.

Field persistence

Bronze beetle mortality of field-aged leaf residues assessed in laboratory bioassays was low compared to the previous laboratory assays and beetle mortality on leaves collected 2 h after treatment reached only 30% compared to 12% on untreated leaves (N.S. $P = 0.06$, Figure 2). Beetle mortality on leaves collected on subsequent days declined rapidly and was similar to that for the control on all other sample days (days 3–7 not shown).

After 7 days of feeding on leaves collected 2 h after application, beetles had consumed $23.6 \pm 4.3\%$ (mean $\pm$ SEM) of untreated leaves compared to $9.4 \pm 2.3\%$ of the leaves treated with Ye MH96 ($P < 0.01$).

Bacterial quantification from the leaves indicates application initially established a population of 5.6×10^5 YeMH96 cells/cm² of leaf area. However, within 24 h of application bacterial

Figure 2 Mean mortality of adult bronze beetle after 7 days of exposure to *Yersinia entomophaga* MH96 residues on apples leaves harvested on Day 0 (immediately the residue was dry), Day 1 and Day 2. Error bars denote SEM.

numbers had dropped to near undetectable levels (20–40 cells/cm²).

DISCUSSION

Ye MH96 was toxic to adult bronze beetles when applied to apple leaves in laboratory bioassays of excised leaves and in the field when the beetles were confined in sleeve cages. The level of mortality, and time to death, were both dependent on the rate of bacteria applied, with high concentrations of bacteria producing 100% mortality in the laboratory within 4–5 days (data not presented). The results are consistent with previous studies where Ye MH96 has produced

Table 2 Mean percentage mortality ($\pm$ SEM) of bronze beetle confined within sleeve cages in the field after exposure to *Yersinia entomophaga* MH96 applied to tagged apple shoots compared to a control spray of adjuvant and water. Single applications were applied to fresh shoots and multiple applications were made to the same shoots each week.

Treatment	Week 1	Week 2	Week 3
Single Application			
Ye MH96	64.4 ± 7.1	40.2 ± 6.3	52.5 ± 7.1
Control	35.3 ± 12.9	9.7 ± 3.0	11.1 ± 4.1
Multiple Application			
Ye MH96	85.7 ± 5.2	57.5 ± 6.1	77.4 ± 5.7
Control	41.0 ± 10.6	16.8 ± 4.5	10.6 ± 2.1

high levels of mortality within a short time period against a wide range of insects (Hurst et al. 2011).

By applying high concentrations of bacteria and maintaining leaves for assay in the laboratory, 100% mortality was consistently reached. When bacteria were applied and bioassayed in the field, mortality averaged 42% after a single application (corrected by Abbott's formula) and 66% after multiple treatments, which demonstrates the potential of Ye MH96 as a biopesticide to control bronze beetle.

The results showed higher levels of mortality in the laboratory than in the field. This may be related to short survival of the bacteria in the field as the persistence study showed a rapid loss of efficacy of Ye MH96 towards bronze beetle with no significant activity at day 3. Loss in efficacy was shown to be associated with cell survival as enumeration of Ye MH96 from leaves treated in the field showed that bacterial numbers had dropped to near undetectable levels within 24 h of application. This suggests that the bacterium is susceptible to environmental parameters, such as desiccation and UV light, and that field performance could be improved by protective formulations.

Currently organic apple growers are using soil cultivation as a means of controlling immature soil-dwelling bronze beetles. However, this is only partially effective and many growers are concerned about damage to their soil structure (Rogers et al. 2009). Pyrethrum has been used by some growers for bronze beetle control; unfortunately this is very expensive because it is applied at ten times the normal field rate and still has only a low efficacy. Consequently a new biological solution for managing bronze beetle in organic apple orchards is required. These trials show that Ye MH96 can consistently kill damaging bronze beetle adults in both the laboratory and field. The next challenge is to improve field performance through improved formulation to enhance field persistence. Once achieved, a Ye MH96-based product could provide an effective biological solution to control this intractable horticultural pest.

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