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Research

Metarhizium Entomopathogenic Fungi Against the Beetle *Brontispa longissima* (Coleoptera: Hispidae): Isolation and Species Identification

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Abstract

Seven entomopathogenic fungi strains (M1–7) were isolated from field-obtained dead coconut hispine beetles *Brontispa longissima* (Gestro), identified to species, and bioassayed for their pathogenicity. According to ITS sequences, all isolates belong in the genus *Metarhizium*, mainly *M. flavoviride* and *M. anisopliae*. Measured median lethal times (LT_{50}) of 1×10^7 conidia/ml of M1–7 against fourth-instar *B. longissima* larvae within 15 d following exposure were, respectively: 5.43, 10.64, 11.26, 10.93, 6.62, 4.73, and 5.95 d. The isolate M6 yielded the highest mortality to fourth-instar larvae, and was thus selected to be tested against other larval instars and adults of *B. longissima,* after Time–Dose–Mortality (TDM) models. M6 proved more pathogenic against larvae than adults. The obtained bioassays data produced a good fit to the TDM models, yielding estimated LC_{50} and LT_{50} for each of the tested developmental stages of *B. longissima*. Both the obtained dose (β) and time effect (ri) parameters from TDM models suggest that first-instar larvae are the most susceptible life stage of the pest insect, while adults are more resistant to M6 infection. Calculated LC_{50} values were, respectively, 1.23×10^3 and 1.15×10^6 conidia/ml for first-instar larvae and adults, on the 15th day following M6 inoculation. Estimated LT_{50} were 3.3 and 5.9 d for first-instar larvae and adults, respectively, at 1×10^8 conidia/ml. Taken together, these results would suggest *Metarhizium* M6 as an option for the biological control of *B. longissima* in the field.

Key words: integrated pest management, pathogenicity, time-dose-mortality model, biological control

Brontispa longissima (Gestro) (Coleoptera; Chrysomelidae) is one of the most serious invasive pests attacking palm trees across Southeast Asia and the Pacific region. This insect can propagate very rapidly generating multiple overlapping generations, thus being difficult to control and presenting a high potential of causing severe damage to the host plants (Takano et al. 2012, Lu and Peng 2017). The larvae and adults of *B. longissima* feed from palm tree leaves, mainly attacking *Cocos nucifera* (L.), *Roystonea regia* (Kunth), *Areca catechu* L., *Archontophoenix alexandrae* H. Wendl. & Drude, *Caryota ochlandra* Hance, *Dypsis lutescens* (H. Wendl.), *Phoenix dactylifera* (L.), and *Livistona chinensis* (Jacq.) (Arecales; Arecaceae) (Tao et al. 2012). *Brontispa longissima* is native to Indonesia and Papua New Guinea, from where it invaded Southeast Asia, Japan, Australia, Singapore, Laos, Thailand, Vietnam, the Philippines, several Pacific islands of Malaysia, and China (Takasu et al. 2010). In China, it was first recorded in June 2002 at the city of Haikou of Hainan province, quickly becoming a major pest throughout southern China (Lu 2004, Ju et al. 2005). There are 4–5 recorded generations each year in the Hainan province of China.

Brontispa longissima takes about 50 d to develop from egg to adult stage: eggs take 3–6 d to hatch; larval development time between first and sixth instars takes about 30–40 d; prepupal stage lasts about 3 d and pupal stage about 6 d; adult life span reaches up to 220 d (Zhou et al. 2004). Each female *B. longissima* can lay about 100 eggs in the first 2 mo following copulation, and eggs are often laid where the female adults will feed from the heart leaves (Jin et al. 2013). Under severe *B. longissima* infestation, the spears of infested palm trees will rot, ultimately causing the death of host plants, particularly of young shoots. Larvae and adults of *B. longissima* feed on unopened buds and mature leaves from coconut palm trees, leading to unappealing brown leaves and decreased fruit production (Supp Fig. 1 [online only]). This way, *B. longissima* infestations bring substantial economic losses from affecting coconut production as well as the local tourism industry in the tropics.

Copious quantities of regularly used pesticides—carbaryl, cypermethrin, trichlorfon, and lindane—have been invested in the management of *B. longissima* in China (Lin et al. 2012, Lu 2005). However, these efforts have proven futile, as *B. longissima* continues to spread rapidly into the country (Zou et al. 2019). Such insecticides are typically sprayed or irrigated through high pressure water jets or similar tools, in such a way that this strategy typically wastes a lot of insecticide, given that palm trees are relatively tall plants. Tang et al. (2006) proposed injecting insecticides into the palm tree stems instead of spraying, reporting that effective concentrations of imidacloprid-dichlorvos (14%), imidacloprid (4.5%), and dichlorvos-oxymoron (30%) would kill *B. longissima* specimens sheltered in between leaves. However, this method of control proved only effective with king coconut plants, and would not be a good choice for controlling *B. longissima* on other palm tree species. Furthermore, due to the '3R' issue—insect pest Resistance, Resurgence, and Residue—caused by large-scale use of insecticides, it soon becomes impossible to control *B. longissima* in the long term because of the ever-growing demand for more chemicals (Lin et al. 2012). There is, therefore, an urgent need for safer, more sustainable control methods.

Entomopathogenic fungi are important assets in the context of Integrated Pest Management (IPM), because of their long environmental persistence and reduced ecological impact (Skinner et al. 2014). Entomopathogenic fungi from the genera *Beauveria*, *Metarhizium*, and *Isaria* have been widely used to control agricultural and forest insect pests (Liu et al. 1989, Campos et al. 2010, Shan and Feng 2010, Nian et al. 2015). The fact that 4–5 generations of *B. longissima* will typically overlap every year in South China makes it more difficult to ensure the control efficacy of individual fungi isolates, which might be more or less pathogenic to different beetle life stages. On top of this, better evaluation methods to assess the potential of fungal isolates as pest control agents are needed. Aimed at integrating the dose and time effects of fungal isolated against pests in synchrony, the Time–Dose–Mortality (TDM) model is considered particularly suitable for evaluating the pest management potential of entomopathogenic fungi (Feng et al. 1999). Nonetheless, as far as we know, no studies in Asia have focused in screening fungal isolates to simultaneously control *B. longissima* larvae and adults using TDM models.

In this study, we have isolated seven entomopathogenic fungi strains from field-obtained samples, identified their species, and quantified their virulence against fourth-instar larvae of *B. longissima*; finally, the most lethal isolate was selected to be tested against other larval instars and adult stages of *B. longissima*, through TDM model.

Material and Methods

Insects

Specimens of *B. longissima* from different life stages were collected from Wenchang county, Hainan province of China, and reared inside plastic boxes (17 cm long × 11 cm wide × 5 cm tall) kept inside a rearing chamber set to 24–26°C, relative humidity 75–80%, and 12:12 (L:D) h photoperiod. They were fed fresh coconut palm leaves, provided every 2 d. Adults and larvae belonging to same instar were sorted, and only seemingly healthy individuals were used in bioassays as described subsequently.

Species Identification of Metarhizium Isolates

Seven fungal isolates labeled M1–7 were obtained from field-collected *B. longissima* dead adults; their original geographical locations in China were: M1 and M2 came from Hainan province (N 19.536644, E 110.865143, 12 April 2015); M3 and M4 from Shenzhen city (N 22.461868, E 114.099597, 8 July 2015); M5–7 from Zhuhai city (N 22.263989, E 113.575311, 17 May 2015). These fungus-infected beetles were found on different occasions. Fungal spores were collected from the surface of infected beetles using an inoculating needle, and transferred as horizontal lines drawn onto Potato Dextrose Agar (PDA: 20 g dextrose, 200 g boiled potato, and 20 g agar per liter) plates. Growing hyphae were selected from a single colony of each isolate, and inoculated to be cultured in a separate PDA plate until a pure *Metarhizium* strain was obtained. The fungal isolates were conserved inside a laboratory of Guangdong Academy of Forestry, Guangzhou, China. They were cultured inside Petri dishes (9 cm in diameter) containing PDA, incubated in an incubator set to 25 ± 1°C, 80 ± 1% RH, and 12-h photoperiod, for 8 d. Based on visual inspection of sporangia, conidia and mycelium (following Humber 1997), all isolates were identified as *Metarhizium* spp.

In this study, we have employed ITS sequences for species identification. DNA from conidia and mycelia of the seven isolates were extracted using the Axygen genomic DNA extraction kit (Axygen Biotechnology, Hangzhou, China). The ITS sequences of these isolates were PCR amplified with primers ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ and ITS5: 5′-GGAAGTAAAAGTCGTAACAAGG-3′. Employed PCR settings were as follows: initial activation step of 95°C for 5 min preceding the cycling program, followed by 37 cycles of denaturation at 95°C, and annealing at 56°C for 30 s and 72°C for 50 s, and a final extension step at 72°C for 10 min. Obtained amplicons were sent to be purified and sequenced at Shenggong, Ltd (Shanghai, China). Resulting ITS gene sequences were aligned using ClustalW in Mega v. 6.0, and their homologies determined by BLASTn algorithm searches within NCBI (National Center for Biotechnology Information) database. A phylogenetic tree of the isolates were constructed using model strains of genus *Metarhizium*: *M. album* (HM055451), *M. flavoviride* var. *lavoviride* (AF138270), *M. flavoviride* var. *type E* (AF139855), *M. flavoviride* var. *pemphigum* (AF139850), *M. flavoviride* var. *minus* (AF138272), *M. flavoviride* var. *novazealandicum* (AF139853), *M. anisopliae* var. *lepidiotae* (EF484924), *M. anisopliae* var. *majus* (EF051727), *M. anisopliae* var. *acridum* (FJ787311), *M. anisopliae* var. *anisopliae* (EF113339); *B. bassiana* (AY510068) was the out group. This phylogenetic tree was constructed with MEGA v. 6.0, by Neighbor-Joining method.

Preparation of Conidial Suspensions of Seven Isolates and Pathogenicity Bioassay Against Fourth-instar Larvae of *B. longissima*

Conidia of each isolate were collected using a fine brush from the PDA cultures, and suspended into 0.05% Tween-80 in water. Final conidial concentrations were prepared with the aid of a hemocytometer into a dilution of 1×10^7 conidia/ml. The conidial viability of all strains was evaluated, yielding germination rates above 95% for all the strains. Therefore, fourth-instar larvae of *B. longissima* obtained as previously described were placed into petri dishes, and had their thoraxes exposed to 2 µl of the conidial suspension using a micro-pipette. Each replicate consisted of 30 larvae, and three replicates were conducted for each fungal isolate (totaling $n = 90$). Negative controls were exposed to 0.2 µl 0.05% Tween-80 solution. The treated larvae were then transferred to plastic boxes (17 cm long × 11 cm wide × 5 cm high) placed inside a rearing chamber as previously described, and provided with fresh coconut leaves as feed every 2 d. Mortalities of larvae were recorded daily for 15 d, where dead larvae were isolated, surface-sterilized by wiping with 75% ethanol and visually surveilled for the growth of mycelia and conidia for extra 7 d.

TDM Model of M6 Fungus Against Other Larval Instars and Adults of *B. longissima*

Out of the seven isolates tested as previously described, strain M6 exhibited the highest lethality against *B. longissima* fourth-instar larvae. This isolate was then selected for further testing pathogenicity against adults and younger larval instars of *B. longissima*. Conidial suspensions at five concentrations of M6 were prepared—1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, and 1.0×10^8 conidia/ml—of which 0.2 µl droplets were applied to the thoracic surface of adults and larvae (from first to fifth instar) as described earlier. In order to evaluate the dose and time effects from M6 application on adults and larvae, TDM models were designed with the bioassays data (Feng 1998, Tang and Zhang 2013). Specifically, obtained data were fitted to the model of conditional death probability using the following equation:

$$q_{ij} = 1 - \exp[-\exp(\gamma_j + \beta\,\lg(d_i))]$$

A mortality model was constructed by superposing the obtained time effect parameterγ_j using the following equation:

$$P_{ij} = 1 - \exp[-\exp(\tau_j + \beta\,\lg(d_i))\ ]$$

In the above equations, the variable q_{ij}—i.e., conditional probability of death—represents the mortality at a given time interval $[T_{j-1}, T_j]$ (where $j = 1, 2, \ldots$ J) following inoculation with a conidial suspension at a given dose d_i (where $i = 1, 2, \ldots$ I). The variable p_{ij} represents mortality at the onset time of the bioassay, with an inoculation dose d_i to a certain time t_j, which gives the cumulative probability of death. β represents the dose effect parameter in both formulas provided above. τ_j is the parameter of cumulative time effect of death, which correlates with γ_j as follows.

$$\tau_j = \ln\left(\sum_{k=1}^{j} e^{\gamma^{i}}\right)$$

Finally, we calculated the logarithm of the dose effect Lg (LC50) and the time effect of dose (LT_{50}) based on the parameters of dose and cumulative time effects from the TDM models.

Statistical Analysis

Data from the mortality bioassays, the median lethal concentration (LC_{50}) and median lethal time (LT_{50}) were submitted to Probit regression analysis using SPSS 20.0 software. Proportional mortality data on 15-d post inoculation were arcsine-transformed, checked for homogeneity of variance using Levene's test, and cross-compared by one-way ANOVA and LSD tests. TDM modeling and associated computations were performed using an updated version of the DPS software (Hangzhou Reifeng Information Technology Co., Ltd, Hangzhou, China).

Results

Species Identification and Cladistics

Results from the ITS sequencing of the seven isolated strains are available in Supp 1 (online only), from which phylogenetic trees were built through Neighbor-Joining of rDNA ITS (Fig. 1). All isolated strains belonged in the genus *Metarhizium*, of which: M1, M3, M4, and M5 fitted within *M. anisopliae* var. *majus*, M6 matched *M. anisopliae* var.

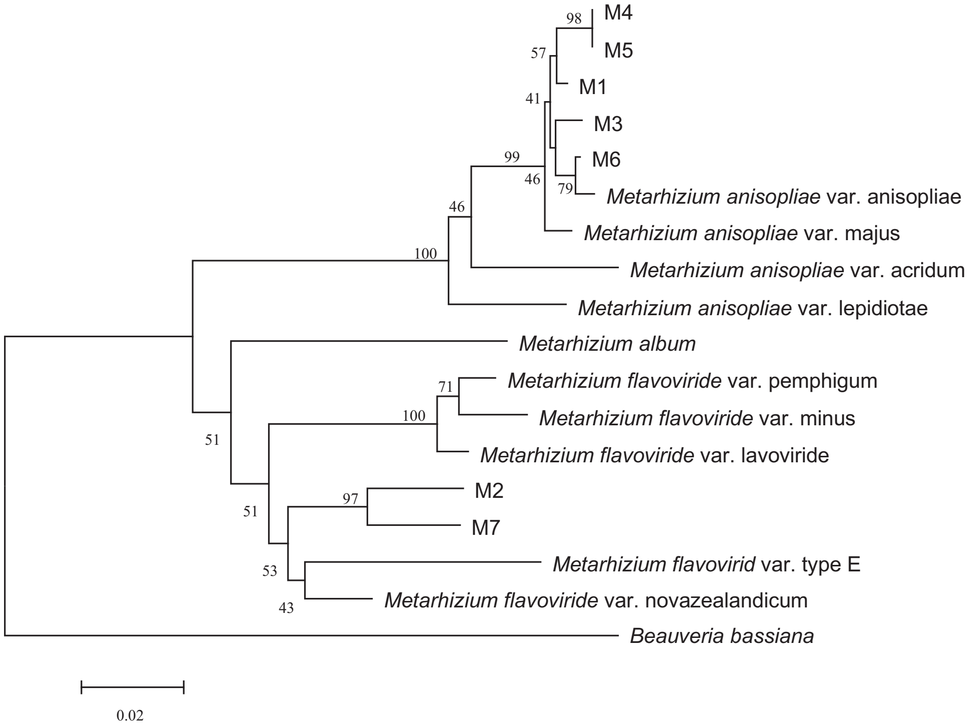

Fig. 1. Representative Neighbor-Joining phylogeny tree of the rDNA ITS region of selected and experimental *Metarhizium* strains. Numbers (%) on branches represent bootstrap support values from 2,000 iterations. *Metarhizium* isolated strains M1 to M7 were obtained during this project.

anisopliae, and M2 and M7 clustered together ambiguously within *M. flavoviride* var. *type E* or *M. flavoviride* var. *novazealandicum*.

Pathogenicity Against Fourth-Instar Larvae of *B. longissima*

Treatment with the different concentrations of conidial suspensions of each isolate resulted in the mortality rates presented in Table 1. The highest obtained mortality came from M1 and M6 isolates at the concentration of 1×10^7 conidia/ml, where M6 delivered the top maximum mortality of 94.73 ± 0.76 %. On the other hand, isolates M2 and M3 caused lower mortality compared with others.

Estimated LT_{50} values at 1×10^7 conidia/ml are also presented in Table 1. Within the same concentrations, the estimated pathogenicity of the isolates would rank as M6>M1>M7>M5>M2>M4>M3.

Pathogenicity of the Selected M6 Strain Against Other Life Stages of *B. longissima*, Under TDM Model

As the most pathogenic against fourth-instar larvae, the isolated strain M6 was selected to be further tested against other instars and adults of *B. longissima* (Supp Fig. 3 [online only]). The measured pathogenicity proved highly variable, depending both on the tested host developmental stages and conidial concentrations, as well as relative to the observation time point postinfection. Tested insects started dying from day 3 post inoculation, and 100% mortality was achieved from 15 d post inoculation, at 1×10^8 conidia/ml. Mycelia and conidia were later observed on the body surface of dead *B. longissima* (Fig. 2 and Supp Fig. 2 [online only]), evidencing death by conidial infection.

The obtained mortality data produced a good fit to TDM models (Table 2). Regarding sample heterogeneity tested with Hosmer–Lemeshow, the following results were observed with data from first-instar larvae to adults, respectively: 11.57 (df = 7, P = 0.12; $\chi^2_{0.05}$ = 14.07), 14.02 (df = 7, P = 0.5; $\chi^2_{0.05}$ = 14.07), 10.66 (df = 8, P = 0.22; $\chi^2_{0.05}$ = 15.51), 13.51 (df = 7, P = 0.61; $\chi^2_{0.05}$ = 14.07), 7.97 (df = 8, P = 0.44; $\chi^2_{0.05}$ = 15.51), and 12.78 (df = 7, P = 0.78; $\chi^2_{0.05}$ = 14.07). Calculated β and γ_j parameters were thus fitted to the effects of conidial concentration and time post inoculation, generating a cumulative TDM relationship for the isolate M6, along with the pertaining LC_{50} and LT_{50} estimations.

Regarding the obtained dose effects (β) against *B. longissima* from first-instar larvae to adults, obtained estimations were 0.80, 0.71, 0.78, 0.67, 0.46, and 0.39 (Table 2), respectively, indicating the pathogenicity of M6 is strongest against first-instar larvae and weakest against adults, within the same conidial concentrations.

Table 1. Median mortalities and lethality (LT_{50}) of seven isolated strains of *Metarhizium* against fourth-instar larva of the coconut beetle *Brontispa longissima*, at the concentration of 1×10^7 conidia/ml. (Data regarding 15 d post inoculation)

Isolates	LT_{50} (days)	R^2	Mortality (±SE)[a] (%)
M1	5.43	0.91	92.95 ± 0.45a
M2	10.64	0.89	64.92 ± 1.23d
M3	11.26	0.93	63.16 ± 3.56d
M4	10.93	0.83	73.68 ± 1.09c
M5	6.62	0.98	75.44 ± 1.12bc
M6	4.73	0.95	94.73 ± 0.76a
M7	5.95	0.96	77.19 ± 0.87b

[a] Means followed by different letters within the same column were significantly different (LSD tests, alpha = 0.05).

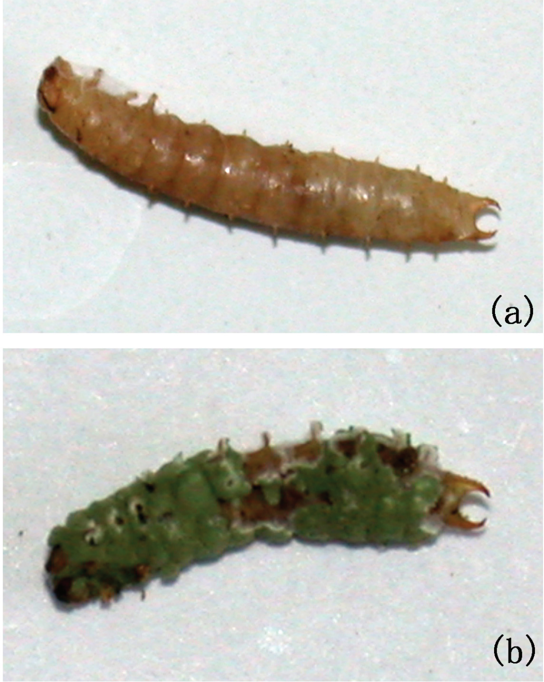

Fig. 2. Naturally dead (a) and *Metarhizium*-infected (b) *Brontispa longissima* fourth-instar larvae which were surveilled to ensure their deaths were caused by *Metarhizium* infection.

Maximum time effect r_i values indicate the death peaks according to time intervals (Table 2). Death peaks for third- and fifth-instar larvae, as well as for adults, were recorded at the fifth time interval (r_5). The obtained r_5 value for adults was −2.66, which is higher than the obtained for larvae, indicating that third- and fifth-instar larvae were more susceptible to pathogen infection than adults, within the same time intervals post inoculation. Following the same pattern, also highest r_i values were obtained for first-, second- and fourth-instar larvae at r_4, where first-instar larvae ranked lowest at −6.26, indicating that first-instar larvae are the most susceptible life stages to the fungal infection.

Based on the TDM model, LC_{50} values obtained for the different life stages were fitted to a function over time post inoculation (Figs. 3 and 4), where LC_{50} estimations decreased over the days following exposure (Fig. 3). The calculated $\log_{10}(LC_{50})$ values for M6 against adults and different instars of *B. longissima* were different: within the same time intervals, calculated $\log_{10}(LC_{50})$ values for adults were always the highest, followed by fifth-instar larvae down to the first-instar larvae, the lowest. These results reinforce that first-instar larva are the most susceptible, and adults the most resistant, to infection by M6.

Estimated LT_{50} values by interpolation to fitted TDM were inversely proportional to conidial concentrations (Fig. 4), where measured LT_{50} values of M6 against first-instar larvae dropped between 3.3 and 4.8 d at the concentrations of 1×10^7 and 1×10^8 conidia/ml. Concomitantly, M6 values against the fifth-instar larvae and adults increased between 5.0–7.4 and 5.9–8.2 d, respectively. The LT_{50} for adults at the concentrations of 1×10^5 and 1×10^6 conidia/ml could not be computed.

Discussion

This study is part of an effort to screen for the most highly virulent entomopathogenic fungal strains already locally adapted to *B. longissima* to be tested as biocontrol agents, within the broader context

Table 2. Estimated parameters for the conditional time–dose–mortality relationships of a *Metarhizium anisopliae* var. anisopliae isolate 'M6' against different life stages of the coconut beetle *Brontispa longissima*

Assays	Conditional mortality model				Cumulative mortality model			
	Parameter[a]	Value	S.E	t^b	Parameter [a]	Value	var (τ_j)	cov (β, τ_j)
First-instar	β	0.80	0.0852	9.43	β	0.80	0.0073	0.0073
	r_1	−7.57	0.667	11.35	τ_1	−6.48	0.410	−0.054
	r_2	−6.89	0.636	10.84	τ_2	−7.57	0.447	−0.053
	r_3	−6.59	0.633	10.41	τ_3	−5.84	0.398	−0.052
	r_4	−6.26	0.609	10.28	τ_4	−5.33	0.378	−0.052
	r_5	−7.58	0.704	10.81	τ_5	−5.23	0.373	−0.052
H-L test[c]				C = 11.57, $P = 0.12$				
Second-instar	β	0.71	0.0497	14.22	β	0.71	0.0028	0.0028
	r_1	−6.26	0.370	16.89	τ_j	−6.25	0.160	−0.0204
	r_1	−5.08	0.343	14.82	τ_j	−4.81	0.138	−0.0195
	r_3	−5.06	0.319	15.84	τ_3	−4.23	0.122	−0.0183
	r_4	−4.93	0.316	15.61	τ_4	−3.83	0.112	−0.0174
	r_5	−5.11	0.332	15.35	τ_5	−3.58	0.105	−0.0169
H-L test				C = 14.02, $P = 0.50$				
Third-instar	β	0.78	0.073	0.57	β	0.78	0.0064	0.0064
	r	−6.72	0.560	12.00	$\tau1$	−6.72	0.375	−0.048
	r_2	−6.12	0.542	11.29	$\tau2$	−5.68	0.350	−0.047
	r_3	−5.48	0.510	10.75	$\tau3$	−4.88	0.319	−0.045
	r_4	−5.24	0.508	10.30	$\tau4$	−4.35	0.298	−0.042
	r_5	−4.78	0.468	10.21	$\tau5$	−3.85	0.253	−0.038
H-L test				C = 10.66, $P = 0.22$				
Fourth-instar	β	0.67	0.0481	13.92	β	0.67	0.0042	0.0042
	r_1	−6.64	0.368	18.08	τ_1	−6.64	0.247	−0.029
	r_2	−4.65	0.331	14.06	τ_2	−4.52	0.200	−0.028
	r_3	−4.57	0.304	15.01	τ_3	−3.85	0.174	−0.026
	r_4	−4.53	0.303	14.96	τ_4	−3.44	0.159	−0.025
	r_5	−4.59	0.323	14.19	τ_5	−3.16	0.149	−0.024
H-L test				C = 13.51, $P = 0.61$				
Fifth-instar	β	0.46	0.042	10.96	β	0.46	0.00104	0.00104
	r_1	−6.43	0.401	16.06	τ_1	−6.43	0.0934	−0.0072
	r_2	−4.15	0.299	13.83	τ_2	−4.05	0.0519	−0.0070
	r_3	−3.69	0.286	12.89	τ_3	−3.15	0.0468	−0.0067
	r_4	−3.66	0.274	12.11	τ_4	−2.54	0.0428	−0.0064
	r_5	−3.32	0.294	12.43	τ_5	−2.26	0.0408	−0.0063
H-L test				C = 7.97, $P = 0.44$				
Adult	β	0.39	0.0561	6.91	β	0.39	0.00522	0.00522
	r_1	−4.47	0.430	10.40	τ_1	−4.47	0.306	−0.037
	r_2	−3.28	0.411	7.98	τ_2	−3.02	0.275	−0.037
	r_3	−3.14	0.412	7.62	τ_3	−2.39	0.267	−0.036
	r_4	−3.06	0.414	7.39	τ_4	−1.97	0.256	−0.035
	r_5	−2.66	0.399	6.68	τ_5	−1.57	0.236	−0.034
H-L test				C = 12.78, $P = 0.78$				

[a] The subscript of γ represents the specific time intervals following inoculation.

[b] The *t*-statistics for all the parameter estimates proved highly significant ($P < 0.0001$).

[c] By *H-L* test: Homogeneity hypothesis for the goodness of fit was accepted when $P \geq 0.05$ in Hosmer–Lemeshow test.

of IPM. Phylogenetic inferences towards species identification of collected *Metarhizium* are typically obtained through sequencing of translation elongation factor (Ef-1alpha) and internal transcribed spacers (ITS) (Driver et al. 2000, Bischoff 2009). According to the ITS analysis, the strains isolated in this study included mostly *M. anisopliae* and *M. flavoviride*, indicating these are two species of *Metarhizium* locally associated with *B. longissima*. *Metarhizium* isolate M6 delivered the top maximum mortality and lowest estimated LT_{50} values than other strains against fourth-instar larvae, meaning this isolate produced the highest virulence and thus was selected to be tested against other life stages, following TDM models. Such further tests indicate M6 proved as more potent against the beetle larvae than adults, which is in agreement with previous observations by Wu et al. (2006). The mortalities of treated *B. longissima* were proportional to M6 conidial concentrations within same life stages, where first-instar larvae were consistently the most susceptible. A possible reason for the observed pattern could derive of enzymes which are secreted during substrate colonization, such as protease, lipase, and chitinase, which are key virulence factors. As a result of lower cuticle schlerotization and a high protein content in the epidermis, the hyphae germinated from conidia could destroy and penetrate the exoskeleton of a younger insect into the body cavity, leading to faster death of *B. longissima* smaller larvae. Furthermore, the still-developing protective tissues and defenses of

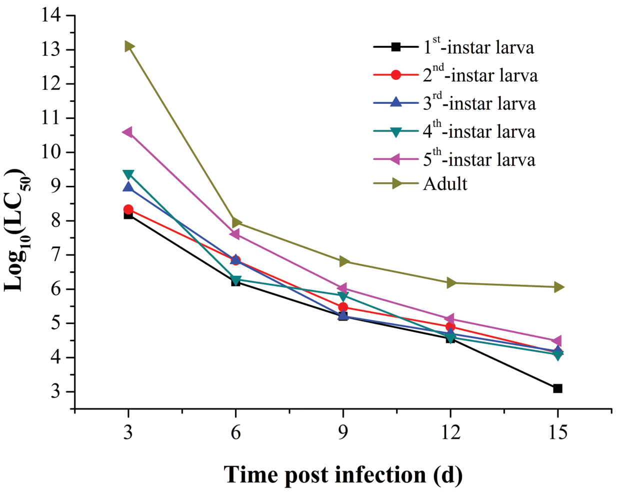

Fig. 3. Plotted log-transformed estimates of LC_{50} for *Metarhizium anisopliae* var. anisopliae isolate 'M6' against different life stages of the coconut beetle *Brontispa longissima*.

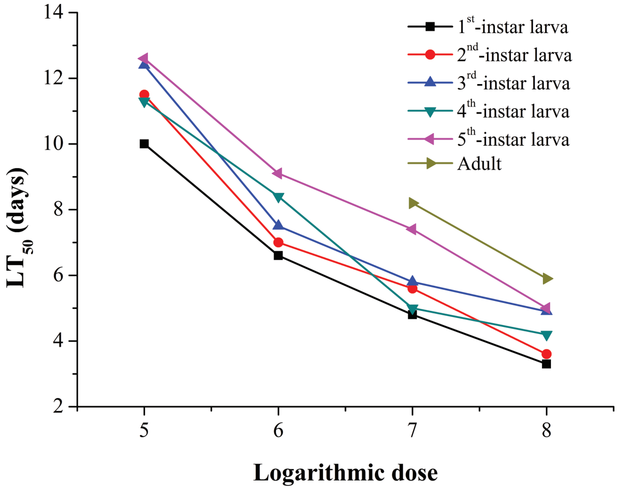

Fig. 4. The LT_{50} at different logarithmic doses of *Metarhizium anisopliae* var. anisopliae isolate 'M6' against different life stages of the coconut beetle *Brontispa longissima*.

younger larvae may also enable *Metarhizium* conidia to kill larvae more easily.

Theoretically, optimal conditions for the biological control of *B. longissima* should be evaluated through early assessment of the developmental stages present and the degree of damage affecting trees, prior to spraying them with conidial suspensions. In the case of M6, the best application opportunity window should be when youngest larvae are predominant. Such a protocol is expected to help reduce *B. longissima* populations throughout the year. Alternatively, whenever fifth-instar larvae and adults seem predominant over other life stages, conidial solutions of higher concentrations should be used. Another promising approach would be combining the conidial solutions with insecticides, the latter adjusted to impairing doses.

Probit analysis has often been used to assess bioassay results for the relative toxicity of pesticides and entomopathogenic fungi (Rahmi and Kenan 2005). However, in the case of the present experiment, entomopathogenic fungal response varied simultaneously according with time, application dose, and mortality; therefore, hampering any accurate estimation using probit analysis (Feng 1998). TDM modeling, on the other hand, enabled integrating dose and time effects into interactions, providing a more comprehensive overview of the virulence of the entomopathogenic fungus *M. anisopliae* var. *anisopliae* strain M6.

Finally, this study screened for local fungal isolates resulting in the acquisition of a potent M6 strain as biocontrol candidate to manage *B. longissima* in China. During the process of practical fungal production and application, however, there has been some serious spore degradation, resulting in decreased efficacy in conidial production after consecutive subculturing of strains. Therefore, a necessary development for this and related studies involving persistence, environmental tolerance, mass production, and field efficacy would be assessing the stability of pathogenic strains after continued culturing cycles, towards better reproducibility for field applications.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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