

BEAUVERIA BASSIANA AS A POTENTIAL BIOCONTROL AGENT AGAINST THE CLOVER ROOT WEEVIL, *SITONA LEPIDUS*

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ABSTRACT

Three clover root weevil (*Sitona lepidus*) adults infected with the entomopathogenic fungus, *Beauveria bassiana*, were collected from pastures in the Waikato in 1997. Genotyping using rDNA comparisons showed the strain from weevils to be a cosmopolitan type. Application of fungal conidia topically and by contaminating leaf, soil and root surfaces showed that both *B. bassiana* isolated from *S. lepidus* in New Zealand and commercial preparations of this species were pathogenic to adult and larval clover root weevil, with up to 100% mortality within two to four weeks of inoculation. Isolates varied in their effectiveness against larvae and adults, with two isolates effective against adults found to be less pathogenic to larvae than a third isolate.

Keywords: *Sitona lepidus*, *Beauveria bassiana*, biological control, clover root weevil.

INTRODUCTION

Two species of the genus *Sitona* Germar (Coleoptera: Curculionidae) have established in New Zealand. *Sitona discoideus* Gyllenhal was first reported in Napier in 1974 (Esson 1975) and rapidly dispersed throughout New Zealand causing serious losses in sustainability and production in lucerne (Goldson *et al.* 1985). A second species, the clover root weevil (*S. lepidus* Gyllenhal), was confirmed to be present in New Zealand in March 1996 (Barratt *et al.* 1996). In the last two years this species has spread at approximately 35 km/year (Willoughby and Addison 1997a), and has contributed to poor clover performance in the rye/clover based pastures of the Waikato (Willoughby and Addison 1997b). It has also established north of Auckland and in the Bay of Plenty causing similar problems. The potential loss of clover as a pasture component poses serious problems for pastoral farmers, who now need to look for costly alternative management regimes to maintain pasture productivity.

Because of the minor importance of the weevil in the northern hemisphere, there is little known about its biology and management. Broad acre use of synthetic insecticides in New Zealand pasture is not an economically or environmentally sustainable option for the control of *S. lepidus*. Conversely, biological control, using predators, parasitoids or pathogens is an attractive option, if agents can be found. Biological control using an introduced parasitoid has been successful in reducing the threat of two other introduced weevil pests, *S. discoideus* (Barlow and Goldson 1993) and *Listronotus bonariensis* (Goldson *et al.* 1994) in New Zealand. However, to date no suitable parasitoids active against *S. lepidus* have been identified. Predators are usually unsuitable in as much as they are polyphagous and thus could pose a threat to indigenous New Zealand fauna. The other group of natural enemies, microbial pathogens, has a potential as a bio-control agent particularly if the pathogen is endemic and has a degree of specificity. Diseases have been used successfully both overseas and in New Zealand to combat weevils and other pasture pests (e.g. Bournier *et al.* 1996).

The fungal genus *Beauveria* Vuillemin (Deuteromycetes) contains species that are often pathogenic to insects. One of the most common species, *B. bassiana*, which has been recorded to attack over 20 insect species in New Zealand (Glare *et al.* 1993) occurs in most parts of the world. *B. bassiana* is one of the few species of fungi developed into a commercial product; a strain is sold in the USA by Mycotech (Butte, Montana) for the control of Colorado potato beetle, whiteflies and aphids.

As the only pathogen found to date attacking the weevil in New Zealand, with potential for development as both an inundative and classical control agent, we initiated research to determine potential of *B. bassiana* for the control of clover root weevil. This contribution describes the isolation, identification and subsequent testing of three strains of *B. bassiana* isolated from three diseased adult clover root weevils that were found in 12,000 individuals examined during the last 2 years. Additionally, two preparations of *B. bassiana* from the USA were also tested against clover root weevil adults and larvae.

MATERIALS AND METHODS

Clover root weevil

Adult clover root weevils were collected in the field using a modified motorised suction device and maintained in insect proof containers in the laboratory prior to experimental use. Larvae were collected from hand-sorted soil samples obtained from the field and stored in soil at ambient temperatures (12°C - 22°C) prior to testing. All adult and larval clover root weevil were used in experiments within 24 hours of collection.

Isolation and identification of entomopathogenic fungus

All weevils collected in the field over the last 2 years were examined for clinical signs of disease. Conidia from weevils which supported fungal sporulation typical of *B. bassiana* were transferred to the selective medium JC (4% D-glucose; 1% Neopeptone, 1.5% agar, 0.0125% cyclohexamide, 0.035% streptomycin and 0.05% tetracycline; modified from Joussier and Catroux 1976) and grown at 22°C. Subsequently, pure cultures were transferred to Potato Dextrose Agar (PDA)(Oxoid), maintained at 20-25°C, and subcultured every two-three weeks. The isolates were placed in the AgResearch Insect Pathogen Culture Collection (Lincoln) and stored at -80°C. Samples of the commercially available *B. bassiana*-based product, BotaniGard ES Strain GHA (Mycotech Corporation, Butte, Montana), and a powder formulation, Mycotech Lot 97-10-1 TGAI, were obtained through Elliott Chemicals Ltd. (Auckland). The Mycotech strain was isolated from BotaniGard onto PDA for molecular comparison.

Determination of species affiliation was by microscopic examination of conidiophores and conidia. Morphological examinations were conducted on PDA-grown material mounted in water. Conidial dimensions were determined using eye-piece micrometers in a Zeiss microscope at 1000x. Initial classifications were based on collection notes for overseas isolates, or conidial morphology for New Zealand isolates (Glare and Inwood 1998).

Molecular characterisation

B. bassiana samples isolated previously in New Zealand have been divided into distinct genotypes based on RAPD analysis and restriction length polymorphism of ribosomal DNA (Glare and Inwood 1998). To determine the genotype(s) of *B. bassiana* from *S. lepidus*, fungal DNA was isolated using a liquid nitrogen grinding method similar to that of Rogers and Bendich (1988), but the CTAB buffer was replaced with an extraction buffer of 50mM Tris-HCl (pH 8.0), 50mM EDTA, 2% Sarcosyl, 150mM NaCl. The region of the ribosomal repeat from the 3' end of 16s rDNA across internal transcribed spacer 1 (ITS 1), the 5.8s rDNA, and ITS 2, to the 5' end of the 28s rDNA were PCR amplified using the primers, TW81 and AB28 as described in Glare and Inwood (1998). Amplified rDNA was digested with restriction endonuclease enzymes using standard methods (Sambrook *et al.* 1989) and visualised on a 2% agarose gel.

Bioassays against clover root weevil adults and larvae

Fungal inoculum: For isolates F267, F268 and F269, conidia were collected directly from the PDA plate cultures grown at 25°C for 1-2 weeks and suspended in

a solution of 0.05% Triton 100 in distilled water. Conidial density was estimated using a haemocytometer prior to use. For the two Mycotech preparations of *B. bassiana* the conidial densities were: BotaniGard -1.8×10^{13} /litre and the Mycotech powder formulation -1.4×10^{11} /g. With the Mycotech powder preparation, it was necessary to resuspend the powder in 0.00025% Citowett (BASF) a non-ionic general purpose wetting agent, containing 1000 g/litre alkylaryl polyglycol ether. A treatment of 0.00025% Citowett without conidia was included in the assay to determine if Citowett had any direct effect on *S. lepidus* larvae.

Adult bioassay: Inverted 120 ml specimen bottles, using their lids as bases, were set up as insect cages. Within the bottles, polystyrene was used to secure 2 ml vials filled with water in which a fresh leaf of white clover was placed. Clover vials and unsterilized soil were placed in the base of the cages. Assessments of disease symptoms, adult survival and feeding were made at regular intervals. Treatment mortality was recorded where it was attributable to disease based on clinical symptoms. Feeding was assessed by the degree of leaf notching on a comparative scale of 0 to 5 with 5 being the most severe feeding. Fresh untreated foliage was replaced at each assessment. Bioassays were conducted at ambient temperature (12°C-24°C). Each insect cage contained one weevil and each treatment was replicated 10 times.

In bioassay 1, isolates F267 and F268 were used against adults by treating leaves and soil with 50 µl of conidial suspension. In bioassay 2, BotaniGard was used to treat both leaves and leaves and soil. Fifty microlitres of BotaniGard was applied to each vial. Water was used as controls in both bioassays.

Larval bioassay: Field-collected late instar clover root weevil larvae were inoculated topically with 5 µl of a *B. bassiana* conidial suspension or fed excised white clover roots that had been dipped in a *B. bassiana* conidial suspension. Three concentrations of conidia were used: 10^4 , 10^6 or 10^8 conidia/ml. The larvae were contained individually in 5 ml vials and assessed at regular intervals for disease symptoms and survival. Each vial contained a single larva and each treatment was replicated 10 times. Bioassays were conducted at ambient temperature (12°C-24°C).

In bioassay 3, isolates F267, F268 and F269 were used to inoculate larvae, while in bioassay 4, BotaniGard and the Mycotech powder formulation (diluted to the appropriate concentrations in 0.00025% Citowett), were used. Both water and 0.0025% Citowett were used as controls.

RESULTS

Isolation and characterisation of fungal pathogens

Three cadavers of *S. lepidus* were found to be attacked by entomopathogenic fungi. Fungus was isolated from each cadaver and accessioned into the AgResearch Insect Pathogens Culture Collection (Lincoln) under the numbers F267, F268 and F269. All were from adult *S. lepidus* collected in the central Waikato in 1997. Microscopic examination confirmed this fungus to be *B. bassiana*, which produced small near-spherical conidia (F267, $2.4 \pm 0.33 \times 2.2 \pm 0.21$; F268, $2.2 \pm 0.28 \times 2.2 \pm 0.28$; F269, $2.4 \pm 0.30 \times 2.2 \pm 0.21$ µm; \pm SD, n=10) on denticulate rachis.

Molecular comparison of the conserved ribosomal DNA region ITS1/5.8s/ITS2 by digestion with *Mse*1, *Tru*91 and *Tha*1 showed the isolates to be identical to a group of *B. bassiana* isolates that occur both in New Zealand and exotically (Glare and Inwood 1998). The BotaniGard strain was shown to also belong to this cosmopolitan genotype on rDNA comparison.

Adult bioassays

Bioassay 1 (F267 and F268): Treating foliage and soil within the insect cages with the equivalent of a high field dose (10^{13} conidia/ha) of *B. bassiana* isolate F267 resulted in 100% mortality of adult weevils by 29 days after application (Table 1). With F268, the same application resulted in only 80% mortality by 48 days, by which time untreated control mortality had reached 40% (Table 1). Both isolates reduced the feeding of the clover root weevil adults considerably in the first week post treatment.

TABLE 1: Bioassay 1; mortality of *Sitona lepidus* adults in the presence of *Trifolium repens* foliage and soil treated with two strains of *Beauveria bassiana* applied at 10^{13} conidia/ha in 100 litre water/ha equivalent. (Feeding is measured on a 0-5 scale where 0= no feeding and 5= all clover consumed).

Days post treatment	Percent mortality (average feeding score)		
	Control	F268	F267
5	0 (2.9)	10 (1.0)	20 (0.6)
7	0 (3.8)	40 (1.7)	40 (1.4)
12	0 (3.0)	60 (2.8)	70 (2.5)
16	0 (3.3)	60 (3.8)	80 (3.0)
22	0 (2.6)	70 (2.0)	80 (3.0)
29	20 (3.1)	70 (2.7)	100 (1.5)
37	30 (3.4)	70 (2.7)	
41	40 (3.1)	80 (2.3)	
48	40	80	

Bioassay 2 (BotaniGard): In a separate bioassay, a commercial formulation of *B. bassiana*, BotaniGard, was used against adults at the equivalent of recommended field rates (10^{13} conidia/ha) (Table 2). After 31 days, mortality reached 90% when BotaniGard was used to treat both leaves and soil, but only 70% mortality when only leaves were treated.

TABLE 2: Bioassay 2; mortality of *Sitona lepidus* adults in the presence of *Trifolium repens* foliage, on foliage and soil treated with a *Beauveria bassiana* commercial formulation (BotaniGard ES) applied at 10^{13} conidia/ha in 100 litre water/ha equivalent. (Feeding is measured on a 0-5 scale where 0= no feeding and 5= all clover consumed).

Days post treatment	Percent mortality (average feeding score)		
	Control	Application to leaves only	Application to leaves and soil
5	10 (1.9)	0 (0.4)	10 (0.8)
9	10 (2.6)	30 (1.4)	30 (1.7)
12	10 (2.6)	50 (2.1)	60 (2.0)
17	10 (2.8)	60 (2.8)	80 (1.3)
24	30 (2.6)	60 (2.5)	90 (3.0)
31	40 (2.8)	70 (2.8)	90 (0.0)

Larval bioassays

Bioassay 3 (F267, F268 and F269): In assays against larvae, all three *B. bassiana* isolates originally from *S. lepidus* adults caused some mortality of larvae (Table 3). Using isolate F269 at concentrations of 10^6 and 10^8 conidia/ml, application by root dipping and topical application to larvae resulted in 100% mortality (Table 3). With this isolate, root dipping also resulted in 100% mortality even at the lowest (10^4 conidia/ml) dose. Isolates F267 and 268, effective against adult weevils in bioassay (Table 1), failed to kill over 50% in any treatment against larvae (Table 3). Level of mortality among treated larvae did not appear sensitive to inoculum concentration in the range 10^4 - 10^8 conidia/ml.

Bioassay 4 (Mycotech preparations): In an additional bioassay, larvae were exposed to two formulations of *B. bassiana*: BotaniGard and a Mycotech powder formulation (diluted to the appropriate concentration in 0.00025% Citowett). Mortality reached 100% by 13 days in all treatments at or above 10^6 conidia/ml (Table 4). The resuspended Mycotech powder caused 100% mortality when used as a root dip at the lowest (10^4 conidia/ml) dose. Citowett had no effect on mortality compared to water.

TABLE 3: Bioassay 3; percent mortality of *Sitona lepidus* larvae in the presence of *Trifolium repens* roots and soil treated with three strains of *Beauveria bassiana* applied at a range of conidial concentrations. (R = root dipping. L = topical application of conidial suspension to larvae.)

Isolate	Days post treatment	Conidial concentration/ml							
		10 ⁸		10 ⁶		10 ⁴		Control	
		R	L	R	L	R	L	R	L
F267	2	0	0	0	0	0	0	0	0
	7	10	10	20	10	20	0	10	0
	15	20	30	40	50	20	40	10	0
F268	2	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	10	0
	15	10	20	30	50	30	0	10	0
F269	2	10	0	0	0	20	0	0	0
	7	10	60	40	60	40	30	10	0
	15	100	100	100	100	60	100	10	0

TABLE 4: Bioassay 4; percent mortality of *Sitona lepidus* larvae in the presence of *Trifolium repens* roots and soil treated with various concentrations of *Beauveria bassiana* formulations, BotaniGard ES and a Mycotech powder. (R = root dipping. L = topical application of conidial suspension to larvae.)

Strain	Days post treatment	Conidial concentration/ml									
		10 ⁸		10 ⁶		10 ⁴		Control ¹		Citowett ¹	
		R	L	R	L	R	L	R	L	R	L
Mycotech	2	40	20	10	10	0	0	0	0	0	0
	6	90	100	50	40	100	10	10	10	0	0
	13	90	100	100	100	100	70	30	30	30	30
BotaniGard	2	10	10	30	20	0	0	0	0	0	0
	6	80	90	40	40	20	0	10	10	0	0
	13	100	100	100	100	30	30	30	30	30	30

¹ Control = water. Citowett = 0.00025% Citowett.

DISCUSSION

The entomopathogenic fungus, *B. bassiana* has shown potential in these bioassays against both adults and larvae. All isolates used belonged to the same subspecific genotype, including the Mycotech preparations. All isolates and preparations may not be as effective against all life stages of the weevil. Two isolates, F267 and F268, were more effective against adults than larvae, while a third unformulated isolate, F269, was highly pathogenic against larvae. Tests against adults with F269 have not been completed. The bioassays against larvae did not show a strong dose response, with little reduction in mortality between 10⁸ and 10⁴ conidia/ml treatments.

The origin of *B. bassiana* infecting clover root weevil naturally in New Zealand is uncertain. It could have been imported on the host, but as *B. bassiana* is ubiquitous in this country and only three clover root weevils have been discovered infected, it could also have resulted from contact after introduction of *S. lepidus*. *B. bassiana* has an extensive host range extending to several insect Orders. While individual isolates are not pathogenic to all recorded hosts for the species, isolates will often infect

multiple hosts to varying degrees. For example, earlier studies have shown that isolates of *B. bassiana* from a grass grub epizootic (Townsend *et al.* 1995) were pathogenic to *L. bonariensis* in the laboratory (Glare, Groden and McNeill, unpubl. data). It would not be surprising, therefore, for isolates that are pathogens of other insects, such as *L. bonariensis*, to have infected *S. lepidus*.

While promising results have been obtained in this study, use of two isolates (F267 and F268) against adults in a single field trial was disappointing (Willoughby, Kettlewell, Glare and Nelson, unpubl. data). This may be related to the rapid inactivation of unformulated conidia. In the laboratory, the viability of the conidia can be lost within 6 hours of exposure to sunlight (Willoughby unpubl. data). This does not necessarily preclude the potential for biopesticides based on *B. bassiana*. The commercial formulations used in this study offer some UV protection, and this opens up the possibility of a weevil pesticide being available in the near future. The commercial formulation of *B. bassiana*, BotaniGard, is based on a single isolate, but marketed for a range of insect pests from Coleoptera to Hemiptera. The product has excellent shelf life and formulation characteristics, including some UV resistance, so its pathogenicity to clover root weevil in the laboratory is encouraging.

Application of any fungal products against weevils needs to be tested in the field and careful consideration given to which life stage is best targeted. The preliminary results from this study have shown that *B. bassiana* is active against both larvae and adults. Application against adults may be possible using an emulsifiable liquid formulation (such as BotaniGard) although UV and desiccation may still reduce the effectiveness of the conidia. Conversely, *Beauveria* spp. conidia can survive for years in soil, so the larvae may be a better target for application. Experimentation has already been undertaken with the application of fungi for grass grub using a modified disc drill, which resulted in excellent survival of the inoculum over two years (Glare *et al.* 1994). Such an approach may be useful with *B. bassiana* against clover root weevil.

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