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Time–concentration–mortality modeling of the synergistic interaction of *Beauveria bassiana* and imidacloprid against *Nilaparvata lugens*

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Abstract: Interactions between the entomopathogenic fungus, *Beauveria bassiana* SG8702, and imidacloprid on *Nilaparvata lugens* (Homoptera: Delphacidae) were studied in laboratory bioassays by spraying suspensions of unformulated conidia (assay 1) and aqueous dilutions of emulsifiable conidia formulation alone (assay 2) or together with imidacloprid at the sub-lethal rates of 0.5, 1.0 and 2.0 μg ml^{-1} (assays 3–5). Each assay consisted of five conidia concentrations plus an appropriate control and three replicates, each including 30–40 third-instar nymphs, so as to generate time–concentration–mortality data for modeling analysis. A mineral oil-based emulsion used to formulate *B bassiana* slightly enhanced fungal activity but had no significant impact on the background mortality of *N lugens*. On the basis of LC_{50} estimates and associated variances on days 4–12 after spraying, synergistic interactions of both agents or formulations were determined by estimating relative potencies of assay 2 over assay 1 (1.2–9.0), assay 3 over assay 2 (1.3–1.7), assay 4 over assay 2 (7.5–9.6), assay 5 over assay 2 (22.7–101), assay 4 over assay 3 (3.8–5.8), assay 5 over assay 3 (16.1–61.0), and assay 5 over assay 4 (3.0–10.5). The time–concentration–mortality modeling method was not only mathematically but also biologically robust to evaluate the interactions of *B bassiana* and imidacloprid on *N lugens*. Compared with their counterparts, enhanced fungal formulations displayed consistently earlier or greater activities against the pest species based on LC_{50} and LT_{50} estimates determined from their time–concentration–mortality relationships. The results highlight a potential for pest control by combined formulation or application of *B bassiana* and imidacloprid.

Keywords: *Nilaparvata lugens*; *Beauveria bassiana*; emulsifiable conidia formulation; imidacloprid; synergistic interaction; time–concentration–mortality modeling; microbial control

1 INTRODUCTION

Entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) have been studied intensively to develop mycoinsecticides for integration into management systems for sucking insect pests.[1,2] As true insect pathogens, all fungal agents have a latent period of infection to develop in hosts, and thus insects are killed slowly compared with conventional insecticides. Because of their slow action, efforts to formulate fungal agents are often suppressed when market potential is expected. Despite the advantages of fungal formulations in insect control, the fact that fungal agents alone lack knockdown action discourages large input into commercial development.[1–3] Since sprays of chemical insecticides on vegetables, fruits and teas have been strictly restricted in China,[4] it has been necessary to search for alternative measures that may reduce insecticide use. Consequently, the development of mycoinsecticides against sucking insects, such as aphids and whiteflies, is again receiving attention.[5–7]

The integration of fungal biocontrol agents and selected insecticides into formulations can potentially reduce the use and residues of chemical insecticides in agriculture. As a systemic insecticide primarily against sucking insects, imidacloprid has been widely used

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in the last decade[8] and currently is one of very few insecticides recommended for protection of vegetables, fruits and teas from insect damage.[4] However, cautious use of this neonicotinoid compound is necessary because of the potential of target insects to develop resistance[9] and mite fertility stimulation.[10–12] Imidacloprid has proved to be biologically compatible with *B bassiana*[13,14] and may enhance fungal infections to insect pests at low application rates in the laboratory[15–17] or in the field.[18–21] The interactions of fungal agents with imidacloprid in previous reports are often synergistic[15–21] but occasionally antagonistic.[22] This conflict perhaps rises from the lack of a biologically sound method to properly quantify the interactions between fungal agents and imidacloprid on a given target pest.

In this study, the interactions of *B bassiana* and imidacloprid on *Nilaparvata lugens* (Stål), a typical sucking pest not responsive to rice lines transformed with toxin genes of *Bacillus thuringiensis* Berliner,[23] were bioassayed under laboratory conditions and quantitatively analysed on the basis of conventional probit analysis with parallelism test for the slopes of the linear concentration–mortality (CM) relationships[24] and a novel method, more complicated but robust, for time–concentration–mortality (TCM) modeling.[25–27] Inclusion of *B bassiana* in the study is based on its prominent status among fungal biocontrol agents, well-developed technology for mass production and formulation,[3,28] and wide application against sucking insect pests.[1,2]

2 MATERIALS AND METHODS

2.1 Production of *Beauveria bassiana* conidia

Aerial conidia of *B bassiana* were produced on steamed rice[3] using an aphid-derived isolate, SG8702.[29] This isolate has proved to be highly infective to aphids,[5,6] leafhoppers,[20] whiteflies[21] and even spider mites.[30] Rice cultures fully covered with a layer of conidia after 7-day incubation at 25 °C and 12:12 h light:dark photoperiod were dried by ventilation at $\leq$35 °C for 24 h. Conidia were then harvested through a 74-μm vibrating sieve. Harvested conidia were dried to a water content of 4.5% at ambient temperature on a vacuum drier (VirTis Company, Gardiner, NY) and preserved at 4 °C for use. The powdery product of *B bassiana* dried as above in our laboratory always had $>1.2 \times 10^{11}$ conidia g^{-1} with a viability of >90%[20,21] and maintained similar viability for $\geq$12 months if preserved at 4 °C.[31]

2.2 Planthopper stock

A laboratory population of *N lugens* was maintained on rice plants grown in nutrient solution in incubators at 25 °C and 12:12 h light:dark photoperiod. To obtain nymphs for bioassays, five brachypterous adults arbitrarily taken from the population were moved onto *ca* 4-cm-high seedlings on a sponge board floating in a plastic cup (7 cm diameter, 9 cm high) individually caged and filled with nutrition solution in which roots grew, and allowed to freely lay eggs on the seedlings for 3 days. The adults were then removed and the eggs laid on the seedlings maintained under the same regime until they had developed to third-instar nymphs (*ca* 20 days after adult removal). At that time, 30–40 nymphs were transferred to new seedlings (*ca* 3 cm high) from the old plants in each cup. Large and small nymphs were discarded to minimize age variation among the nymphs to be assayed.

2.3 Bioassays

Five bioassays (assays 1–5) were conducted. Each assay included five concentrations plus a corresponding control and was replicated three times (30–40 nymphs per replicate per concentration treatment). Assay 1 used aqueous *B bassiana* suspensions (1×10^8, 5×10^7, 1×10^7, 5×10^6 and 1×10^6 conidia ml^{-1}) containing 0.2 g $litre^{-1}$ Tween-80 (the control, denoted by CK_1, was 0.2 g $litre^{-1}$ Tween-80 only). Assay 2 included treatments of 100-fold aqueous dilutions of emulsifiable formulations (1×10^{10}, 5×10^9, 1×10^9, 5×10^8 and 1×10^8 conidia ml^{-1}) of *B bassiana* conidia in a mineral oil (paraffin)-based liquid (MOBL) containing 50 g $litre^{-1}$ emulsifier (a technical preparation provided by Xiaoshan Chemical Additives Co, Hangzhou, China), 3 g $litre^{-1}$ suspension stabilizer (sodium carboxymethyl cellulose) and 1 g $litre^{-1}$ UV protectant (vitamin C).[32] In assays 3–5, 100-fold aqueous dilutions of emusifiable formulations of conidia (2×10^{10}, 1×10^{10}, 5×10^9, 1×10^9 and 5×10^8 conidia ml^{-1}) were separately supplemented with imidacloprid at the rates of 0.5, 1.0 and 2.0 μg ml^{-1}. The control for assay 2 (CK_2) was 100-fold MOBL aqueous dilution containing neither conidia nor imidacloprid, and those for assays 3–5 (CK_3, CK_4 and CK_5) were MOBL aqueous dilutions containing imidacloprid at 0.5, 1.0 and 2.0 μg ml^{-1}, but no conidia. All emulsifiable formulations were prepared the day before each assay began but diluted immediately before spraying.

In each assay, prepared suspensions or aqueous dilutions were sprayed, from low to high concentrations, onto nymphs dwelling on seedlings in the cups. To ensure that sprays were even, three cups (replicates at a given concentration) were gently placed on the bottom of a large bucket (48 cm diameter and 60 cm high), uncaged and exposed to a spray from a hand-held Micro Ulva sprayer (Micron Sprayers Limited, Bromyard, Herefordshire, UK). The sprayer was held 1 m above the bucket bottom and operated at 11 000 rev min^{-1}, yielding a mist of 50–60 μm droplet diameter (manufacturer's guidance). The exposure lasted for 5 s, followed by 3-min deposition. Cups were then gently removed from the bucket and caged again before being moved to incubators at 25 °C and 12:12 h light : dark photoperiod. All treated nymphs were examined daily and survival and death records made. Cadavers which appeared in the cages were removed and transferred to moist Petri dishes for

3 days or longer. Deaths were attributed to fungal infection if cadavers had outgrowths of *B bassiana*.

To determine the concentration of conidia to which nymphs and seedlings were exposed, nine plastic sheets (3×3 cm) were placed in a triangle on the bottom of the bucket to collect conidia while the nymphs were exposed to a spray. The sheets were then washed twice in a total volume of 3 ml of 0.2 g litre^{-1} Tween-80, which was then centrifuged at 10 000 rev min^{-1} for 10 min. The deposits were resuspended in 0.2 ml of 0.2 g litre^{-1} Tween-80 and three 10-µl samples were pipetted into counting chambers of a standard hemocytometer for counts of conidia under a microscope. An average conidia concentration under each spray was then estimated as the number of conidia mm^{-2}.

2.4 Data analysis

Bioassay data in the TCM form were analyzed for interactions between *B bassiana* and imidacloprid using two methods. Linear regression of probit-transformed mortality against $\log_{10}$-transformed concentration[24] was performed to generate a CM relationship for each assay and a test for parallelism for the slopes of all assays. If a parallelism hypothesis was accepted, a common slope was estimated and differences between intercepts among the CM relationships were used to illustrate their interactions by computing a LC_{50}, a lethal concentration for *B bassiana* to cause 50% planthopper mortality. Since this classic method ignores the time variable that obviously affects the effect of a given concentration on tested insects, a TCM modeling method[24–27] was used to analyze each of the data sets from assays 1–5. Briefly, with this method the planthopper mortalities (q_{ij}) occurred at a given concentration of *B bassiana* (C_i) at specific time intervals after spray (t_j) were corrected with background mortalities observed from the CK_0 and then fitted to the conditional TCM model

$$(q_{ij} = 1 - \exp[-\exp(\gamma_j + \beta \log_{10}(C_i)])$$

by approaching to a maximum likelihood equation. The β and γ_j estimates were used to determine the cumulative TCM relationships in the form of

$$p_{ij} = 1 - \exp[-\exp(\tau_j + \beta \log_{10}(C_i))]$$

where $\tau_j = \ln(\sum_{k=1}^{j} e^{\gamma_k})$. The parameter estimates and associated variances and covariances were then used to compute the LC_{50} as a function of the time after treatment or LT_{50} (a lethal time for a given concentration to cause 50% planthopper mortality) as a function of the conidial concentration. Finally, the relative potencies of one bioassay over others were calculated as 10^a with approximate 95% confidence limits of $10^{a \pm 2\sigma}$ $\{a = \theta_i - \theta_j, \sigma = [\mathrm{var}(\theta_i) + \mathrm{var}(\theta_j)]^{1/2}$, where θ_i and θ_j were the estimates of $\log_{10}$ (LC_{50}) for paired assays and var(θ_i) and var(θ_j) were the variances associated with θ_i and θ_j}. The LC_{50} estimates for paired assays were significantly different if the lower limits of 95% confidence intervals (CI) exceeded 1.0.[24] All analyses including probit analysis, the TCM modeling and associated computations were performed using DPS software.[33]

3 RESULTS

3.1 Trends in mortalities of planthoppers

The mortality of *N lugens* observed in assays 1–5 was concentration- and time-dependent (Fig 1). Final mortalities observed on day 12 after exposure to *B bassiana* alone or together with imidacloprid were 32–91% in assay 1, 31–88% in assay 2, 29–93% in assay 3, 46–95% in assay 4, and 50–98% in assay 5. Final background mortalities observed in controls differed significantly ($F_{4,8} = 77.17, P < 0.01$) with a maximum of 36.0 (±3.5)% in CK_5, followed by 30.2 (±1.4)% in CK_4. These two observations were significantly greater than those in CK_{1-3} (11.9–16.3%). This indicates that a spray of 100-fold aqueous dilution of the emulsifiable MOBL as a control in assay 2 did not cause significantly higher background mortality than a spray of 0.2 g litre^{-1} Tween-80 in assay 1.

Despite the concentration-dependent mortality trends in assays 1–5 during a 12-day period after sprays (Fig 1a–e), the proportions of cadavers with typical outgrowths of *B bassiana* at five conidia concentrations were 56.6–71.1% in assay 1, 53.4–64.5% in assay 2, 58.2–69.3% in assay 3, 56.4–71.5% in assay 4 and 58.8–74.9% in assay 5. Thus, inclusion of the sub-lethal rates of imidacloprid in sprays of *B bassiana* may elevate the mortality trends of *N lugens* (Fig 1c–e) but did not obviously affect fungal outgrowth pattern on the cadavers killed by the fungal infection.

3.2 The CM relationships

The cumulative mortalities observed at different conidia concentrations on day 12 (p_{i12}) were corrected as $(p_{i12} - p_{ck})/(1 - p_{ck})$ using the corresponding CK_1 or CK_2 mortality (p_{ck}) for assay 1 or assays 2–5. Probit analysis with parallelism test was then run by linearly regressing the probit-transformed corrected mortalities against the $\log_{10}$-transformed conidia concentrations, generating parameter estimates for the CM relationship of each assay (Table 1). Since the parallelism test accepted a hypothesis of parallelism for the slopes ($F_{4,15} = 1.06, P = 0.41$), a common slope of 1.069 (±0.062) was obtained for all the assays. Thus, the estimates of intercepts (2.193–3.742) clarified differences in the CM relationships. The larger the estimated intercept, the higher trend the linear CM relationship. The LC_{50} values on day 12 were then computed as 422, 312, 227, 46 and 15 conidia mm^{-2} for assays 1–5, respectively. Obviously, the lethal effect of unformulated *B bassiana* on *N lugens* was smaller than that of the emulsifiable formulation with

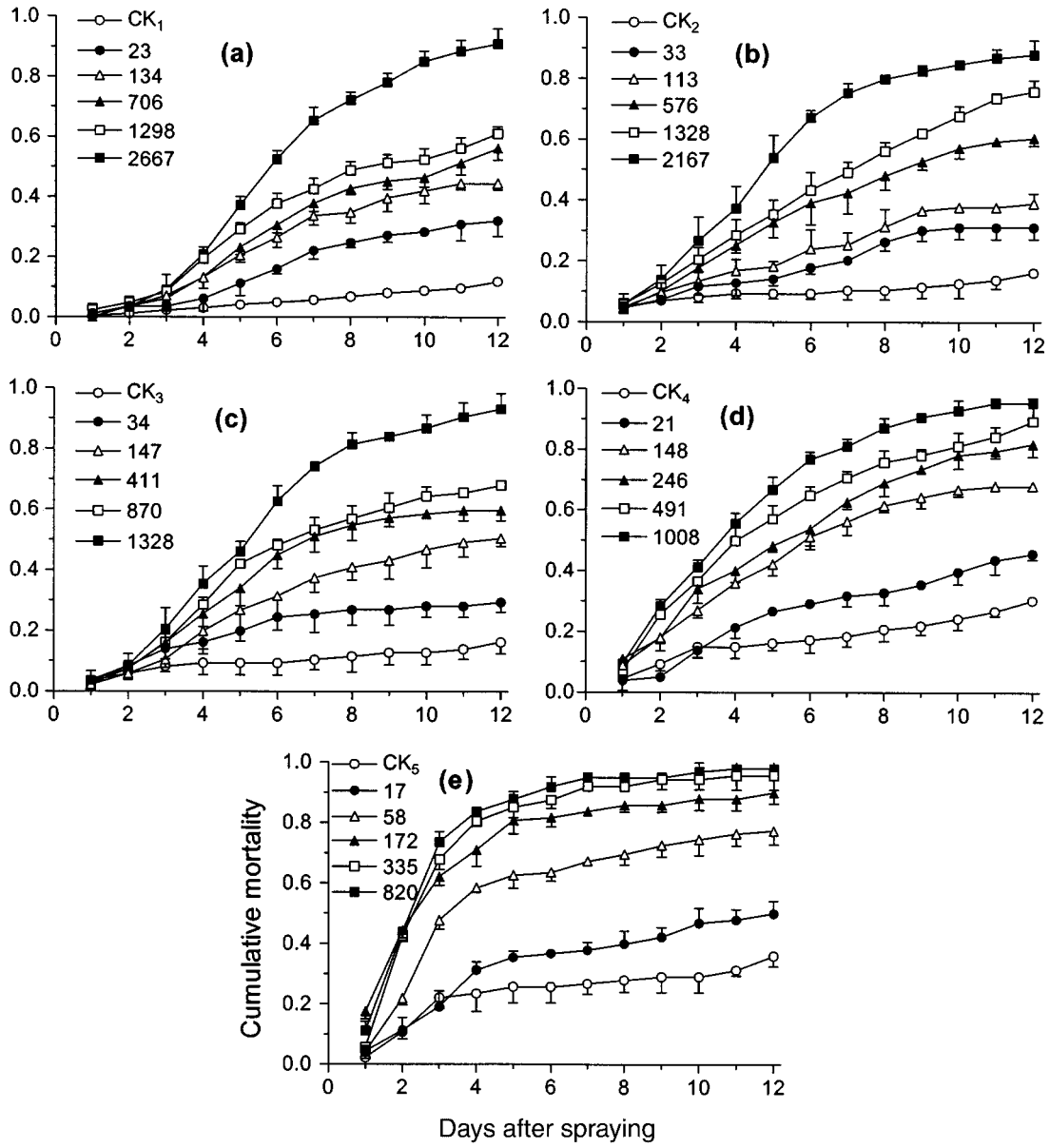

Figure 1. Trends in mean mortalities (±SE) of *Nilaparvata lugens* after exposure to different concentrations (number of conidia mm^{-2}, denoted by symbols) of *Beauveria bassiana* (Bb) alone or together with imidacloprid (Im). (a) Assay 1: Bb suspended in 0.2 g $litre^{-1}$ Tween-80. (b) Assay 2: 100-fold aqueous dilutions (AD) of Bb emulsifiable formulations (BbEF) in mineral oil-based liquid (MOBL). (c) Assay 3: 100-fold AD of BbEF plus Im 0.5 μg ml^{-1}. (d) Assay 4: 100-fold AD of BbEF plus Im 1.0 μg ml^{-1}. (e) Assay 5: 100-fold AD of BbEF plus Im 2.0 μg ml^{-1}. CK_1 = 0.02% Tween-80; CK_2 = 100-fold AD of MOBL; $CK_3 = CK_2$ + Im 0.5 μg ml^{-1}; $CK_4 = CK_2$ + Im 1.0 μg ml^{-1}; and $CK_5 = CK_2$ + Im 2.0 μg ml^{-1}.

MOBL. As imidacloprid in sprays increased from 0.5 to 2.0 μg ml^{-1} (assays 3–5), the LC_{50} greatly decreased, suggesting a positive interaction between the fungal and chemical agents. However, the CM relationships for assays 1 and 3 did not pass the test for goodness of fit ($P < 0.05$), indicating uncontrolled bias in the estimates for both assays.

3.3 The TCM relationships

All data in Fig 1a–e fitted well to the TCM model with an acceptable goodness of fit (Table 2) although the CM relationships based on probit analysis were not acceptable for assays 1 and 3 (Table 1). The estimates of the parameters for the effects of time and concentration and their variances and covariances were used to determine the TCM relationships. The TCM data observed in CK_3, CK_4, and CK_5 (Fig 1c–e) were also well fitted, resulting in a TCM relationship for the sub-lethal application rates of imidacloprid alone. In all TCM modeling analyses, planthopper mortalities at different concentrations at specific time intervals were corrected for the corresponding background mortalities of CK_1 for assay 1 and those of CK_2 for assays 2–5 and the sub-lethal rates of imidacloprid in order to exclude the potential effect of the mineral oil (used to formulate *B bassiana*) on the planthoppers.

Based on the TCM relationships established, the LC_{50} values varying with days after spray were computed for each assay (Fig 2a). Note that the LC_{50} varied in downward curved trends over days after spraying and differed from one assay to another. On

Table 1. Concentration–mortality relationships for *Beauveria bassiana* alone (Bb) or together with imidacloprid (Im) against *Nilaparvata lugens* based on probit analysis (parallelism test for slopes: $F_{4,15} = 1.06$, $P = 0.41$)

Bioassay (Bb + Im μg ml^{-1})	Number of nymphs treated	Intercept	Slope (±SE)	χ^2 ($df = 3$)	P[a]	LC_{50} (Number of conidia mm^{-2}) (95% CL)
Unformulated Bb	499	2.193	1.069 (±0.062)	19.756	0.0002	422 (164–1682)
Formulated Bb	511	2.333	1.069 (±0.062)	4.026	0.2587	312 (222–432)
Formulated Bb + Im 0.5	495	2.483	1.069 (±0.062)	9.862	0.0198	227 (158–311)
Formulated Bb + Im 1.0	489	3.227	1.069 (±0.062)	2.293	0.5138	46 (24–71)
Formulated Bb + Im 2.0	550	3.742	1.069 (±0.062)	0.675	0.8790	15 (6–26)

[a] Homogeneity hypothesis for the goodness of fit was accepted if $P \geq 0.05$.

Table 2. Estimates of parameters obtained for conditional time–concentration–mortality relationships of *Beauveria bassiana* alone (unformulated in assay 1 or formulated in assay 2) or together with imidacloprid at the sub-lethal application rates of 0.5, 1.0 and 2.0 μg ml^{-1} (assays 3–5) against *Nilaparvata lugens*

	Mean (±SE)					
Parameter[a]	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Imidacloprid
$\hat{\beta}$	0.86 (±0.12)	1.07 (±0.13)	1.11 (±0.14)	0.99 (±0.12)	0.98 (±0.10)	2.46 (±0.75)
$\hat{\gamma}_1$	−6.81 (±0.57)	−5.91 (±0.42)	−6.36 (±0.48)	−4.91 (±0.36)	−4.66 (±0.28)	−3.90 (±0.42)
$\hat{\gamma}_2$	−6.00 (±0.45)	−6.28 (±0.45)	−6.77 (±0.52)	−4.69 (±0.36)	−3.47 (±0.25)	−3.32 (±0.34)
$\hat{\gamma}_3$	−6.65 (±0.55)	−5.54 (±0.41)	−5.47 (±0.44)	−4.33 (±0.34)	−3.10 (±0.25)	−3.23 (±0.34)
$\hat{\gamma}_4$	−4.97 (±0.39)	−5.62 (±0.42)	−5.11 (±0.43)	−4.30 (±0.35)	−3.36 (±0.26)	−7.22 (±2.47)
$\hat{\gamma}_5$	−4.58 (±0.38)	−5.28 (±0.41)	−4.86 (±0.42)	−4.28 (±0.35)	−3.74 (±0.29)	−4.39 (±0.60)
$\hat{\gamma}_6$	−4.45 (±0.37)	−4.96 (±0.39)	−4.88 (±0.42)	−4.23 (±0.36)	−4.71 (±0.42)	−5.47 (±1.09)
$\hat{\gamma}_7$	−4.62 (±0.38)	−5.63 (±0.45)	−5.14 (±0.45)	−4.40 (±0.37)	−4.04 (±0.35)	−6.68 (±1.86)
$\hat{\gamma}_8$	−4.83 (±0.41)	−4.93 (±0.39)	−5.28 (±0.46)	−4.27 (±0.38)	−4.71 (±0.46)	−4.06 (±0.51)
$\hat{\gamma}_9$	−5.39 (±0.47)	−5.42 (±0.43)	−5.98 (±0.54)	−4.74 (±0.43)	−4.66 (±0.47)	−6.51 (±1.74)
$\hat{\gamma}_{10}$	−5.43 (±0.49)	−5.63 (±0.47)	−5.87 (±0.53)	−4.50 (±0.41)	−4.03 (±0.37)	−5.30 (±1.03)
$\hat{\gamma}_{11}$	−4.81 (±0.41)	−5.79 (±0.51)	−5.95 (±0.56)	−4.54 (±0.43)	−4.61 (±0.50)	−4.54 (±0.67)
$\hat{\gamma}_{12}$	−5.29 (±0.50)	−7.05 (±0.82)	−6.61 (±0.73)	−4.86 (±0.51)	−5.48 (±0.76)	−4.02 (±0.53)
H-L test[b]	$\hat{C} = 13.29$ $P = 0.10$	$\hat{C} = 3.01$ $P = 0.93$	$\hat{C} = 9.06$ $P = 0.34$	$\hat{C} = 2.90$ $P = 0.94$	$\hat{C} = 15.59$ $P = 0.06$	$\hat{C} = 1.96$ $P = 0.98$

[a] The numbered subscript for $\hat{\gamma}$ represents the specific day after spraying.
[b] Homogeneity hypothesis for the goodness of fit was accepted if $P \geq 0.05$ in the Hosmer–Lemeshow test ($df = 8$ for all tests).

day 12, for example, the LC_{50} with 95% CI was computed as 406 (296–556) conidia mm^{-2} for assay 1, 329 (256–423) for assay 2, 244 (192–310) for assay 3, 44 (30–64) for assay 4, and 15 (9–23) for assay 5. These estimates were well in accord with those determined by the simple CM relationships (Table 1) but had much narrower CIs. The LT_{50} estimates were estimated as a function of conidia concentration for each assay (Fig 2b). A minimum conidia concentration for a computable LT_{50} was 410 conidia mm^{-2} for assay 1, 330 for assay 2, 244 for assay 3, 44 for assay 4, and 15 for assay 5, corresponding well to the LC_{50} estimates on day 12. The LT_{50} estimates decreased with the increasing conidia concentrations within each assay and also were clearly differentiated among the five assays. At 500 and 1000 conidia mm^{-2}, for instance, the LT_{50} estimates were 10.9 and 8.2 days for assay 1, 8.8 and 7.0 days for assay 2, 7.0 and 5.5 days for assay 3, 4.3 and 3.5 days for assay 4, and 2.2 and 1.9 days for assay 5, respectively. Therefore, both LC_{50} and LT_{50} (Fig 2) determined by the TCM relationships demonstrated positive interactions of *B bassiana* with the sub-lethal application rates of imidacloprid against *N lugens*. The interaction was most conspicuous in assay 5, intermediate in assay 4, but relatively weak in assay 3.

Overall, *B bassiana* conidia formulated into the MOBL had a greater effect on *N lugens* than unformulated conidia despite little difference in background mortalities between CK_1 and CK_2. The LC_{50} for the emulsifiable formulation on day 12 was smaller than the corresponding estimate for unformulated *B bassiana* in either the simple CM or the complicated TCM relationships. Adding the sub-lethal rates of imidacloprid to sprays of *B bassiana* further decreased the LC_{50} over days after spray (Fig 2a). Similarly, the formulation also reduced the LT_{50} compared with unformulated *B bassiana* and inclusion of the sub-lethal rates of imidacloprid in the formulation greatly reduced the LT_{50} (Fig 2b).

Of greater interest, the TCM relationship for the sub-lethal sprays of imidacloprid against *N lugens* also led to the estimates of LC_{50} or even LC_{90} over days after spray (Fig 3) although a spray of the aqueous MOBL dilution containing 2.0 μg ml^{-1} imidacloprid (CK_5) caused a maximal mortality of

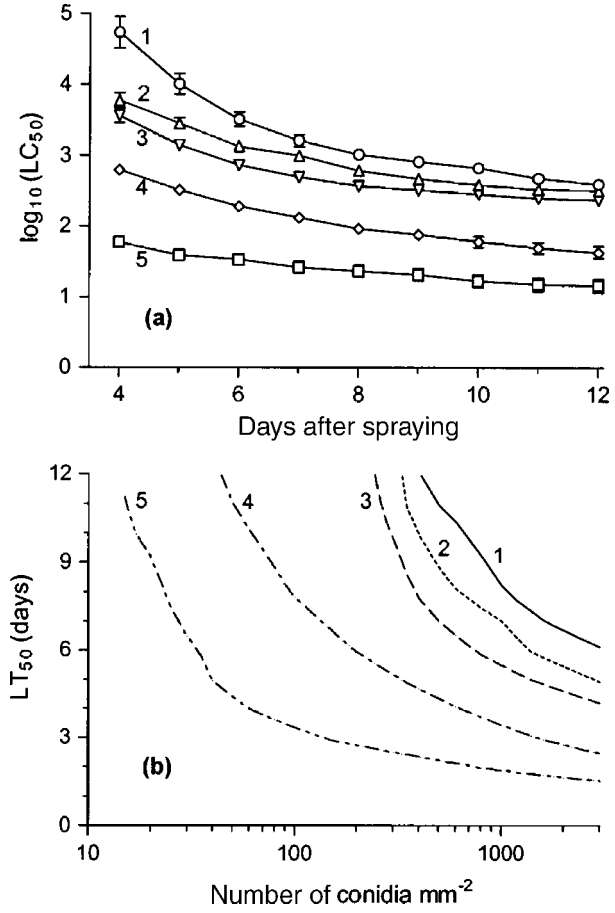

Figure 2. (a) Curved trends in the logarithmic estimates of LC_{50} (bars: SE) over days after spraying (b) LT_{50} at different concentrations of *Beauveria bassiana* conidia on *Nilaparvata lugens* in assays 1–5 (lines 1–5), based on the TCM relationships. See Fig 1 for details of each assay.

36% only (Fig 1e). Probit analysis could not provide such estimates if maximal mortality in an assay was less than 50%.[24] The LC_{50} values (with 95% CIs) determined by this TCM relationship against *N lugens* were 10.5 (4.2–26.3) μg imidacloprid ml^{-1} on day 2, 5.5 (2.9–10.2) on day 6, and 3.8 (2.4–6.1) on day 12. A comparison of the latter LC_{50} with those determined

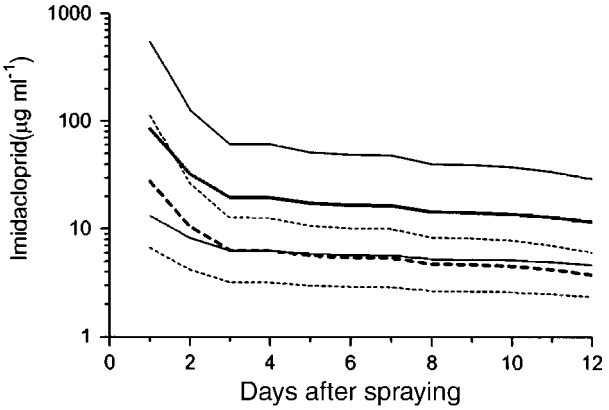

Figure 3. Curved trends in the estimates of LC_{50} (bold dashed line: mean; dashed: 95% CIs) and LC_{90} (bold solid line: mean; solid: 95% CIs) of imidacloprid at the sub-lethal rates of 0.5–2.0 μg ml^{-1} against *Nilaparvata lugens* over days after spraying, based on the TCM relationship for the data of CK_{3-5}.

by the TCM relationships for the interactions between *B bassiana* and imidacloprid (Fig 2a) indicates again that the inclusion of imidacloprid in assays 3–5 at the rates of 0.5–2.0 μg ml^{-1} was responsible for the enhanced effects of the formulation on the pest species.

3.4 Relative potencies

The relative potencies based on the LC_{50} estimates and associated variances determined by the TCM relationships for assays 1–5 are listed in Table 3. The relative potency of an enhanced formulation over its counterpart generally decreased over days after spraying, indicating that the enhanced formulation acted earlier or faster on the pest species than its counterpart. For instance, the relative potency of the emulsifiable formulation over unformulated *B bassiana* decreased from 9.0 on day 4 to 1.2 on day 12. Inclusion of the sub-lethal imidacloprid rates (0.5, 1.0 and 2.0 μg ml^{-1}) into sprays of the emulsifiable conidia formulation elevated the relative potencies to respectively 14.9, 86.9 and 911 on day 4, and 1.7, 9.3 and 28 on day 12. The interactions between *B bassiana* and imidacloprid on *N lugens*, although considerably

Table 3. Relative potencies and 95% confidence intervals for the interactions between *Beauveria bassiana* and imidacloprid at the sub-lethal application rates of 0.5, 1.0 and 2.0 μg ml^{-1} against *Nilaparvata lugens*, based on the time–concentration–mortality relationships fitted for assays 1–5

Paired assays	Relative potency at days after treatment (95% CI)[a]				
	Day 4	Day 6	Day 8	Day 10	Day 12
1:2	9.0 (0.2–425)	2.4 (0.1–47.8)	1.7 (0.1–23.7)	1.7 (0.1–20.8)	1.2 (0.1–12.9)
1:3	14.9 (0.4–540)	4.4 (0.3–69.1)	2.8 (0.2–31.2)	2.4 (0.2–23.4)	1.7 (0.2–14.3)
1:4	86.9 (2.5–3080)*	16.8 (1.1–257)*	11.2 (1.0–121)*	11.2 (1.2–106)*	9.3 (1.1–74.9)*
1:5	911 (29.4–28205)*	96.0 (7.1–1300)*	44.9 (4.6–438)*	40.6 (4.7–349)*	28.0 (2.6–306)*
2:3	1.7 (0.2–15.0)	1.9 (0.3–12.0)	1.6 (0.3–9.0)	1.4 (0.3–6.9)	1.3 (0.3–6.6)
2:4	9.6 (1.1–84.5)*	7.0 (1.1––44.1)*	6.6 (1.3–34.3)*	6.5 (1.4–30.6)*	7.5 (1.7–33.7)*
2:5	101 (14.5–704*)	40.0 (7.6–210)*	26.4 (5.9–118)*	23.5 (5.7–96.3)*	22.7 (5.7–89.9)*
3:4	5.8 (1.1–30.5)*	3.8 (0.9–15.4)	4.0 (1.1–14.4)*	4.7 (1.4–16.0)*	5.6 (1.7–18.0)*
3:5	61.0 (15.9–233)*	21.6 (6.8–68.5)*	16.1 (5.5–47.1)*	17.2 (6.1–48.2)*	16.8 (6.1–46.1)*
4:5	10.5 (2.9–38.0)*	5.7 (1.9–17.3)*	4.0 (1.5–10.9)*	3.6 (1.4–9.2)*	3.0 (1.3–7.3)*

[a] The LC_{50} estimates for paired assays differed significantly if the lower limit of the 95% CI of a given relative potency exceeded 1.0 (marked with *).

variable over time, were apparently synergistic at the sub-lethal application rates of the chemical but were significant only at $\geq 1.0\,\mu g\,ml^{-1}$ (Table 3).

4 DISCUSSION

As shown above, the emulsifiable formulation of *B bassiana* conidia exhibited a slightly greater lethal effect on *N lugens* than unformulated conidia, but the MOBL used to formulate the fungal agent did not substantially increase background mortality of the pest species. This supports observations on the activities of fungal biocontrol agents enhanced by oils.[34–36] The formulation containing sub-lethal rates of imidacloprid further enhanced fungal activity against *N lugens* (Fig 2). The synergistic interactions between the fungal and chemical agents increased as the sub-lethal rate of imidacloprid applied increased within the range concerned. The relative potencies of an imidacloprid-inclusive formulation over the *B bassiana* formulation alone varied over days after spray in a range of 1.3–1.7 at the rate of $0.5\,\mu g\,ml^{-1}$, 7.5–9.6 at $1.0\,\mu g\,ml^{-1}$, and 22.7–101 at $2.0\,\mu g\,ml^{-1}$. These results strongly support interpretations of synergistic interactions of fungal agents and imidacloprid on other insect pests.[15–19] Thus, sprays of the *B bassiana* formulation including imidacloprid at a rate of $1–2\,\mu g\,ml^{-1}$ would be of high potential for practical purposes.

In this study, the synergistic interactions of *B bassiana* and imidacloprid on *N lugens* were quantitatively demonstrated by the TCM relationships much better than by the CM ones. Although the linear CM relationships from probit analysis were simple and easy for use in estimating LC_{50} in this study and elsewhere,[15,16] their failure to include time–concentration interaction may result in uncontrolled bias in evaluating interactions of both agents, as seen in assays 1 and 3 (Table 1). A solution to this problem lies in the robustness of the TCM relationship that integrates both variables into a single model,[25–27] enabling one to estimate not only separate effects of both variables but also their interactions (Table 2). Thus, paired comparisons of the LC_{50} estimates (Fig 2) determined by the TCM relationships provide not only mathematically but also biologically robust assessment for the relative potency of one imidacloprid-inclusive *B bassiana* formulation over another (Table 3).

However, conventional variance analysis of mortality data from factorial experiments is not suitable for revealing fungal/chemical interactions since it is unable to estimate continuous time–concentration interactions that play an important part in the lethal activity of both agents. This may have led to a conflicting interpretation of 'antagonism' between *B bassiana* and imidacloprid against whiteflies because the final whitefly mortality of a full-rate *B bassiana* × full-rate imidacloprid treatment did not exceed that of a full-rate imidacloprid treatment alone.[22] We do not agree with using 'antagonism' to describe that phenomenon. Although final mortalities of target insects caused by fungal/chemical interactions may not necessarily be additive of separate effects of the two agents in our study (Fig 1a–e) or elsewhere,[15–19] the LC_{50} or LT_{50} values determined by the TCM relationships including time–concentration interactions can be used to clarify the interactions of both agents or formulations on target insects. Since a TCM relationship generates the LT_{50} estimates by linear interpolation with no computable variances, we prefer to use the LC_{50} estimates for calculating relative potencies among the tested combinations of both fungal and chemical agents (Table 3).

Finally, a range of application rates of a selected insecticide must be scaled carefully in studies to evaluate its interaction with fungal biocontrol agents. Low sub-lethal rates should first be taken into consideration with a maximal rate alone causing no more than 50% mortality of target insects. Including too much insecticide in treatments may easily overwhelm the activity of the fungal counterpart. Taking imidacloprid $100\,g\,kg^{-1}$ WP for example, a recommended application rate for control of sucking insects on vegetables is *ca* $300\,g\,ha^{-1}$ in China.[4] From our experience, adding 30 g of this product to each litre of *B bassiana* formulation containing 1×10^{10} conidia ml^{-1} may satisfy requirements for the control of aphids, whiteflies and leafhoppers if a 1000-fold aqueous dilution (containing imidacloprid at $3\,\mu g\,AI\,ml^{-1}$) is sprayed in the field.[20,21] In such a case, imidacloprid $100\,g\,kg^{-1}$ WP is applied together with *B bassiana* at 14% of its labeled rate. In this study, imidacloprid was added at rates in a range of $0.5–2.0\,\mu g\,ml^{-1}$, leading to a good detection of synergistic interactions between *B bassiana* and imidacloprid against *N lugens*, although the interactions were significant only at rates of $\geq 1.0\,\mu g\,ml^{-1}$. For practical purposes, the level of imidacloprid that is added to a fungal formulation should be based not only on relative potencies, best determined by the TCM relationships, but also on a strategy for insect pest management (eg faster or slower), a tolerance to insecticide use and residues, and a balance between cost and loss.

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