

# INVESTIGATIONS INTO THE FORMULATION AND EFFICACY OF ENTOMOPATHOGENIC FUNGI AGAINST LARVAE OF YELLOW MEALWORM (*TENEBRIO MOLITOR* L., COLEOPTERA: TENEBRIONIDAE)

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## Summary

A new invert emulsion formulation of entomopathogenic fungi was developed using non-toxic ingredients readily available in Australia and tested for compatibility with two Australian strains of *Beauveria bassiana* (Bals.). Laboratory bioassays were conducted to determine the efficacy of these fungi when applied in an invert emulsion formulation against yellow mealworm larvae, *Tenebrio molitor* L. The entomopathogenic fungi consistently caused higher mortality of *T. molitor* larvae when the invert emulsion was used compared with aqueous conidia suspensions. When *B. bassiana* strain BG1 was applied as an invert emulsion using different concentrations, the LC<sub>50</sub> (measured as conidia mL<sup>-1</sup>) was > 600x lower than the LC<sub>50</sub> of aqueous suspension of the conidia. The invert emulsion formulation tested improved the consistency and efficacy of the fungal strains tested against *T. molitor* under controlled conditions and has the potential to increase their efficacy when used on a commercial scale.

**Keywords:** *Beauveria bassiana*, invert emulsion

## INTRODUCTION

There are very few registered myco-insecticides available in Australia despite the presence of local entomopathogenic fungi (EPF) strains that are active against economic pests. A commercial product containing *Metarhizium anisopliae* (Metschnikoff) Sorokin is available that targets locusts (DAFF 2009) but attempts to develop a commercial myco-insecticide from *Beauveria bassiana* (Balsamo) Vuillemin for the Australian market have not been successful yet (Pilkington, pers. comm.). Germination of EPF conidia (whether from a natural source or a commercial product) is essential for the infection process and requires specific microclimate conditions, particularly temperature and humidity, at the germination site (Van Driesche and Bellows 1996). Environmental conditions are often a limiting factor when EPF are applied in the field (Ferron *et al.* 1991, Glare and Milner 1991, Vandenberg *et al.* 1998). The formulation used to apply EPF products can be manipulated to enhance the conidial performance (Hidalgo *et al.* 1998). Invert emulsion formulations (an emulsion in which oil is the continuous or external phase and water is the internal phase) of EPF can increase pest mortality by improving the conidial viability (Batta 2003, 2007).

The type of invert emulsion formulation developed by Batta (2003) has not been tested against Australian strains of EPF. Use of an invert emulsion may increase efficacy of a particular EPF strain and/or reduce the quantity of conidia needed to achieve the desired level of mortality. This study determined the effect of an invert emulsion formulation on the performance of two Australian strains of *B. bassiana* under laboratory conditions. It was a preliminary investigation to

demonstrate the potential of these formulations for future research.

## MATERIALS AND METHODS

### Isolation and formulation of entomopathogenic fungi

Strain BG1 of *B. bassiana* was isolated from infected diamondback moth larvae (*Plutella xylostella* L.) found on cabbage crops in Adelaide, South Australia. Strain BF1 of *B. bassiana* was isolated from infected adults of elephant weevil borer (*Orthorhinus cylindrirostris* F.) found on blueberries in Corindi, New South Wales. Each strain was isolated first from its host insect then cultured using Sabouraud's Dextrose Agar (SDA, supplied by Difco, Detroit, MI). All strains were then subcultured using SDA to obtain a pure culture for experimental use.

Subcultures of these strains were incubated at 25±1.0° C on SDA for 10 days before harvesting their conidia. Conidial harvesting was carried out by scraping the conidia and mycelium from the surface of the SDA using sterile scalpel blades followed by suspension of the scrapings in sterile de-ionized water. Suspended conidia were separated from mycelium of each strain by sieving them through a 75 µm mesh cheese cloth. The concentration of conidia in the sieved suspensions was then determined using a haemocytometer. It was 4.0 x 10<sup>8</sup> conidia mL<sup>-1</sup> for the strain BG1 (became 1.0 x 10<sup>8</sup> conidia mL<sup>-1</sup> of prepared invert emulsion) and 1.2 x 10<sup>7</sup> conidia mL<sup>-1</sup> for the strain BF1 (became 3.0 x 10<sup>6</sup> conidia mL<sup>-1</sup> of prepared emulsion). The difference in conidia concentrations between the two strains was attributed to differences in growth rate and hence in the

quantity of conidia produced during the 10 day growth period. To check for the continued presence of viable conidia, samples (100  $\mu\text{L}$  each) of each strain (formulated and unformulated) were taken at weekly intervals following preparation, spread on the surface of SDA plates and incubated at  $25 \pm 1.0^\circ\text{C}$  for five days. The plates were then assessed for growth of *B. bassiana* colonies.

The invert emulsion used here consisted of an oil phase and an aqueous phase (50:50). These phases were mixed mechanically at 20000 rpm for 1.5 min using a homogenizer (Milk Shaker, Breville model MS-200, Australia). The aqueous phase of the formulation consisted of sterile de-ionized water ( $450 \text{ g kg}^{-1}$ ), glycerin ( $42.5 \text{ g kg}^{-1}$ ) and water-soluble emulsifier ( $7.5 \text{ g kg}^{-1}$ , Dehymuls<sup>®</sup> LE, Carechemicals, Cognis, Germany). The water-soluble emulsifier was first melted into the sterile de-ionized water at  $75^\circ\text{C}$  in a water bath for 15 min before adding the glycerine. Introduction of standardized conidial suspensions of each strain into the invert emulsion was achieved by mixing each suspension with the aqueous phase of the invert emulsion after it had cooled to about  $40^\circ\text{C}$ . The quantity of sterile de-ionized water + standardized conidial suspension in the prepared final emulsion should equal to  $450 \text{ g kg}^{-1}$ . The oil phase of the formulation consisted of canola oil ( $480 \text{ g kg}^{-1}$ , Home Brand, Coles, Sydney) and oil-soluble emulsifier: Tween 20 ( $20 \text{ g kg}^{-1}$ ). After homogenization of the two phases as described above, the invert emulsion was left to stand in a sealed container (i.e. 100 mL graduated cylinders or in 500 mL screw capped glass bottles) for at least 24 h before use (to ensure the mixture of the two phases was stable). The invert emulsion was stored at  $25 \pm 1.0^\circ\text{C}$  until used.

### Bioassays

One of the *B. bassiana* strains was isolated from a native weevil pest (*Orthorhinus cylindrirostris* (Fabricius)) but it was not possible to use *O. cylindrirostris* larvae for laboratory bioassays because they require living trees as a habitat (Murdoch unpublished). Instead *Tenebrio molitor* L. was chosen as a model insect for the bioassays because it was readily available in large quantities and invert emulsion formulations have shown promise for control of stored grain pests (Batta 2005). Larvae (3<sup>rd</sup> or 4<sup>th</sup> instar) were purchased from Pisces Enterprises (Kenmore, Queensland). The larvae were kept in wheat bran contained in round plastic tubs (120 mm diameter) with perforated lids until used.

The first bioassay compared mortality of *T. molitor* larvae treated with the maximum concentration of each EPF strain formulated in invert emulsion (see previous section) and with unformulated aqueous suspensions of conidia. Control treatments for this bioassay were

undiluted invert emulsion without EPF (blank formulation) and sterile de-ionized water. The second bioassay compared the mortality of *T. molitor* larvae treated with successive ten-fold dilutions of the maximum concentration of strain BG1. Formulated and unformulated forms of this strain, in addition to blank formulation (also subjected to successive ten-fold dilutions) were included in this bioassay.

There were three replicates of each treatment (EPF strain or concentration) in each bioassay. A replicate consisted of 10 larvae in a 90 mm diameter Petri dish, which were sprayed with 1.0 mL of the appropriate treatment solution (formulated or unformulated strain or blank formulation or sterile water) using a small calibrated sprayer. After spraying, 10g of wheat bran was added to each dish. Treated dishes were then kept in an incubator at  $25 \pm 1.0^\circ\text{C}$  and 14:10 light:dark for up to 7 days. Dead larvae were counted and the data converted into percentage mortality.

### Data analysis

Percentage data on larval mortality were subjected to transformation (inverse sine square root) before conducting one-way ANOVA to compare mortality between treatments for each strain in the first bioassay followed by Tukey's HSD to separate means when significant treatment effects were detected (JMP version 8). Probit analysis was used to determine the median lethal concentration ( $\text{LC}_{50}$ ) of formulated and unformulated concentrations of strain BG1 in the second bioassay.

## RESULTS

Samples from the formulated EPF strains showed consistent growth on SDA over the five-week storage period whereas samples from aqueous suspensions did not produce viable colonies on media after one week because the conidia germinated in the suspension. This suggests that the formulation was not detrimental to the conidia although quantitative analysis of conidial viability is needed to confirm this.

Larval death occurred within five days of EPF treatment but another two days were required for the appearance of typical white mycelium growth of the fungus species on the outer surfaces of dead larvae. This mycelium growth confirmed that death was caused by fungal attack. Formulation in invert emulsion increased mortality of *T. molitor* larvae by about 50% for both strains tested in comparison with unformulated treatments (Strain BG1:  $F_{3,8} = 122.1$ ,  $P < 0.0001$ , Strain BF1:  $F_{3,8} = 172.0$ ,  $P < 0.0001$ , Table 1). The application of undiluted blank formulation alone caused about 20% mortality, indicating that use of the invert emulsion increased efficacy of the EPF by approximately 30%.

**Table 1.** Mean ( $\pm$  se) mortality (%) of *Tenebrio molitor* larvae (3<sup>rd</sup> to 4<sup>th</sup> instar) treated with formulated and unformulated strains of *Beauveria bassiana* (3 replicates per treatment, n = 10 larvae per replicate). Means (in each column) followed by the same letter are not significantly different at  $P = 0.05$ .

Formulation Treatment	Mortality (mean $\pm$ se)	
	Strain BG1 ( $1 \times 10^8$ conidia mL <sup>-1</sup> )	Strain BF1 ( $3 \times 10^6$ conidia mL <sup>-1</sup> )
Invert emulsion	96.6 $\pm$ 5.7 a	100.0 $\pm$ 6.6 a
Aqueous suspension	40.0 $\pm$ 3.3 b	53.3 $\pm$ 0 b
Blank formulation (control)	23.3 $\pm$ 3.3 b	20.0 $\pm$ 0 c
Sterile de-ionized water (control)	0 $\pm$ 0 c	0 $\pm$ 0 d

**Table 2.** Probit analysis of formulated (invert emulsion) and unformulated (aqueous suspension) *Beauveria bassiana* strain BG1 (tenfold dilutions from  $1 \times 10^8$  to  $10$  conidia mL<sup>-1</sup>) tested against *Tenebrio molitor* larvae (3<sup>rd</sup> to 4<sup>th</sup> instar, 3 replicates per treatment, n = 10 larvae per replicate).

Active Ingredient	LC <sub>50</sub> (conidia mL <sup>-1</sup> )	95% Fiducial limits LC <sub>50</sub> (conidia mL <sup>-1</sup> )	Slope	Chi square	Heterogeneity	Degrees of freedom
Formulated	$8.1 \times 10^5$	$3.0 \times 10^5 - 2.2 \times 10^6$	0.68	2.38	0.39	6
Unformulated	$5.1 \times 10^8$	$8.3 \times 10^6 - 3.1 \times 10^{10}$	0.26	1.29	0.21	6

Although the concentration of BF1 spores used was lower by two orders of magnitude than the concentration of BG1 spores, the observed mortality was similar between treatments for each strain. This suggests that strain BF1 may be more virulent towards *T. molitor* than strain BG1. However strain BG1 was selected for further testing at different concentrations because its cultures produced a higher yield of fresh conidia per petri dish than strain BF1 (Batta and Murdoch, personal observation) so it was easier to harvest sufficient conidia to prepare the formulations of this strain.

Mortality declined with decreasing conidial concentrations for both the unformulated and formulated strain BG1 (Table 2). The LC<sub>50</sub> (measured as conidia mL<sup>-1</sup>) was substantially lower (637.5x) for formulated ( $8.0 \times 10^5$  conidia mL<sup>-1</sup>) than unformulated ( $5.1 \times 10^8$  conidia mL<sup>-1</sup>) strain BG1 (Table 2).

## DISCUSSION

Formulation as an invert emulsion improved substantially the performance of the two *B. bassiana* strains. Larval mortality of *T. molitor* five days after treatment was at least 10% higher than the 80% mortality reported by Rodriguez-Gomez *et al.* (2009) using a different strain of the same fungal species. Use of an invert emulsion formulation also improved the performance of a *B. bassiana* strain against the almond bark

beetle, *Scolytus amygdali* Guerin-Meneville (Batta 2007). This increase in efficacy is attributed primarily to increased viability of the conidia within the invert emulsion, which in turn increases the infection rate of the target insects (Batta 2003).

Important advantages of this particular formulation are firstly that the ingredients are relatively cheap and readily available within Australia; secondly that these ingredients are either food additives or used in cosmetics and so are non-toxic; thirdly preparation of the invert emulsion is not complicated and does not need sophisticated tools or procedures. However several points need to be addressed before an EPF product using this formulation can be developed. Firstly, in these experiments fresh spores were harvested directly from fungal cultures but the use of dried spores may be preferable for large scale production. The response of dried spores to the invert emulsion has not been established nor has conidia viability been quantified within the invert emulsion for Australian EPF strains. Secondly, the insect host range of strains BG1 and BF1 may not be sufficient to support a commercial market in Australia for an EPF product so testing of EPF strains that are active against polyphagous pests would be desirable. Finally, the performance of formulated EPF needs to be measured at a larger scale under Australian field conditions.

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