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Development of mycoinsecticide formulations with *Beauveria bassiana* and *Metarhizium anisopliae* for the control of lesser mealworm, *Alphitobius diaperinus*, in chicken broiler houses

Steven J. Rice · Dalton K. Baker · Diana M. Leemon

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Abstract Entomopathogenic fungi are promising alternatives to synthetic chemicals for controlling lesser mealworm populations in broiler houses. Granular formulations of two isolates of the fungi *Beauveria bassiana* and *Metarhizium anisopliae* based on ground chicken feed pellets produced 98 and 87% larval mortality, respectively, after 11 days' exposure to $\approx$ 0.2 g conidia/54 g formulation m^{-2} in the laboratory. Oil based formulations with canola oil and Codacide™ were generally slower to produce mortality but still significantly effective. Granular formulations were effective on a range of broiler-house substrates (new and used litter, soil) of varying pH and moisture content. The efficacy of the *M. anisopliae* isolate appeared enhanced and the *B. bassiana* isolate inhibited on damp new litter (40% moisture). Substrate pH (3.45–6.38) did not discernibly inhibit fungal efficacy. Granular formulations of both fungal isolates tested at average broiler-house temperatures were ineffective at 35 °C but became active when re-tested at 30 °C. Granular mycoinsecticide formulations based on the two isolates are now ready for testing in commercial broiler houses.

Keywords Biopesticide · Biocontrol · Litter · Poultry · Darkling beetle

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S. J. Rice (✉) · D. K. Baker · D. M. Leemon
Queensland Department of Agriculture and Fisheries, Ecosciences Precinct, Dutton Park, QLD 4102, Australia
e-mail: Steven.Rice@DAF.qld.gov.au

Introduction

Lesser mealworm (*Alphitobius diaperinus*) (Panzer, 1797) (Coleoptera: Tenebrionidae) is a significant, worldwide pest of chicken broiler houses. Very large numbers can occur in the flock's bedding litter, mostly under feed pans and along walls (Lambkin et al. 2007, 2008). Lesser mealworm vectors avian pathogens including fowl pox and *Escherichia coli* and food safety threats such as *Salmonella* and *Campylobacter* (De Las Casas et al. 1976; McAllister et al. 1996; Poole and Crippen 2009; Strother et al. 2005). It also damages broiler-house earth floors and insulation with its tunnelling behaviour (Despins et al. 1987; Hinton and Moon 2003; Japp et al. 2010). Application of chemical insecticides to the floor and walls are the usual management practice for lesser mealworm in broiler houses, but selection of insecticide resistant populations and poor application methods have compromised their efficacy (Cogan et al. 1996; Lambkin and Rice 2006; Tomberlin et al. 2008). Insecticide residues in litter are also of concern, particularly in a

time of increased public scrutiny regarding production of chemical free, safe food (Ong et al. 2016).

Entomopathogenic fungi have been suggested as alternatives to synthetic insecticides for control of insect pests (Lacey et al. 2015). The conidia of these fungi attach to insect surfaces, and under favourable conditions germinate, penetrate, and proliferate throughout the insect body, ultimately causing insect death (Boucias and Pendland 1998; Shang et al. 2015). Over 170 insecticides based on entomopathogenic fungi (mycoinsecticides) have been registered worldwide, with most using the fungi *Metarhizium anisopliae* sensu lato (s.l.) (Metschnikoff) Sorokin or *Beauveria bassiana* s.l. (Balsamo-Crivelli) Vuilleman (de Faria and Wraight 2007).

Many published studies have confirmed the susceptibility of lesser mealworm to specific isolates of both *M. anisopliae* s.l. and *B. bassiana* s.l. (Chernaki-Leffer et al. 2007; Gindin et al. 2009; Rohde et al. 2006; Santoro et al. 2008). This susceptibility is affected by many external factors including ambient conditions, the formulation in which the fungi are applied, and the substrate to which the fungi are applied (Alexandre et al. 2006; Alves et al. 2008; Crawford et al. 1998; Geden et al. 1998; Gindin et al. 2009; Steinkraus et al. 1991). Substrates inside broiler houses, like soil and litter, are potential sites for application of fungi to control the lesser mealworm populations, but they have several properties that potentially affect fungal efficacy including incompatible pH levels, fungi-static volatiles, and variable particle size (Alves et al. 2008; Crawford et al. 1998; Garbeva et al. 2011; Geden et al. 1998).

In the present study, several mycoinsecticide formulations based on two isolates of the fungi *M. anisopliae* or *B. bassiana* were developed for control of lesser mealworm larvae. Larvae were targeted because they are the most prevalent life-stage in meat-chicken broiler houses in Australia (Lambkin et al. 2007), where the study was conducted. A number of oil-based and granular carriers for the fungal conidia were tested, with emphasis on producing economical and practical formulations for application in a prospective broiler house field trial. The most efficacious formulations of each isolate were then tested on a number of substrates of different pH and moisture content collected from broiler houses, to assess their compatibility. In addition, the formulations were tested for efficacy under temperatures analogous to those in broiler houses.

Materials and methods

Fungal isolates

Isolates B27 and M81 were used in this study to generate mycoinsecticide formulations of *B. bassiana* and of *M. anisopliae*, respectively, because of their high virulence to lesser mealworm larvae in preliminary studies (Supplementary Table S1). The isolates were kept in a reference collection at the Ecosciences Precinct (Brisbane, Australia) and lodged with the Queensland Plant Pathology Herbarium (accession numbers BRIP#61361 and BRIP#61339 for B27 and M81, respectively). The putative identities of the isolates were confirmed via molecular characterisation: B27 was confirmed *Beauveria bassiana* sensu stricto (s.str.) by partial sequences of both the loci translation elongation factor-1α *TEF* (GenBank accession # MK532368) (Rehner et al. 2011) and the nuclear intergenic region *Bloc* (GenBank accession # MK573630) (Bischoff et al. 2006), and M81 was confirmed *Metarhizium anisopliae* s.str. by *TEF* alone (GenBank accession # MK532369).

Isolate production

Conidial powder of both isolates was produced for tests, using similar methods: Yeast-peptone broth (BD DIFCO^{TM}) was inoculated with conidia and incubated for five days at 28 °C on an orbital shaker (120 rpm). Culture bags (Unicorn Bio control bags, UNICORN Imp & Mfg Corp.) containing 1.5 kg sterilised chicken feed pellets for M81 (Ridley Agri Products PTY LTD) or 1.5 kg of rolled oats (Uncle Toby's PTY LTD) for B27 were inoculated with 150 ml of the liquid culture. Oats and chicken feed pellets were used because they were potential substrates for mycoinsecticide granules; M81 grew better on the pellets, and B27 only grew on the oats. After inoculation, bags were incubated at 28 °C for seven days after which the solid cultures were broken up and incubated a further 14 days. The solid cultures were transferred to paper bags to dry for 4–5 days at 19 °C in a de-humidified room. After drying, conidia were harvested from the solid cultures using a Myco-harvester 6^{TM} (VBS Ltd,

UK). Conidia powder was stored at 4 °C until required (≈ 6 months). Average percent viability and conidia per gram of powder respectively were estimated at 88.7% and 5.7×10^{10} for M81 and 99.5% and 1.34×10^{11} for B27 using methods of Goettel and Inglis (1997).

Test insects

The lesser mealworm larvae used in the tests came from a laboratory strain held at the Ecosciences Precinct and were cultured as described in Rice and Lambkin (2009). The instars of the larvae (4–5th instar) used in the tests were determined by using the lifetables generated by Wilson and Miner (1969).

Mycoinsecticide formulations and tests

Oil-based formulations

Isolates M81 and B27 were each formulated in canola oil (Crisco®, Goodman Fielder Pty Ltd) and in an aqueous oil emulsion (Codacide™ 860 g l^{-1} organic oil adjuvant, Kendon Chemical and Manufacturing Pty Ltd) at a rate of 0.01 g conidia ml^{-1}. A 2.5% Codacide™ emulsion in tap water was used for M81 and a 5% emulsion was used for B27, due to differences in the wettability of their conidia.

Oil-based formulation tests

Oil-based formulations of M81 were evenly applied to filter papers (55 mm Whatman No.1) in Petri dishes (60 mm) at a rate of 0.125 ml per paper (0.5 g conidia/53 ml m^{-2}), using a micropipette. Five replicates were prepared for each M81 formulation, for the positive controls (oil carriers only, no conidia), and for a negative control (untreated filter paper). Five replicates of an application of pure M81 conidial powder at 1.25 mg per filter paper (0.5 g m^{-2}) were also included for comparison. After treatment, the wet filter papers were dried in a fume hood for 2 h before use in tests. Groups of ten larvae (4–5th instar) and one chicken feed pellet for food were added to the Petri dishes, which were then sealed with rubber bands, and incubated at 30 °C and 55% RH (TRH-460 environmental cabinet, Thermoline Scientific™) in semi-darkness. Larvae were assessed for mortality at days 7 and 11. Another oil-based formulation test was performed as described, but with isolate B27 in place of M81. Tests with both isolates were conducted twice.

Granular formulations

The media used to produce granular formulations were prepared two ways: (1) by growing solid cultures of M81 and B27 each to maturity on chicken feed pellets and rolled oats, respectively, as previously described (see "Isolate production") and (2) by mixing harvested dry conidia (40 g) of M81 or B27 with fresh chicken feed pellets (300 g). Samples (100 g) of each medium were modified by sieving (hard or soft) to remove loose conidia and then ground in a blender or left whole to produce a variety of granular formulations for testing (Table 3). The hard sieving was undertaken using a 1.15 mm pore-size sieve (Endecotts LTD, London) on an automated vibratory sieve shaker (Analysette Spartan 3 Pulverisette™, Fritsch International Pty Ltd) operating for 20 min. Soft sieving was done manually with a 1 mm pore-size hand sieve for 5 min. Blending (Breville® blender, BBlL300) was performed until samples consisted of homogenous fine particles.

Granular formulation tests

The granular formulations were applied evenly onto filter papers (55 mm Whatman No. 1) in Petri dishes (60 mm) at a rate of 0.15 g per paper (54 g m^{-2}). Positive controls with blank granules (no conidia) and a negative control (untreated filter paper) were included. Five replicates were used with all treatments. Larvae (4–5th instar) and feed pellets were added to the Petri dishes, which were then sealed and incubated as previously described (see "Oil-based formulation tests"). Mortality was assessed at days 7 and 11. The testing of granular formulations was conducted sequentially over three assays, with the outcomes of each assay informing the methodology of the next assay. Assays 1 and 2 assessed M81 whole and ground formulations, respectively, and assay 3 assessed B27 granular formulations. Assays were each conducted twice.

Conidia quantities in granular formulations

Estimations of the quantities of conidia per mass of each granular formulation were made using the method of Goettel and Inglis (1997). Approximately 0.1 g of formulation was suspended in 10 ml of 0.1% Tween® 80 (Merck Pty Ltd). The suspension was pipetted onto a haemocytometer and then the number of conidia counted. Conidia per ml of solution was determined and subsequently the number of conidia per gram. The procedure was conducted three times for each granular formulation.

Evaluation of granular formulations on broiler house substrates

Substrates

The granular formulations of M81 and B27 based on admixed soft-sieved ground pellets were considered the most promising and were selected for testing against larvae on a number of substrates sampled from a broiler house complex in Carbrook, Queensland (Table 1). For each substrate, ≈ ten sub-samples of variable volume were taken randomly from across several broiler houses at the complex and combined as one sample. Substrates were stored in sealed plastic bags at 5 °C until use.

Substrate pH and moisture content

The pH of each substrate was determined by making five replicate dilutions in tap water (1:5 w:w), agitating the suspensions for 1 h on an orbital shaker, and then taking measurements using a pH meter (Rayment and Higginson 1992). The moisture content of each substrate was determined by heating five replicate samples (1 g) of each substrate in glass Petri dishes until dry (24 h at 100 °C), allowing them to cool in a desiccator for 1 h, and then measuring their dry mass.

Substrate tests

Substrates (10 ml quantities) were evenly distributed in 60 mm Petri dishes, and treated with M81 or B27 granular formulations (admixed soft-sieved ground pellets) as previously described (see "Granular formulation tests"). Positive controls were blank granules (no conidia) applied to empty plates and to substrates. Five replicates were used for all treatments. Larvae (4–5th instar) and feed pellets were added to the Petri dishes, which were then sealed and incubated as previously described (see "Oil-based formulation tests"). Mortality was assessed at Day 7 and 11. The test was conducted twice.

New litter comparison tests

The results of the initial substrate assays indicated that the B27 granular formulation performed poorly on new litter (A). To confirm this result, another sample of new litter (B) was taken from the broiler houses at the next flock for comparison to new litter (A). In addition, a commercial pine wood shaving product (Hysorb®, East Coast Wood shavings™) purchased

Table 1 Broiler house substrates used in tests of granular formulations of isolates M81 (*M. anisopliae*) and B27 (*B. bassiana*) against lesser mealworm larvae

Substrate	Description
New litter (A)	First sample of freshly spread bedding litter (composite of soft and hard wood shavings) taken from the floor before flock placement in the broiler house
New litter (B)[a]	Second sample of freshly spread bedding litter (composite of soft and hard wood shavings) taken before flock placement in the broiler house
Soil	Floor soil recently washed and disinfected (≈ 48 h ago) in situ by the broiler company as part of broiler husbandry practices. Washing was done with a hose using label rates of Total Kleen™, Sea Jay Industries PTY LTD and disinfected by boom spray with Bactercide liquid Sanitiser™(active ingredients 15% Glutaraldehyde/10% quaternary ammonium) Chemical Systems Australia PTY LTD
Used litter	bedding litter sampled from the floor of a broiler house with a 56 day-old flock

[a]New litter sample (B) was taken from a subsequent flock to new litter (A), to compare test results obtained with new litter (A)

from a pet supplier was also included in the test to provide a sample of analogous bedding litter that had never been exposed to a broiler house. The moisture content and pH of new litter (B) and the pine wood shaving product were measured as previously described (see "Substrate pH and moisture content"). The two samples of new litter (A and B) and the pine wood shaving product were then compared in assays testing the B27 granular formulation against larvae, using the same assay method as previously described (see "Substrate tests"). The test was conducted twice.

Heat-treated new litter tests

The efficacy of the B27 granular formulation was much greater on new litter (B) than on new litter (A), and the moisture contents and pH levels of new litter (A) (40% and 3.45) and new litter (B) (18% and 4.47) were found to be different. To help demonstrate if pH or moisture content alone were contributing to the difference in the granular formulation's efficacy, the new litters (A) and (B) were heat-treated until dry (100 °C for 24 h) to standardise their moisture content. The dried new litters (A) and (B) were then compared in assays testing the B27 granular formulation against larvae, using the same assay method as previously described (see "Substrate tests"). The test was conducted twice.

Efficacy of granular formulations at broiler house temperatures

The granular formulations of M81 and B27 based on admixed soft-sieved ground pellets were tested against lesser mealworm larvae at 25 and 35 °C. These temperatures were found to be the average median and maximum broiler-house temperatures as measured over five flocks in two commercial broiler houses in Carbrook, Queensland, Australia. Granular formulations were applied evenly to filter paper in Petri dishes as previously described (see "Granular formulation tests"). A negative control consisting of untreated filter was included. Five replicates were used for all treatments. Larvae (4–5th instar) and feed pellets were added to the Petri dishes, which were then sealed and incubated as previously described (see "Oil-based formulation tests"). Mortality was assessed at days 7 and 11. After day 11, the larvae were discarded and the Petri dishes of granular formulations were kept and re-tested with new batches of larvae at 30 °C, to measure if the previous thermal exposure affected the efficacies of the granular formulations. Mortality was assessed at days 7 and 11. Tests were conducted twice.

Statistical analysis

The binary survival data from replicate tests were pooled and subjected to a generalised linear model (McCullagh and Nelder 1989) using the Binomial distribution and logit link, using GenStat (2016). Adjusted mean proportions, and their SE, were estimated. Mean counts were compared using Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

The masses of conidia in granular formulations were compared by one-way ANOVA using (GenStat 2016). Mean masses were compared using Fisher's protected LSD test ($P \leq 0.05$).

Results

Mycoinsecticide formulation tests

Oil-based formulations

There was a significant treatment effect with the M81 oil formulations at day 7 (deviance $\text{ratio}_5 = 20.83$, $P < 0.001$) and Day 11 (deviance $\text{ratio}_5 = 47.26$, $P < 0.001$). At day 7, the observed mortality in all treatments was below 50%, and the M81 Codacide formulation was not significantly different to the carriers alone (Table 2). At day 11, M81 conidial powder had the highest mortality value, but was not significantly different to the M81 canola formulation. The M81 Codacide formulation was not significantly different to the canola carrier alone at day 11. There was a significant treatment effect with the B27 oil formulations at day 7 (deviance $\text{ratio}_5 = 68.19$, $P < 0.001$) and day 11 (deviance $\text{ratio}_5 = 64.6$, $P < 0.001$). All the formulations of B27 were significantly different to the controls by day 7, with the conidia powder most effective (Table 2). By day 11, all B27 formulations had produced high mortality, with B27 Codacide not significantly different to B27 powder. Control mortality was high in the negative control.

Table 2 Mean percent mortality (± SE) at day 7 and day 11 for 4–5th instar lesser mealworm larvae exposed to isolate M81 (*M. anisopliae*) and to B27 (*B. bassiana*) in oil-based formulations (canola oil, and Codacide™ emulsion) and conidial powder applied to filter paper at 0.5 g conidia/53 ml m^{-2} and 0.5 g conidia m^{-2}, respectively

Isolate	Treatments	Day 7		Day 11	
M81	No treatment	7^{a}	(± 2.55)	13^{a}	(± 3.35)
	Codacide	6^{a}	(± 2.37)	5^{a}	(± 2.18)
	Canola	7^{a}	(± 2.55)	17^{ab}	(± 3.73)
	M81 powder	46^{b}	(± 4.98)	83^{c}	(± 3.73)
	M81 + codacide	12^{a}	(± 3.25)	32^{b}	(± 4.62)
	M81 + canola	41^{b}	(± 4.91)	67^{c}	(± 4.66)
B27	No treatment	15^{a}	(± 3.56)	39^{b}	(± 4.87)
	Codacide	9^{a}	(± 2.85)	28^{b}	(± 4.85)
	Canola	8^{a}	(± 2.71)	22^{a}	(± 4.14)
	B27 powder	94^{c}	(± 2.37)	100^{d}	(± 0.01)
	B27 + codacide	69^{b}	(± 4.41)	99^{d}	(± 0.99)
	B27 + canola	73^{b}	(± 4.59)	87^{c}	(± 0.34)

Mortality data for each day was analysed separately for each isolate. Means with the same letter within days are not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Granular formulation tests

In assay 1, M81 tested as whole chicken feed pellet formulations produced a significant treatment effect against larvae at day 7 (deviance $\text{ratio}_3 = 43.74$, $P < 0.001$) and at day 11 (deviance $\text{ratio}_3 = 46.22$, $P < 0.001$). The admixed soft-sieved pellets were the most effective on both assessment days (Table 3). There was no significant difference in efficacy between production hard-sieved pellets and admixed hard-sieved pellets on either assessment day.

In assay 2, M81 tested in ground-pellet formulations produced a significant treatment effect against larvae at day 7 (deviance $\text{ratio}_2 = 57.15$, $P < 0.001$) and at Day 11 (deviance $\text{ratio}_2 = 67.92$, $P < 0.001$). An admixed hard-sieved formulation was not tested in assay 2 because this type showed no significant difference to production hard-sieved formulation in Assay 1 (Table 3). There was no significant difference in efficacy between production ground pellets and admixed ground pellets on either day.

In assay 3 of the granular formulation tests, isolate B27 in ground oat and ground pellet formulations produced a significant treatment effect against larvae at day 7 (deviance $\text{ratio}_5 = 97.35$, $P < 0.001$) and day 11 (deviance $\text{ratio}_5 = 96.27$, $P < 0.001$). There was no significant difference between the soft sieve pellet formulation and the no sieve oat formulation at both days (Table 3).

There was considerable variation between the different granular formulations with respect to conidial content ($F_{5,11} = 71.47$, $P < 0.001$) (Table 3). Hard-sieved production formulations of M81 on pellets (assays 1 and 2) contained significantly more conidia per gram than the equivalent hard-sieved production formulations of B27 grown on oats (assay 3). The soft-sieved admixes of M81 and B27 isolates on pellets were not significantly different in conidia per gram (assays 2 and 3). As expected, the no-sieve ground oats with B27 had the largest amount of conidia per gram (assay 3).

Broiler house substrates

Substrate tests

The admixed soft-sieved ground pellet granular formulations of M81 and B27 applied to a variety of broiler house substrates produced a significant treatment effect against larvae at day 7 (deviance $\text{ratio}_{11} = 68.16$, $P < 0.001$) and day 11 (deviance $\text{ratio}_{11} = 68.97$, $P < 0.001$). Both the M81 and B27 formulations performed effectively on soil producing mortalities ≥ 90% at day 7 (Table 4). The M81 formulation was least effective on used litter, but still produced significant mortality. There was no significant difference in efficacy between B27 formulations on soil, used litter, and alone, either assessment day. A disparate result occurred on new litter (A), where the M81 formulation produced the highest mean mortality and B27 formulation the lowest (apart from controls). The mean moisture content and pH of new litter (A) were the most numerically different to the other substrates (Table 4). By day 11, the mortalities had increased and the effects of the isolate formulations were not significantly different on most substrates, with B27 on new litter least effective (Table 4).

New litter comparison tests

The granular formulation of B27 applied to new litter (A) and (B) and wood shavings substrates produced a significant treatment effect against larvae at both day 7

Table 3 Mean percent mortality (± SE) at day 7 and day 11 for 4–5th instar lesser mealworm larvae exposed to isolates M81 (*M. anisopliae*) and B27 (*B. bassiana*) in a range of granular formulations based on modified chicken feed pellets and rolled oats, each applied to filter paper at 54 g m^{-2}, and the mean mass of conidia (± SE) per 100 g for each formulation

Assay	Treatments (formulations)	Mean g conidia per 100 g		Day 7		Day 11	
1	No treatment	–		4^{a}	(± 1.96)	12^{a}	(± 3.24)
	M81 production hard-sieved whole pellets	0.24^{b}	(± 0.03)	42^{b}	(± 4.88)	69^{b}	(± 4.61)
	M81 admixed hard-sieved whole pellets	0.09^{a}	(± 0.02)	36^{b}	(± 4.75)	61^{b}	(± 4.86)
	M81 admixed soft-sieved whole pellets	0.32^{bc}	(± 0.02)	77^{c}	(± 4.17)	87^{c}	(± 3.34)
2	No treatment	–		6^{l}	(± 2.36)	14^{l}	(± 3.46)
	M81 production hard-sieved ground pellets	0.24^{b}	(± 0.03)	67^{m}	(± 4.54)	87^{m}	(± 3.33)
	M81 admixed soft-sieved ground pellets	0.32^{bc}	(± 0.02)	63^{m}	(± 4.65)	74^{m}	(± 4.36)
3	No treatment	–		5^{z}	(± 2.18)	27^{y}	(± 4.39)
	Ground oats	–		7^{z}	(± 2.55)	11^{z}	(± 3.11)
	Ground pellets	–		9^{z}	(± 2.86)	15^{z}	(± 3.54)
	B27 production no-sieve ground oats	0.96^{d}	(± 0.08)	95^{w}	(± 2.17)	100^{w}	(± 0.02)
	B27 production hard-sieve ground oats	0.09^{a}	(± 0.004)	68^{y}	(± 4.66)	93^{x}	(± 2.54)
	B27 admixed soft-sieved ground pellets	0.36^{c}	(± 0.008)	97^{w}	(± 1.68)	98^{xw}	(± 1.39)

Masses with the same superscript letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Mortality data for each day in each assay was analysed separately. Means with the same letter within days and assays are not significantly different according to Fisher's LSD test ($P \leq 0.05$)

– No conidia applied

Table 4 Mean percent mortality (± SE) at day 7 and day 11 for 4–5th instar lesser mealworm larvae exposed to isolates M81 (*M. anisopliae*) and B27 (*B. bassiana*) in granular formulations (chicken feed pellet) each applied at 54 g m^{-2} to broiler house substrates of different mean (± SE) moisture content (%) and pH

Substrate	% Moisture		pH		Day 7		Day 11	
No substrate	na				6^{a}	(± 2.37)	17^{a}	(± 3.73)
Soil	16.17	(± 0.10)	6.38	(± 0.15)	4^{a}	(± 1.96)	14^{a}	(± 3.45)
New litter (A)	41.03	(± 0.01)	3.45	(± 0.02)	8^{a}	(± 2.71)	17^{a}	(± 3.73)
Used litter	14.65	(± 0.05)	5.94	(± 0.02)	9^{a}	(± 2.86)	21^{a}	(± 4.04)
M81 no substrate	na				71^{cd}	(± 4.53)	82^{c}	(± 3.82)
M81 + soil	*				90^{ef}	(± 2.99)	96^{d}	(± 0.02)
M81 + new litter (A)	*				94^{f}	(± 2.37)	96^{d}	(± 1.96)
M81 + used litter	*				63^{b}	(± 4.82)	82^{c}	(± 3.82)
B27 no substrate	na				89^{ef}	(± 3.12)	98^{d}	(± 1.39)
B27 + soil	*				92^{ef}	(± 2.71)	100^{d}	(± 0.04)
B27 + new litter	*				36^{b}	(± 5.62)	57^{b}	(± 4.89)
B27 + used litter	*				82^{de}	(± 3.84)	97^{d}	(± 1.70)

Mortality data for each day was analysed separately. Means with the same letter within days are not significantly different according to Fisher's LSD test ($P \leq 0.05$)

na not applicable

*Moisture content and pH as per substrate alone

(deviance $\text{ratio}_5 = 51.77$, $P < 0.001$) and day 11 (deviance $\text{ratio}_5 = 61.03$, $P < 0.001$). The formulation was most effective on new litter (B) and pine wood shavings, with mortalities > 90% by day 11 (Table 5, Section 5a). The B27 formulation on new litter (A) performed similar to the initial tests, with

lower mortalities at both days, particularly day 7. The mean moisture content of new litter (A) was over twice that of new litter (B) and over four-fold the value for wood shavings (Table 5). The mean pH value of new litter (A) was the most acidic.

Heat-treated new litter tests

There was a significant treatment effect against larvae at both day 7 (deviance ratio$_3$ = 43.4, $P < 0.001$) and day 11 (deviance ratio$_3$ = 59.65, $P < 0.001$) when the B27 granular formulation was applied to heat-treated new litter (A) and (B). There was no significant difference in the efficacy of B27 formulation on either new litter (A) or (B), despite differences in pH before and after heat treatment (Table 5, Section 5b).

Efficacy of granular formulations at broiler house temperatures

There was a significant treatment effect against larvae at day 7 (deviance ratio$_5$ = 40.82, $P < 0.001$) and day 11 (deviance ratio$_5$ = 49.78, $P < 0.001$) when the B27 and M81 formulations were tested at 25 and 35 °C. However, only the effect of the formulations assayed at 25 °C was significantly different to the untreated (Table 6, Section 6a). When the granules tested at 25 and 35 °C were re-tested with new larvae at 30 °C, there was a significant treatment effect at day 7 (deviance ratio$_5$ = 42.55, $P < 0.001$) and day 11 (deviance ratio$_5$ = 44.27, $P < 0.001$). There was no significant difference between the sets of formulations within fungal species at either day 7 or 11 (Table 6, Section 6b). Control mortality was higher in the granules previously tested at 25 °C.

Discussion

The oil-based formulations with B27 and M81 tested in the present study were all effective against lesser mealworm larvae, except M81 in Codacide™. In the literature, most of the wet formulations tested against lesser mealworm have been aqueous emulsions with surfactants Tween® or Triton™ X and were primarily used for susceptibility testing (Geden et al. 1998; Rohde et al. 2006; Steinkraus et al. 1991). Oil-based emulsions are thought to offer an advantage over pure aqueous due to greater adhesion of conidia to lipophilic insect cuticles and increased conidial persistence (Prior et al. 1988). Unformulated conidial powder of M81 and B27 used as comparative treatments in the oil formulation tests performed as well as the best oil formulations and produced the highest mortality values. Other research comparing dry and wet formulations applied to surfaces found that dry

Table 5 Mean percent mortality (± SE) at day 7 and day 11 for 4–5th instar lesser mealworm larvae exposed to isolate B27 (*B. bassiana*) in a granular formulation (chicken feed pellet) applied at 54 g m^{-2} to three substrates of different mean (± SE) moisture content (%) and pH (section 5a) and to the new litter substrates with standardised moisture content via heat-treatment (HT) (section 5b)

Section	Substrate	% moisture		pH		Day 7		Day 11	
5a	New litter (A)	41.03	(± 0.01)	3.45	(± 0.02)	3^{a}	(± 1.70)	26^{a}	(± 4.39)
	New litter (B)	18.19	(± 0.09)	4.47	(± 0.02)	8^{ab}	(± 2.71)	19^{a}	(± 3.92)
	Wood shavings	9.10	(± 0.10)	4.63	(± 0.02)	16^{b}	(± 3.66)	20^{a}	(± 0.04)
	B27 + new litter (A)	*				38^{c}	(± 4.84)	71^{b}	(± 4.54)
	B27 + new litter (B)	*				74^{d}	(± 4.38)	93^{c}	(± 2.55)
	B27 + wood shavings	*				77^{d}	(± 4.20)	95^{c}	(± 2.18)
5b	HT new litter (A)	0		3.36	(± 0.02)	14^{z}	(± 3.45)	25^{z}	(± 4.27)
	HT new litter (B)	0		4.29	(± 0.02)	9^{z}	(± 2.85)	28^{z}	(± 4.42)
	B27 + HT new litter (A)	*				61^{y}	(± 4.82)	90^{y}	(± 3.09)
	B27 + HT new litter (B)	*				67^{y}	(± 4.65)	89^{y}	(± 3.09)

Mortality data for each day in each section was analysed separately. Means with the same letter within days are not significantly different according to Fisher's LSD test (P ≤ 0.05)

*Moisture content and pH as per substrate alone

Table 6 Mean percent mortality (± SE) at day 7 and day 11 for 4–5th instar lesser mealworm larvae exposed to isolates M81 (*M. anisopliae*) and B27 (*B. bassiana*) in granular formulations (chicken feed pellet) applied at 54 g m^{-2} to filter paper, at two temperatures (25 and 35 °C) (section 6a) and for a new batch of 4–5th instar larvae exposed to the same formulations previously tested at 25 and 35 °C, re-tested at 30 °C (section 6b)

Section	Treatment	Test temperature (°C)		Day 7		Day 11	
6a	No treatment	25		11^{a}	(± 3.13)	12^{a}	(± 3.25)
	M81	25		54^{b}	(± 4.98)	80^{c}	(± 0.04)
	B27	25		83^{c}	(± 3.75)	98^{d}	(± 1.69)
	No treatment	35		13^{a}	(± 3.36)	33^{b}	(± 4.70)
	M81	35		15^{a}	(± 3.57)	38^{b}	(± 4.85)
	B27	35		16^{a}	(± 3.67)	30^{b}	(± 4.58)
		New test temperature (°C)	Previous test temperature (°C)				
6b	No treatment	30	25	18^{y}	(± 3.83)	42^{y}	(± 4.92)
	M81	30	25	44^{y}	(± 4.95)	75^{x}	(± 4.32)
	B27	30	25	79^{d}	(± 4.06)	97^{w}	(± 1.68)
	No treatment	30	35	4^{z}	(± 1.96)	18^{z}	(± 3.84)
	M81	30	35	49^{x}	(± 4.98)	70^{x}	(± 4.57)
	B27	30	35	77^{w}	(± 4.19)	91^{w}	(± 2.86)

Mortality data for each day in each section was analysed separately. Means with the same letter within days are not significantly different according to Fisher's LSD test ($P \leq 0.05$)

formulations were often more effective (Alves et al. 2008; Geden et al. 1998; Steinkraus et al. 1991).

Chicken feed pellets and rolled oats were used as media in the granular formulations because they are potential substrates for mass conidia production and sufficiently friable for conversion to granules. Furthermore, both substrates are known food sources for insects, suggesting potential as bait (Jagadeesan et al. 2013; Rice and Lambkin 2009). Ultimately, oats were omitted as potential granules because they were costly and after granulation were too damp and sticky to pass through granule dropping equipment under investigation for field trial. These issues potentially stymied the use of B27 as a granular formulation, since it grew optimally on oats. However, results showed B27 could be harvested from oats and mixed with pellets to create an efficacious formulation. Admixed granular formulations were considered advantageous, as large quantities of formulation could be generated quickly by combining small quantities of conidia with fresh chicken pellets. Using spent production media (post conidia harvest) as formulation would still be a viable option for commercial fungal production systems, as it would be routinely generated in large quantities as a by-product of conidia production.

The granular formulations based on chicken feed pellets were chosen for the broiler-house substrate studies because they were most efficacious, economic, and contained the lowest doses of conidia. Both the granular formulations of M81 and B27 performed very well on soil, despite the soil being previously treated with disinfectant containing active ingredients (glutaraldehyde/quaternary ammonium) known to be biocidal to some fungi (Bundgaard-Nielsen and Nielsen 1995; Gorman and Scott 1977; Terleckyj and Axler 1993). The present results corroborate Alves et al. (2016) who found that poultry house disinfectants reduced germination but did not affect the efficacy of *B. bassiana* in mortality assays.

The B27 formulation showed no discernible reduction in efficacy when applied to used litter. Contrastingly, Alexandre et al. (2006) reported that lesser mealworm larvae dipped in *Beauveria* suspensions had lower mortalities on old litter. Furthermore, Geden et al. (1998) needed a ten-fold dose of *Beauveria* on used litter as they did on soil to achieve similar larval control. The M81 formulation appeared slightly inhibited on used litter, but was still effective. The pH and moisture content of the used litter was similar to those of the soil, suggesting that any inhibition of M81 was caused by other factors.

Volatile levels, particularly high ammonia gas levels often present in poultry litter (Atapattu et al. 2008; Moore et al. 1996), have been shown to be major inhibitors of fungal germination and growth (Bacon 1986; Garbeva et al. 2011; Schippers et al. 1982). Volatile levels were not measured in this study, but should be considered in future work.

The two formulations had contrasting efficacies on new litter, with the M81 granular formulation appearing enhanced and the B27 formulation inhibited. Subsequent tests with heat-treated new litter suggested that higher moisture content in the new litter was the likely reason B27 was inhibited. Given that litter in broiler houses is sometimes wet due to misting or leaks, applying B27 formulation to the litter might compromise its efficacy. Despite the success of the M81 formulation on new litter, soil application seemed best for both granular formulations, due not only to efficacy but also for practicality. Soil applications in the field can be made between flocks, after the broiler house has been cleaned and before the litter is spread. Moreover, current control insecticides for lesser mealworm are applied to soil, so applying fungal treatments there too would make it easier for industry to adopt mycoinsecticides. In addition, formulations applied to floor soil would be covered by the bedding litter, hence minimising exposure of the flock to the formulation. Under bedding litter, the small granules ($\leq$ 3 mm diam.) comprising the formulation would likely breakdown and combine with the soil substrate quickly. However, even if the formulations were accessible to birds, it is not considered a significant risk, as extensive reviews have concluded that both fungi are safe to birds, and to humans (Zimmermann 2007a, b).

High larval mortality was sometimes recorded in controls by day 11 in the present study (e.g. 39% mortality, Table 3). Rueda and Axtell (1996) showed natural mortality for lesser mealworm larvae (all instars) in the laboratory was $\approx$ 27% at 30 °C and $\approx$ 50% RH over a 26-day stage duration. This suggests limitations in assay duration with larvae if useful mortality data are to be obtained. However, the numerous tests in the present study showed significant treatment effects at day 11 and were therefore considered valid.

Air temperatures fluctuate within broiler houses during the time of a chicken flock, but follow predictable patterns influenced mostly by the husbandry. In Australia, the standard commercial broiler-house temperature regime is for air to be heated to a minimum of 32 °C for the new chicks at day 1 of the flock and then lowered 0.5 °C daily until approximately 22 °C, at day 21 (ACMF 2018). The temperature at floor level is comparable to that of the air and follows a similar temporal pattern (Lambkin et al. 2007), but rises slightly in the latter stages of the flock presumably due to increasing bird mass. In the present laboratory studies, the two granular formulations were ineffective at 35 °C but very effective at 25 °C. These temperatures were the average maximum and median temperatures, respectively, recorded over five flocks at two broiler houses from a commercial broiler farm in a parallel study. Retesting of granules exposed to 35 °C at the lower temperature of 30 °C showed they were still active, producing mortality consistent with fresh formulations. These results suggest that initial exposure to the high temperatures early in the flock should not inhibit the overall effectiveness of the formulations. After the short heating period has finished, the granular formulations would return to activity before the larval numbers increase a few weeks into the flock as observed in other studies (Lambkin et al. 2008).

The present study indicated that granular formulations of M81 (*M. anisopliae* s.str.) and B27 (*B. bassiana* s.str.) consisting of ground chicken feed pellets with conidial powder were the most effective and the most practical for application against lesser mealworm larvae. Moreover, these formulations were compatible with broiler house temperatures and substrates. These findings are promising for the poultry industry, as mycoinsecticide granules could be applied quickly, easily, and safely to broiler house floors between flocks, with minimal risk to operators and livestock. The authors' future research entails field-testing both M81 and B27 granular formulations at a broiler house complex in southeast Queensland, Australia, in comparison to industry standard insecticides. A demonstration of the efficacy of these mycoinsecticide formulations in the field could potentially provide the broiler industry with a new tool for lesser mealworm population management.

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Compliance with ethical standards

Conflict of interest The authors S.J. Rice, D.K. Baker, and D.M. Leemon declare that there are no conflicts of interest.

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Steven J. Rice is a technical officer with Agri-Science Queensland. He specialises in the development of management strategies for lesser mealworm populations in poultry houses and is currently developing myco-pesticides for arthropod pests. He has particular interest in the interactions between chemical insecticides and entomopathogenic fungi.

Dalton K. Baker is part of an integrated pest management team within Agri-Science Queensland. He is involved in biological control research of pests in animal production in Australia.

Diana M. Leemon is a principal scientist for bee health and arthropod pest management in Agri-Science Queensland. She leads a research group investigating the non-chemical control of livestock pests and parasites, primarily through the development of myco-pesticides.