FIELD APPLICATION OF BIOPOLYMER-COATED *BEAUVERIA BASSIANA* F418 FOR CLOVER ROOT WEEVIL (*SITONA LEPIDUS*) CONTROL IN WAIKATO AND MANAWATU

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ABSTRACT

Clover root weevil (CRW) (*Sitona lepidus*) continues to have a severe impact on white clover in pastures in the North Island of New Zealand. Trials were established in October 2005 as part of a continuing evaluation of the insect-pathogenic fungus *Beauveria bassiana* F418 strain for management of this pest. F418 was applied as a rice + biopolymer formulation, targeting CRW larvae in the clover root feeding zone (3-5 cm below the surface). Overall, there was a reduction in CRW numbers at all of the test sites. The number of larvae recovered was significantly lower in F418-treated than control plots after 3 weeks, and the number of pupae was similarly lower in F418 than control plots after 6 weeks. Higher levels of fungal propagules were recovered from 0-6 cm in the soil profile than 6-10 cm.

Keywords: clover root weevil, *Beauveria bassiana*, biopolymer-coated rice, formulation, biological control.

INTRODUCTION

The clover root weevil (CRW) (*Sitona lepidus* Gyllenhal) has had a severe impact on the productivity of white clover-ryegrass pastures throughout New Zealand's North Island (Eerens et al. 1998; P.J. Gerard, unpubl. data) and now appears to be established, although not yet widespread, in the South Island (M.R. McNeill, pers. comm.). White clover underpins low-input pastoral systems in New Zealand owing to its nitrogen fixing ability and its high nutritional value. Preferential feeding by CRW adults on newly germinated seedlings reduces establishment or regeneration of clover, while larval feeding on nodules, roots and stolons has a direct impact on plant growth, vigour, persistence and N fixation (Hardwick & Harens 2000; Gerard 2001). Application of fertilisers can compensate for reduced levels of fixed nitrogen in the soil but their use represents an additional cost to farmers, can lead to pollution of waterways through excess nitrate run-off, and can adversely affect root density, effectively reducing the capacity for nitrogen uptake by pastures (Brock 2004).

Two natural enemies are currently under consideration for control of the pest, the parasitoid *Microctonus aethiopoides* and the entomopathogenic fungus *Beauveria bassiana* F418 strain (Goldson et al. 2005; Nelson et al. 2004). The parasitoid was released in the North Island in January 2006, with the goal of it becoming established within the CRW population to provide long-term suppression, similar to the approach used for the Argentine stem weevil (McNeill et al. 2002). However, it is imperative to continue development of additional biological control agents. The combined utilisation of both natural enemies is likely to have a greater and complementary impact on CRW, due to differences in the biological parameters of the two agents. Also, use of *B. bassiana* as a

mycoinsecticide offers potential for controlling 'local' populations of CRW, and against high density infestations found along the leading edge of this pest.

Major challenges remain in devising efficacious field use practices, including effective formulation and delivery techniques. Earlier research demonstrated the virulence of this fungus for CRW and that it can be readily mass-produced on rice. Application of rice-based granules in the field showed some promise in terms of extended persistence (Nelson et al. 2004). This paper reports on paddock-scale trials established in October 2005, using a novel rice + biopolymer formulation of F418, targeting CRW larvae in the clover root feeding zone.

MATERIALS AND METHODS

Production and formulation of *B. bassiana* F418

The strain F418 was originally isolated from a CRW adult collected in Europe and is held in the AgResearch Culture Collection. Conidia were produced on rice at AgResearch, Lincoln, by inoculating 500 g of autoclaved long-grained rice in ventilated plastic autoclave bags with 50 ml of broth (40 g D+Glucose (BDH) and 10 g Neopeptone (Difco) in 1 litre of sterile distilled water) plus conidia scraped from 2-week-old PDA cultures (final inoculum concentration approximately 1×10^8 conidia/ml). Rice was held at 25°C for 8 days. Rice plus conidia was then removed from the bags and allowed to air dry for 24 h prior to coating with a biopolymer (patented method NZ 539962, AgResearch, Lincoln). The formulated rice plus conidia was bagged and stored at ambient temperature until use.

Field trial sites and application of rice granules

Treatments were applied at three trial sites in the Waikato region (Cook's Farm, Tauhei, sites 1 and 2; Wilson's Farm, Hoe O Tainui, site 3) and two in the Manawatu region (Aorangi Farm, Kairanga, sites 4 and 5). Sites were selected on the basis of good (>15%) clover cover uniformly distributed over the site, minimal topographical variation within each site (i.e. flat smooth paddock) and a high uniform population of CRW larvae, as determined by pre-treatment sampling (see below for details of CRW sampling methods). Each trial site consisted of one paddock (approximately 1.5 ha). Paddocks were divided into four strips (plots) with two plots allocated to either a control (no rice) treatment or F418-rice according to a randomised block design. The biopolymer-coated rice was drilled to a depth of 3–5 cm, coulter spacing 150 mm, using a John Deere 750A single disc drill (Waikato) or an Aitchison drill (Manawatu). The rice was applied at 36 kg/ha, which provided a spore loading of approximately 2×10^{13} spores/ha.

CRW sampling plan

Resident CRW populations were determined by taking 50 core samples (10 cm diameter × 10 cm deep) over clover plants in a zig-zag pattern across paddocks 2 days before the F418 treatment was applied. Sites were re-sampled 3, 6 and 12 weeks later. Post-application soil core samples were taken in each plot by randomly locating five "crossways" transects within each plot (using numbers 00 to 99), then taking 10 core samples equally-spaced across each transect. The start point for each transect was randomly selected for each sampling date. Core samples were hand sorted in the field and CRW larvae and pupae counted. Approximately 25 larvae were collected from treated and control plots at sites 1 and 4, and were taken to the laboratory where they were held at ambient temperature in soil collected from their respective field plots. Larvae were examined for symptoms of infection every 2-3 days for 2 weeks.

Recovery and enumeration of *Beauveria* from soil

Pre-treatment samples were taken 2 days prior to drilling in the same zig-zag pattern as described above. Soil core samples were collected immediately after drilling (time 0) and 3, 6 and 12 weeks later. The same five transects used for CRW sampling were used to randomize collection of samples for *B. bassiana* enumeration. Two soil cores (2.5 cm diameter × 10 cm deep) were taken at just the third and seventh position along each transect. Each core was divided into 0-3 (upper), 3-6 (mid), and 6+ (lower) cm sections and soils from each depth were pooled for each treatment plot.

Beauveria bassiana was enumerated from samples by dilution plating onto PDA (Merck) containing streptomycin sulphate (350 mg/litre, Sigma), tetracycline hydrochloride (50 mg/litre, Sigma) and cycloheximide (125 mg/litre, BDH, UK). Plates were incubated at 20°C for 14 days before counting *B. bassiana* colony forming units (CFUs).

Data analysis

CRW sample means for each treatment were $\log_{10}$ transformed and statistically analysed using analysis of variance, with 5 sites and 2 treatments, treating sites as "blocks" (GenStat version 8.11). For *B. bassiana* CFU counts, data were too variable to facilitate analysis between all core section depths; therefore, data were averaged over the 0-3 and 3-6 cm depths (to obtain CFU counts for 0-6 cm across treatments), $\log_{10}$ transformed and analysed as for the CRW counts. However, this analysis was done on data sets from the three Waikato sites (sites 1-3) only, as few fungal propagules were recovered from the Manawatu soils at most time points. Data from 6+ cm were not analysed. All mean values and LSDs were back-transformed for presentation in the results. Each back-transformed LSD is a least significant ratio (LSR), meaning that two treatment means differ significantly only if their ratio is greater than the LSR.

RESULTS

Effect on CRW

The mean number of CRW larvae/m^2 fell by >70% in both the control and F418-treated plots in Waikato, and by 30-69% in Manawatu, from time 0 (pre-treatment) to week 3 (Table 1). While there was considerable site-to-site variation in the scale of the decline, the trend was consistent across all sites and treatments. This decrease may have been a result of larval development through to pupae, as it is accompanied by a corresponding increase in pupal numbers observed at all sites by week three (Table 1). However, larval numbers in the F418-treated plots were 34, 43, 25 and 26% lower than their corresponding controls at sites 1, 2, 3 and 5, respectively. There was no difference in larval numbers at site 4. After six weeks, CRW populations were 62, 44, 52 and 23% lower in the F418-treated than control plots at sites 1, 2, 4 and 5, respectively; there was no difference at site 3. By week 12, the trend was maintained at the three Waikato sites, and larval numbers in the F418 plots were 76, 65 and 58% lower than the controls at sites 1, 2 and 3, respectively. However, larval numbers in the F418 plots were 95 and 20% higher than control at the two Manawatu sites (sites 4 and 5) at week 12.

TABLE 1: Mean number of CRW larvae and pupae/m^2 at three Waikato and two Manawatu experimental sites at 0, 3, 6 and 12 weeks after treatment with *Beauveria bassiana* F418.

		Larvae/m^2 at week				Pupae/m^2 at week		
Site	Treatment	0	3	6	12	0	3	6
1	Control	420	81	27	99	13	57	29
1	F418	420	53	10	24	13	28	15
2	Control	484	104	23	112	0	32	18
2	F418	484	60	13	39	0	27	14
3	Control	782	204	23	48	137	171	27
3	F418	782	153	27	20	137	143	19
4	Control	316	210	378	59	0	59	90
4	F418	316	220	182	115	0	83	42
5	Control	540	274	209	109	0	101	81
5	F418	540	202	162	131	0	202	79

The overall mean number of CRW larvae/m^2 at all sites decreased over the course of the experiment in both the control and F418-treated plots. Larval numbers were lower in plots treated with F418 than control plots at 3 weeks (117 versus 158 larvae/m^2, LSR(5%)=1.32, P<0.05, since the ratio of the means, 158/117 = 1.35, is greater than the LSR(5%) of 1.32), 6 weeks (40 versus 64 larvae/m^2, LSR(5%)=1.72, P<0.10) and 12 weeks (49 versus 81 larvae/m^2, LSR(5%)=2.98, not significant). All larvae recovered from the F418-treated plots died due to mycoses caused by *B. bassiana*.

By week 3, the mean number of pupae/m^2 was higher than pre-treatment levels in all plots and at all sites (Table 1). However, by week 6 pupal numbers were 48, 21, 29 and 54% lower in F418 than control plots at sites 1, 2, 3 and 4, respectively; there was no difference between treatments at site 5. This trend probably reflects events occurring in the larval population, with a significant knock-down of larvae by week three, resulting in significantly fewer pupae in F418 plots by week six. *Beauveria bassiana*-infected pupae were also recovered at these sample times. Data are not presented for week 12 as very few pupae were recovered and numbers were too low for analysis. Overall, fewer pupae were recovered in plots treated with F418 than the control at 3 weeks (71 versus 139 pupae/m^2, LSR(5%)=1.96, not significant) and 6 weeks (27 versus 40 pupae/m^2, LSR(5%)=1.45, P<0.05).

Fungal establishment

Substantially more *B. bassiana* CFUs were recovered from F418-treated plots than their respective controls in the 0-6 cm samples immediately after drilling at sites 2, 3, 4 and 5 (Table 2). This trend was maintained at week 3 for these sites. By week 6, CFUs were higher in 0-6 cm samples in treated than control at sites 3 and 4 only. At site 2, CFUs

TABLE 2: Mean number of *Beauveria* CFUs/g dry soil from 0-6 cm soil samples (0-3 and 3-6 cm combined) and 6+ cm soil core depth at three Waikato and two Manawatu experimental sites at 3, 6 and 12 weeks after treatment with *Beauveria bassiana*.

		Soil *Beauveria* levels (CFU/g) at week:				
Site (depth)	Treatment	Pre-drill	0[1]	3	6	12
1 (0-6)	Control	1052	2186	3322	5225	4240
1 (0-6)	F418	3216	3011	823	1215	1644
2 (0-6)	Control	0	314	659	3480	6088
2 (0-6)	F418	1252	3078	9151	1539	1685
3 (0-6)	Control	1189	594	1030	10647	623
3 (0-6)	F418	71	9502	6948	27769	1257
4 (0-6)	Control	0	0	0	0	0
4 (0-6)	F418	0	4822	296	5049	0
5 (0-6)	Control	0	0	0	0	0
5 (0-6)	F418	0	335	26	0	0
1 (6+)	Control	0	0	683	783	0
1 (6+)	F418	0	2915	0	71	1594
2 (6+)	Control	0	0	0	0	826
2 (6+)	F418	0	1933	0	0	1796
3 (6+)	Control	0	0	621	0	0
3 (6+)	F418	0	1342	0	0	618
4 (6+)	Control	0	0	0	0	0
4 (6+)	F418	0	1597	0	0	0
5 (6+)	Control	0	0	56	0	0
5 (6+)	F418	0	2357	1085	0	0

[1]Soil sample taken immediately after drilling.

in the control samples were twice as high as those in the F418-treated soils at week 6, and numbers had increased again by week 12 in the control plots while they remained stable in the F418-treated plots. At site 5, fungal counts were very low throughout the trial at all depths, and very low/no CFUs were recovered from control or fungal-treated soils from week 6 on.

It is interesting to note that at site 1, fungal colonies in 0-6 cm samples from the control plots exceeded the CFU estimates for the F418-treated plots from time 0 (immediately after drilling) until week 12. It is unclear why this phenomenon appeared to be confined to the control plots within each of the paddocks. Soil type and vegetation cover were consistent across the experimental area. Assuming a relatively homogenous fungal population initially existed in the paddock, the increase in CFUs would be expected to be consistent across the paddock, and should have complemented the loadings added to the soil via the fungal treatment.

Fungal populations were higher at 0-6 cm in the F418-treated soils than the 6+ cm portion of the core samples at 0, 3 and 6 weeks post-treatment across all sample sites (Table 2). By week 12, differences between soil CFUs at different depths were less marked, although the same trend was maintained for soils collected at sites 1, 3, 4 and 5. In control soils, higher CFUs were generally also found in the 0-6 cm samples across all sample times, although most propagules were concentrated in the top 3 cm of the core (data not shown). Overall, higher CFUs were found in the 0-6 cm portion of the soil profile than at 6+ cm across all treatments and sites.

Immediately after application, more *B. bassiana* was recovered from treated soils than control samples, ranging from a high of approximately 9.5×10^3 and a low of 3.3×10^2 CFU/g dry soil at the various sites. By 6 weeks post-treatment, *B. bassiana* populations were higher in the treated than control plots at sites 3 and 4 only. In treated sites, loadings fell to approximately 1.6×10^3 CFU/g by week 12 at sites 1-3. Loadings in the 6+ cm sections reached ~2×10^3 CFU/g dry soil immediately after application, but relatively few propagules were recovered thereafter. When data from the three Waikato sites were combined, differences in fungal loading over time between control and treated plots was not statistically significant, largely as a result of the wide variation in CFU counts between sites (Table 3).

TABLE 3: Mean number of *Beauveria* CFUs/g dry soil (Waikato sites only) for 0-6 cm soil samples (combined). Data were log transformed for analysis then means and LSR were back-transformed for presentation.

	Soil *Beauveria* levels (CFU/g) at week:			
Treatment	0[1]	3	6	12
Control	742	1311	5785	2524
F418	4449	3740	3731	1516
LSR ($P<0.05$)[2]	25	207	22	14
Significance	ns	ns	ns	ns

[1]Soil sample taken immediately after drilling.
[2]The LSR is the least ratio of two means that is significant at $P<0.05$.

DISCUSSION

Results indicated that application of *B. bassiana* F418 as a biopolymer-coated granular formulation had an impact on CRW larval and pupal numbers in a clover-grass paddock. Clearly, there is a large natural decline in larval numbers over time, which could have resulted from insect development to the pupal stage, natural mortality and effects of indigenous natural enemies. However, in F418-treated plots, CRW numbers were generally lower than in their respective control plots, and were significantly lower at

weeks 3 and (P=0.10) week 6. By week 12, small larvae from the second generation were found in soil core samples, so that effects of the fungal treatment on the resident population at the time of application was no longer being measured. Subsequent activity against the young larvae is contingent upon there being high enough residual levels of inoculum in the soil, and contact between infective propagules and larvae feeding in the clover root zone (0-3 cm). Insect mortality is dose-related, so if there are too few infective conidia in the soil, insect infection is unlikely to occur. The reasons for the observed differences in efficacy in Waikato versus Manawatu soils need to be assessed further, although there appears to be a good correlation between soil loadings of *B. bassiana* and effects against CRW. The best method of using fungal biopesticides remains to be determined. If high inoculum levels can be maintained for at least 6 weeks, it may be sufficient to protect the clover from damage by CRW at a key stage in its growth and development, e.g. during spring/early summer, leading to improved clover health and productivity.

There was considerable variation in CFU counts by site, treatment and time, making it difficult to draw general conclusions about fungal establishment at all of the treatment sites, and is probably indicative of differences between the sites. Fungal inoculum levels were elevated up to 6 weeks post-treatment in the Waikato soils, but declined more rapidly in the sandy Manawatu soils. The loadings reached around 10^4 spores/g soil by week 6, but were not maintained. Undoubtedly, there is a natural equilibrium point that will be reached over time as inoculum is lost and the data presented suggest this is around 10^3 propagules/g. This is slightly lower than that reported in previous trials in New Zealand (Nelson et al. 2004) but is consistent with the findings of other workers (Hu & St. Leger 2002; Rath 2002). Soil conditions, timing of application and formulation play a major role in the level of establishment/persistence of inoculum. The use of a granular formulation rather than a spray drench would have resulted in a less uniform distribution of inoculum in the soil, and soil cores taken between rather than along the original drill rows could have missed localised 'high inoculum zones' around the original rice granules. This could account, in part, for the high variation in CFU counts between samples.

At two sites (1 and 2), both on the same farm, higher CFUs were recovered from samples collected in the control plots. The reasons for this differential increase are unclear. Since the control plots were simply a sub-section of the same paddock as the F418-treated plots, a concurrent increase across the site (assuming homogenous distribution of the fungus) would have been anticipated. It is possible that the increase may have been a result of 'hot spots' of host insects in the control plots, leading to localised high levels of inoculum on infected cadavers. The identity of selected *B. bassiana* isolates from soil washes and larvae will be confirmed by DNA sequencing of conserved ITS-5.8s gene regions (Glare & Inwood 1998). Ultimately, this will allow differentiation between native and the applied strain of *B. bassiana*, and definitively assign a 'cause' (i.e. application of F418) and 'effect' (higher CRW mortality) for the treatment applied. However, in spite of the elevated levels of inoculum recovered from control plots at weeks 6 and 12 at sites 1 and 2, CRW larval numbers were consistently lower in the F418 plots. This suggests that the fungus in the control plots was inactive against *Sitona lepidus*. F418 is highly virulent against *Sitona* spp. and shows considerable host specificity, with limited activity against other coleopterans (T.L. Nelson et al., unpubl. data). Similarly, other *B. bassiana* isolates will have a limited host range, and may be relatively inactive against CRW. Simply having high levels of *B. bassiana* in the soil does not automatically translate into high levels of activity against a specific insect.

Higher levels of inoculum were recovered from the top 6 cm of the soil profile than from the 6+ zone. This top zone is where the inoculum was placed during application, but may also be considered as the major bioactive zone in the soil profile, where insects and plant roots occur. Association with either or both of these resources could influence *B. bassiana* soil populations. Infection and conidiation on a susceptible insect, for example, will produce localised high concentrations of conidia. In addition, rhizosphere competence has been demonstrated for certain *Metarhizium anisopliae* strains (an entomopathogenic

species related to *B. bassiana*), suggesting that the soil/root interface may also play a critical role in the survival and multiplication of entomopathogenic fungi (Hu & St. Leger 2002; Bruck 2005). It is not clear how widespread this capacity is among *M. anisopliae* or *B. bassiana* strains, and an association between clover/grass roots and *B. bassiana* has yet to be demonstrated. Such capacity would enhance fungal persistence in the root zone and potentially offers additional ways of specifically introducing fungal inoculum into the environment where pest damage occurs.

For insect infection to occur, conidia must not only remain viable in the soil, but must also be infective. Conidia from virulent pathogens survive for extended periods in soil and germination is suppressed, e.g. by root exudates and humic acids, in the absence of a suitable host (Li & Holdom 1995; Rath 2002). Over time they will use up energy reserves and their ability to successfully infect a suitable host gradually declines (Shah et al. 2005). Persistence is adversely affected by temperature extremes (at the soil surface), drying or saturation (Ekesi et al. 2003). In addition, various biotic agents (other micro-organisms and microarthropods) utilise fungi as a food source, further contributing to a decrease in inoculum levels over time (Dromph 2003). Future research will include refinements in application techniques, timing and placement of the fungal formulation, and overcoming effects of biotic and abiotic factors to achieve greater persistence and efficacy. A better understanding of the fate and dynamics of inoculum following application will aid this, and further improvements may be achieved through use of fungi together with materials that attract CRW larvae to a source of infection (Johnson et al. 2005).

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