ESTABLISHING THE FUNGUS *BEAUVERIA BASSIANA* IN PASTURE FOR CLOVER ROOT WEEVIL *(SITONA LEPIDUS)* CONTROL

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ABSTRACT

The fungus *Beauveria bassiana* is a virulent pathogen of the clover root weevil, a major introduced pest of clover in New Zealand. Trials to investigate establishment of fungal inoculum in pasture were conducted in the Waikato region of New Zealand. Granular formulations of conidia based on biopolymer technology successfully allowed the established of fungus in pasture. However, it did not support survival of inoculum into the second year. Conidia on rice and conidial emulsions resulted in more successful establishment. These results have implications for development of a biopesticide for clover root weevil based on *B. bassiana*.
Keywords: *Beauveria bassiana*, clover root weevil, conidia, biopesticide, biopolymer.

INTRODUCTION

Clover root weevil (CRW) (*Sitona lepidus* Gyllenhal (Coleoptera: Curculionidae)) was first identified in New Zealand in 1995 (Barrett et al. 1996) and has since become established in pasture from Northland to as far south as Palmerston North. It is considered one of New Zealand's most serious pasture pests, severely affecting both the sustainability and the ability of clovers to fix nitrogen.

Early disease surveys of CRW populations in New Zealand found the fungus *Beauveria bassiana* infecting at very low levels (<0.03%) and was the only pathogen detected (Willoughby et al. 1998). Laboratory bioassays of the local strains of *B. bassiana* from the field infected insects along with a commercial *Beauveria* sp.-based biopesticide (Botanigard, Mycotech USA) showed the fungus to have good activity against CRW. In late 2000 several *B. bassiana* were isolated at AgResearch, Lincoln, from CRW cadavers collected in Europe and the United Kingdom. Subsequent laboratory bioassays against New Zealand field-collected CRW showed that one strain (F418) was a particularly effective pathogen, and a candidate for development as a potential biopesticide against CRW for use in New Zealand.

Selection of a highly virulent strain does not automatically guarantee practical field control of a pest. For a microbial control agent to be effective against a soil dwelling pest it is essential for the inoculum to establish and survive within the pasture.

Three trials to investigate establishment of *B. bassiana* in pasture soil were carried out during 2002 and 2003 using various formulations of strain F418. These included granular formulations of commercially produced spore powder, spore suspensions of the same spore powder, and conidia growing on rice produced at AgResearch, Lincoln.

METHODS

Production and formulation of *Beauveria bassiana*

The *B. bassiana* strain F418 was bulk-produced by Seed and Grain Biotechnology, Wodonga, Victoria, Australia to provide a spore powder with a spore count of

1-3x10^{10} spores/g. The spores were then formulated into granules using a patented biopolymer-based method developed at AgResearch, Lincoln (Johnson & Pearson 2001). Liquid suspensions were formulated by mixing the spore powder into water plus Tween 80 (BDH) as a wetting agent, and into a commercially produced nutrient emulsion concentrate from Seed and Grain Biotechnology. Conidia on rice was produced at AgResearch, Lincoln by inoculating 200 g of autoclaved long grained rice with 50 ml of broth (40 g D+Glucose (BDH) and 10 g Neopeptone (Difco)) plus conidia scraped from 2 week old PDA plate cultures.

Recovery, enumeration and identification of *Beauveria* from soil

Beauveria bassiana was enumerated from soil samples by dilution plating onto PDA (Merck) containing streptomycin sulphate (Sigma: 350 mg/litre), tetracycline hydrochloride (Sigma: 50 mg/litre) and cycloheximide (BDH, UK; 125 mg/litre). Plates were incubated at 20-25°C for 14 days. *Beauveria bassiana* isolates were identified by sequencing of extracted DNA as described by Glare & Inwood (1998).

Field evaluation trial

The trial was laid down in April 2002 in clover pasture in Otorohanga. The trial design was a randomised block with five replicates. The plot size was 10 m x 10 m with a 10 m buffer between blocks. The total area treated was 0.05 ha. The treatments were applied in the form of granules (three slow-release formulations: small, medium and large size) containing *B. bassiana* spores at 10^9/g and the conidia covered rice grains at 10^9 spores/g. They were drilled to a depth of 3–5 mm at a sowing rate of 30 kg product/ha using a modified Aitchison 12 coulter drill. Plots were sampled by taking fifty 10 cm diameter cores to a depth of 5 cm over clover plants. The soil cores were taken along two diagonal transects across the total area of the trial 2, 11, 28, 32 and 52 weeks after application.

Maximum challenge trial

Sixty paired areas (0.09 m^2) of white clover plants exhibiting similar growth form were identified in mature pasture on the Tokanui Research Farm in January 2003. The plants were marked with a metal peg with a numbered plastic tag attached for re-identification and the location measured from a central transect. Treatments of either spore suspension or water were applied randomly between paired areas within a 330 mm metal pipe section driven into the soil to a depth of 3–5 cm. Separate rings were used for treated and untreated plants. The ring remained in place until the suspension/water had fully infiltrated. Untreated control plants received a single application of 1000 ml of water. *Beauveria bassiana* spores were applied in 500 ml of water with 1 ml of Tween 80 to act as a surfactant/spreader to achieve a soil spore loading of 10^6 spores/g of soil. This was followed immediately by 500 ml of water applied by watering can to wash the spore suspension 3–5 mm into the soil profile. A third of treated and untreated plants selected as stratified random pairs were destructively harvested at 9, 24 and 34 weeks post application and *B. bassiana* from the soil surrounding the root zones enumerated.

Application technology trial

Forty paired white clover plants were marked for re-identification with a suitably coloured (treated vs untreated) metal peg in mature pasture on the Tokanui Research Farm in May 2003. *Beauveria bassiana* spores in both a fast-release granule formulation and a spore suspension (applied with a watering can) were applied to the soil surface within a 300 mm radius of individual white clover plants. Both treatments were applied to give a soil spore loading of 10^6/g in the top 10 cm of soil and 20 replicates of each treatment were established. Soil samples were taken from treated and untreated plants one week after application, and *B. bassiana* from the soil enumerated. Treated and untreated plants were destructively harvested in September 2003 and *B. bassiana* from the soil surrounding the root zones enumerated.

Statistical analysis

Numbers of colony forming units (cfu)/g soil were compared by analysis of variance using GenStat.

RESULTS

Field evaluation trial

Soil enumeration two weeks after application showed that the *B. bassiana* had established in the soil for all four treatments (Fig. 1). The rice treatment established best at $2x10^5$ spores/g of soil while granule formulations A (small) and B (medium) were at $1x10^3$ spores/g, and granule formulation C (large) was lower again at $5x10^2$ spores/g. Low levels of a background *Beauveria* were detected in the control plots at $1x10^2$ spores/g. Eleven weeks after application the levels of *Beauveria* in the rice treatment had dropped to $3x10^3$ spores/g of soil while the three granule treatments had increased in level to be present at $1x10^3$ spores/g. At the same time the background levels in the control plots had increased to $1x10^4$ spores/g. By week 28, *B. bassiana* was detected at $8x10^2$ spores/g of soil for granule formulation A and $5x10^2$ spores/g for formulation B, while formulation C and the control had declined to 10^2 spores/g. The rice treatment levels only showed a small decline to $1x10^3$ spores/g of soil.

At 32 weeks the rice treatment remained constant at 10^3 spores/g of soil while the granule treatments were now also detectable at 10^3 spores/g. The background levels in the control plots remained constant at 10^2 spores/g of soil. However, the final sampling at 52 weeks only isolated *B. bassiana* from the rice treatment, where it was detected at 10^3 spores/g of soil. No background *B. bassiana* was detected in the controls at this sampling date (Fig. 1).

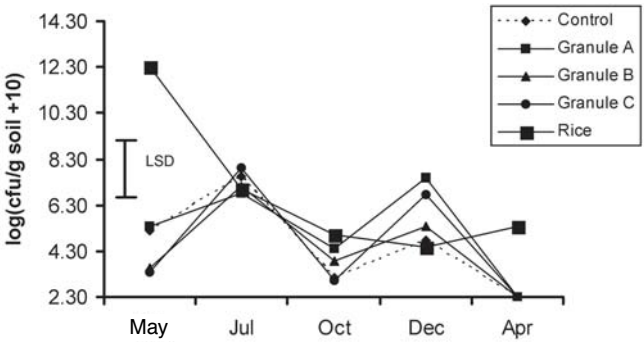

FIGURE 1: Numbers of *B. bassiana* after subsurface application in April 2002 to pasture using three different granular spore formulations and rice inoculated with conidia. Values are the mean of five replicates and the LSD value is indicated.

Maximum challenge trial

The first soil enumeration 9 weeks after application showed that the *Beauveria* had established at levels between 10^3 and 10^4 spores/g of soil in 17 out of the 20 replicates in the treated clover plants (Fig. 2). Background *B. bassiana* was only detected in one of the 20 control replicates, at 10^2 spores/g of soil. The second sampling at 24 weeks confirmed that the *B. bassiana* was established in all but one of the treatment replicates and was detectable at levels between 10^3 and 10^4 spores/g of soil, while only two of the 20 control replicates had background *B. bassiana* present at levels of 10^2 spores/g. The final sampling 34 weeks post application showed the levels of *B. bassiana* detected remained at between 10^3 and 10^4 spores/g of soil in the treatments, while background *B. bassiana* was isolated at between 10^2 and 10^3 spores/g in six of the 20 control replicates.

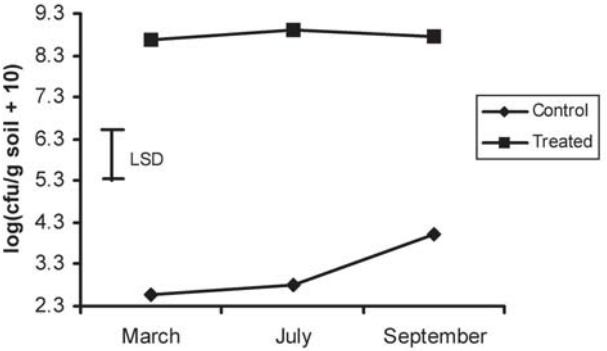

FIGURE 2: Numbers of *B. bassiana* in the soil root zone after application of a spore suspension or water to paired white clover plants in January 2003. Values are the mean of 20 replicates and the LSD value is indicated.

Application technology trial

One week after application soil enumeration showed the granules had established on average at between 10^3 and 10^4 spores/g of soil in 16 out of 20 replicates, while the emulsion treatment had established at between 10^4 and 10^5 spores/g in all the replicates. Background *B. bassiana* at between 10^3 and 10^4 spores/g of soil was detected in nine out of 20 control replicates (Fig. 3). A second sample was taken after 19 weeks and showed that the granules were now established in 18 of the 20 replicates at between 10^3 and 10^4 spores/g of soil, and the emulsion treatment was detectable at between 10^4 and 10^5 spores/g in 17 of the 20 replicates. Background levels in the controls were at 10^3 spores/g of soil in three of the 20 replicates.

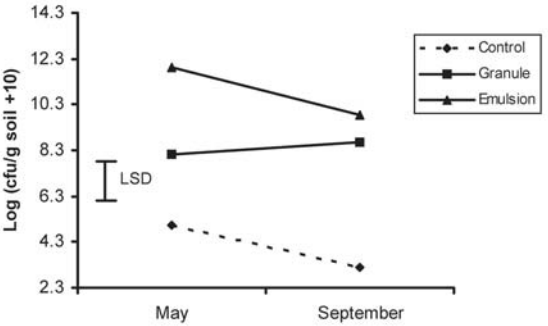

FIGURE 3: Numbers of *B. bassiana* in the soil root zone after application of a granule formulation or an emulsion suspension to paired white clover plants in May 2003. Measurements were made one week after application and in September. Values are the mean of 20 replicates and the LSD value is indicated.

DISCUSSION

The initial field evaluation trial in 2002 showed that *B. bassiana* applied into soil as a slow-release granular form took longer to establish than conidia-laden rice and did not persist at suitable concentrations (>10^4 spores/g of soil) to be effective as a biopesticide. While the initial low detection levels could be explained by the nature of the slow-release formulations, the granules were unable to establish and persist at target levels after 6 months when compared with the rice treatment. This gave the basis for exploring alternative methods of formulation and application techniques.

The achievement of target establishment rates (10^4 spores/g in soil) in the 2003 maximum challenge trial assessment of liquid application of conidia suggests there is potential for surface application technology. The satisfactory survival and detection rates in the top 5 cm of soil 34 weeks after an autumn application demonstrated that unformulated conidia are able to survive in soil over winter and remain viable in spring.

The 2003 application technology trial combined factors from both previous trials, using surface application of a fast-release granule formulation and a nutrient-based spore suspension of *B. bassiana*. The suspension provided higher and more uniform establishment in soil across the replicates than the granules. The establishment level of the fast-release granules was still at an acceptable level and certainly far superior to the slow-release version used in the field evaluation trial.

Contrary to uncertainties over survival of conidia by means of surface application as opposed to subsurface soil inoculation, these results show that not only does the inoculum survive but it establishes and persists at adequate levels in the soil to cause infection in the target insect. This has important implications for development of *B. bassiana* as a biopesticide for the control of CRW. The production of conidia is a relatively expensive process and the additional costs of formulating conidia and using machinery to drill it into soil makes it commercially prohibitive using current production techniques. While results to date for liquid applications of conidia are pleasing, it will be important to determine a year after a spore suspension application whether the soil inoculum levels are still at high enough levels to infect CRW larvae.

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REFERENCES

Barratt, B.I.P.; Barker, G.M.; Addison, P.J. 1996: *Sitona lepidus* Gyllenhal (Coleoptera: Curculionidae), a potential clover pest new to New Zealand. *N. Z. Entomol. 19:* 23-30.

Glare, T.R.; Inwood, A. 1998. Morphological and genetic characterisation of *Beauveria* spp. from New Zealand. *Mycol. Res. 102*: 250-256.

Johnson, V.W.; Pearson, J.F. 2001: NZ Patent 506487.

Willoughby, B.E.; Glare, T.R.; Kettlewell, F.J.; Nelson, T.L. 1998: *Beauveria bassiana* as a potential biocontrol agent against the clover root weevil, *Sitona lepidus*. *Proc. 51st N. Z. Plant Prot. Conf.:* 9-15.