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Screening of High-Virulence and High-Yield *Beauveria bassiana* Strains for the Biocontrol of *Cylas formicarius* (Fabricius)

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Abstract

Cylas formicarius (Fabricius) is a major pest of sweet potato in Taiwan, where no microbial control agents are currently available. This study aimed to identify *Beauveria bassiana* strains with both high virulence and high spore productivity using a rice-based solid-state fermentation (SSF) system. Eleven field isolates were characterized using morphological traits and multilocus sequence analyses (Bloc, *RPB1*, *RPB2*, and *TEF*). Laboratory assays compared conidial production on 1/2 potato dextrose agar (PDA) and rice substrates and evaluated pathogenicity against adult *C. formicarius* by estimating median lethal time (LT_{50}) and the onset of mortality (t_0) based on cumulative mortality data. Substantial variation was observed among isolates in growth performance, sporulation, and virulence. Strain TyEf0054 consistently exhibited high pathogenicity, causing more than 80% mortality with conidia produced from both 1/2 PDA and rice, and showed rapid killing activity, with LT_{50} values of approximately 5.5–5.9 days and t_0 values ranging from 3.55 to 3.99 days. In contrast, some strains exhibited high sporulation and virulence on 1/2 PDA but failed to produce conidia or showed severe clumping during rice-based solid-state fermentation, indicating poor suitability for large-scale production. These results demonstrate that high laboratory virulence alone is insufficient for strain selection and highlight the importance of integrating killing speed, final mortality, and SSF compatibility as joint selection criteria. Overall, TyEf0054 represents a promising locally adapted candidate for the development of commercial mycoinsecticides for sustainable management of sweet potato weevil in Taiwan.

Keywords: *Beauveria bassiana*; *Cylas formicarius*; mycoinsecticide; solid-state fermentation; strain screening

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1. Introduction

Cylas formicarius (Fabricius) is a major pest in sweet potato (*Ipomoea batatas* Lam.) production. While adult weevils cause little damage by feeding on the roots, stems, and leaves, their larvae severely reduce the quality and marketability of sweet potato tubers [1]. Female weevils dig small holes, about 1.4 mm deep, in the stems or tubers to lay their eggs. After hatching, the larvae burrow into the tubers and stems, preventing normal development. Infested tubers turn brown, release a strong odor, and accumulate toxic secondary metabolites, including furano-terpenoids (e.g., ipomeamarone) and coumarins, which are harmful to animals [2–4]. Ultimately, these damaged tubers become inedible and

lose all economic value [5]. For instance, in Malawi, sweet potato weevil damage has been reported to range from 15–27% depending on the cultivar [6]; in different regions of Ghana, from 33–49.8% [7]; and in Chiayi, Taiwan, up to 73.1% [8].

Conventional control strategies for *C. formicarius* still rely mainly on chemical insecticides and pheromone trapping, yet these methods provide only temporary suppression and are insufficient for sustainable management [9,10]. However, entomopathogenic fungi (EPF) are capable of infecting and killing insects or other arthropods, and several EPF have been developed as commercial strains to control agricultural pests in many countries. The most widely used EPF include *Beauveria* spp., *Metarhizium* spp., and *Cordyceps* spp. [11–16]. *B. bassiana* is widely distributed in natural environments [17] and can be isolated from soil [18,19], insect hosts [20–22], and plant tissues [23,24]. Its insecticidal activity is mediated through fungal propagules that invade hosts either via ingestion [25,26] or by directly penetrating the insect cuticle [27]. When the host immune response fails to suppress infection [28], EPF colonize the hemocoel to reproduce and acquire nutrients, eventually breaking through the insect's epidermis to produce new propagules as sources of transmission [29,30]. Importantly, *B. bassiana* has been reported to pose low risk to several non-target beneficial arthropods, including *Cryptolaemus montrouzieri* [31], *Chrysoperla lucasina* [32], and *Stratiolaelaps scimitus* [33]. In addition to their direct insecticidal effects, *B. bassiana* can also colonize plant tissues as an endophyte, thereby promoting plant growth [34], enhancing tolerance to salinity [35], heat [36], and water deficit [37], and reducing the incidence of plant diseases [38–40]. Nevertheless, the biological activity and field performance of *B. bassiana* are often constrained by environmental stressors, including high temperature, ultraviolet radiation, and moisture stress [41–44]. These limitations can be effectively mitigated through formulation strategies, such as the incorporation of photoprotectants or oil-based carriers, which enhance conidial stability, persistence, and tolerance to adverse environmental conditions [45–48].

Previous studies have shown that *Beauveria* spp. and *Metarhizium* spp. exhibit strong pathogenicity and effective control against adult *C. formicarius* [49–52]. Field trials have demonstrated that applications of entomopathogenic fungi can significantly reduce sweet potato tuber damage caused by *C. formicarius* [53,54]. Infected insects are also capable of horizontally transmitting EPF to healthy conspecifics, as demonstrated in *C. formicarius* and other insect systems [55–57]. Moreover, applying *Beauveria* spp. in combination with pheromone traps in the field can establish multiple infection sources, whereby pheromone-attracted male pests become contaminated with fungal conidia and subsequently facilitate indirect infection of females through contact or mating, ultimately leading to mortality [58,59]. Notably, integration of pheromone-based trapping and plastic mulching with EPF as part of an integrated pest management strategy has been shown to provide improved control compared with locally adopted management practices [60].

Currently, there are still few EPF-based products available on the market for controlling *C. formicarius* or other coleopteran pests. Consequently, pest management in Taiwan still relies primarily on chemical insecticides and pheromone traps. To address this limitation, the present study aimed to develop *B. bassiana* strains suitable for commercial solid-state fermentation, which is widely used for the large-scale production of fungal biopesticides [15]. Field isolates were collected and identified, followed by laboratory evaluations of key indicators, including growth performance on plates, virulence against *C. formicarius*, and spore production under solid-state fermentation, to screen for strains with potential for large-scale development.

2. Materials and Methods

2.1. Strain Collection and Preservation

The collection locations and host species of the fungal strains used in this study are listed in Table 1. Fungal strains were isolated and purified from field-collected insect samples and subsequently subcultured on potato dextrose agar (PDA) (BD Difco, Franklin Lakes, NJ, USA) after single-spore isolation. The preservation method followed the protocols described by [61,62]. Mycelial plugs from 7 to 14-day-old cultures were excised and immersed in 2 mL centrifuge tubes containing 0.7 mL of 10% glycerol (Honeywell, Seelze, Germany). Samples were preserved using a cell freezing container CoolCell LX BCS-4050 (Biocision, San Rafael, CA, USA) and stored at 4 °C overnight before being transferred to a −70 °C freezer (PHCbi, Tokyo, Japan) for long-term preservation. The function of the cell freezing box is that when it is placed at a low temperature, the temperature inside the box will cool down at a rate of −1 °C/min to prevent the cooling rate from being too slow or too fast, which will cause excessive dehydration of the intracellular solution or the formation of ice crystals, thereby damaging the cells. After the strain was taken out from freezing, it was placed in a 37 °C water bath for 1.5 min, and the mycelial plugs were then cultured on PDA. Based on experimental requirements, the strains were subcultured on different media to serve as experimental inoculum sources.

Table 1. Strain information of *B. bassiana* for phylogenetic analysis and bioassay against *C. formicarius*.

Strain	Species	Isolation Host	Locality	GPS
TyEf0003	*Beauveria bassiana*	*Lissorhoptrus oryzophilus*	Emei Township, Hsinchu County, Taiwan	24°41′27.6″ N, 121°02′05.8″ E
TyEf0011	*Beauveria bassiana*	*C. formicarius*	Xinwu District, Taoyuan City, Taiwan	24°56′57.8″ N, 121°01′52.9″ E
TyEf0012	*Beauveria bassiana*	*Blosyrus herthus*	Sanzhi District, New Taipei City, Taiwan	25°12′15.1″ N, 121°30′38.9″ E
TyEf0013	*Beauveria bassiana*	*Blosyrus herthus*	Sanzhi District, New Taipei City, Taiwan	25°12′15.1″ N, 121°30′38.9″ E
TyEf0015	*Beauveria bassiana*	*Blosyrus herthus*	Sanzhi District, New Taipei City, Taiwan	25°12′15.1″ N, 121°30′38.9″ E
TyEf0053	*Beauveria bassiana*	*Colasposoma auripenne*	Sanzhi District, New Taipei City, Taiwan	25°12′15.1″ N, 121°30′38.9″ E
TyEf0054	*Beauveria bassiana*	*Colasposoma auripenne*	Sanzhi District, New Taipei City, Taiwan	25°12′15.1″ N, 121°30′38.9″ E
TyEf0055	*Beauveria bassiana*	*Colasposoma auripenne*	Sanzhi District, New Taipei City, Taiwan	25°12′15.1″ N, 121°30′38.9″ E
TyEf0058	*Beauveria bassiana*	*Nisotra* sp.	Xinwu District, Taoyuan City, Taiwan	24°57′08.9″ N, 121°01′52.8″ E
TyEf0059	*Beauveria bassiana*	*C. formicarius*	Xinwu District, Taoyuan City, Taiwan	24°56′57.8″ N, 121°01′52.9″ E
TyEf0060	*Beauveria bassiana*	Hemiptera	Xinpu Township, Hsinchu County, Taiwan	24°50′04.1″ N, 121°03′59.2″ E

2.2. Strain Identification and Phylogenetic Analysis

The insect hosts of the isolated fungal strains are summarized in Table 1. The strains were morphologically identified by light microscopy (Zeiss, Oberkochen, Germany) following [63], and genomic DNA was extracted by the Food Industry Research and Development Institute following the method described by [63]. Amplification and sequencing were conducted for the nuclear intergenic region Bloc (*B locus*), RNA polymerase II largest subunit (*RPB1*), RNA polymerase II second largest subunit (*RPB2*), and translation elongation factor 1-alpha (*TEF*) gene loci. Phylogenetic analysis followed the method described by [64], with analysis performed using IQTREE 2 (ver. 2.2.2.6) [65]. The sequences of the four gene loci were aligned using BioEdit (ver. 7.7.1) and concatenated using MEGA 11. Maximum Likelihood (ML) analysis was conducted using IQTREE2, with the four loci partitioned into four regions. The optimal models for analysis were selected using ModelFinder [66], with Bloc using the HKY + F + G4 model, *RPB1* and *RPB2* using the TNe + G4 model, and *TEF* using the TN + F + I model. ML analysis applied Ultrafast bootstrap (UFBoot) [67] and SH-aLRT tests (approximate likelihood ratio test and Shimodaira-Hasegawa) [68], with 1000 replicates to estimate clade confidence. Bayesian analysis (BS) was performed using MrBayes (ver. 3.2.7) with the GTR + I + Γ model to perform the Markov Chain Monte Carlo (MCMC) algorithm with 1,000,000 replicates to estimate clade confidence. The phylogenetic tree was visualized using FigTree (ver 1.4.4), with *Cordyceps fumosorosea* as the outgroup. GenBank accession numbers for gene sequences used in the phylogenetic analysis are listed in Appendix A Table A1.

2.3. C. formicarius Rearing

Adult *C. formicarius* were collected from Xinwu District, Taoyuan City, Taiwan. The rearing procedure was modified from the method described in [69]. They were reared in an incubator (Fame engineering, Taichung, Taiwan) maintained at 70 ± 10% RH, a 12L:12D photoperiod, and 25 ± 2 °C, with sweet potato tubers as food. For experimental purposes, sweet potato tubers were placed in the adult colony for 1–2 days to allow oviposition and then removed. The egg-laden tubers were left undisturbed for 40–50 days, during which the adults emerged after pupating within the tubers. Once the exoskeletons had hardened and the adults had exited the tubers, individuals within 15 days of emergence were randomly selected for experiments, regardless of sex.

2.4. Plate Cultivation of Beauveria Strains

The plate cultivation procedure was modified from the method described in [70]. Using 1/2 PDA as a nutrient source, colony growth diameter and sporulation were measured. Conidial suspensions were adjusted to a concentration of 1×10^6 conidia/mL using a hemocytometer (Marienfeld, Lauda-Königshofen, Germany). A 5 mm filter paper was placed at the center of the 1/2 PDA plate, and 5 µL of the conidial suspension was dropped onto the filter paper. After the medium fully absorbed the conidial suspension, the plates were incubated at 26 ± 2 °C in a dark environment. Colony diameters and sporulation were recorded on days 7 and 14 after cultivation. For measuring sporulation, the colonies were scraped into a 50 mL centrifuge tube containing 15 mL of 0.05% Tween 20 (J.T. Baker, Phillipsburg, NJ, USA). The tube was vortexed for 1 min, and the mycelia were filtered out using filter paper. Finally, the conidial suspension was adjusted to a volume of 15 mL, and the conidial count was determined using a hemocytometer.

2.5. Solid-State Fermentation of Beauveria Strains

The solid-state fermentation (SSF) procedure was modified from the method described in [71]. Rice and RO water were mixed at a 2:1 (*w*/*v*) ratio and cooked in a rice cooker. An

additional 250 mL of RO water was added to the outer pot to generate steam. After cooking, the rice was stirred thoroughly to ensure even heating, covered with the lid, and left to rest for 30 min. The pot was sealed with plastic wrap and cooled to room temperature. The cooled rice was transferred to sterilization bags and autoclaved (Tomin, Taoyuan, Taiwan) at 121 °C for 20 min. After cooling, the rice was apportioned aseptically in a laminar-flow hood. For each plate, 15 g of sterilized rice was placed into a petri dish (9 cm diameter, 2 cm height). Each plate was inoculated with 1 mL of conidial suspension (1×10^6 conidia/mL) and mixed evenly with a sterile spatula. Five replicate plates were prepared for each strain and incubated at 26.5 °C in the dark for 10 days. After incubation, 1.5 g of fermented rice from each plate was transferred into a 50 mL centrifuge tube containing 15 mL of 0.05% Tween 20. The mixture was vortexed for 1 min, and the mycelial residues were removed using filter paper. The filtrate was adjusted to 15 mL, and the conidial concentration was determined using a hemocytometer. The final spore yield was expressed as the number of conidia per gram of rice substrate.

2.6. Virulence Assay of B. bassiana Strains Against C. formicarius

The virulence assay procedure was modified from the method described in [72]. The fungal strains were separately cultured on 1/2 PDA medium for 7–14 days or on sterilized rice grains for 10 days before harvesting conidia. Based on the conidial yield from rice fermentation, strains with a production lower than 1×10^9 conidia/g were excluded. Six high-yield strains, TyEf0012, TyEf0013, TyEf0015, TyEf0053, TyEf0054, and TyEf0058, were selected for the pathogenicity assay against *C. formicarius*.

Conidial suspensions were prepared using 0.05% Tween 20, and the concentration was adjusted to 1×10^7 conidia/mL using a hemocytometer. For the virulence assay, fifteen adult *C. formicarius* (within 15 days after emergence) were placed into an immersion cage, which was modified from a 2 mL centrifuge tube by creating 0.8 cm and 0.5 cm diameter holes in the bottom and lid, respectively. The holes were covered with 24-mesh metal screens and secured with hot glue. For the bioassay, the immersion cage was submerged in a glass test tube containing 10 mL of the conidial suspension for 10–15 s. The glass test tube measured 6.4 cm in height and 1.7 cm in diameter. This method ensured that the insects were fully immersed in the spore suspension. After immersion, the cage was quickly removed, and excess liquid was drained. The cage was then placed on clean paper towels to absorb residual moisture. The *C. formicarius* were subsequently transferred to a petri dish (2 cm height, 9 cm diameter) containing a clean filter paper and a piece of unpeeled sweet potato tuber as food. The petri dishes were maintained in an incubator at 70 ± 10% RH, a 12L:12D photoperiod, and 25 ± 2 °C. Each treatment was performed in five replicates. Mortality was recorded daily for 10 consecutive days, and dead insects were removed from the dishes. Cumulative survival data were fitted to an exponential decay function according to [73]:

$$Y = (100 - S)e^{-k(t-t_0)} + S; \quad If\ t > t_0$$

$$Y = 100; \quad If\ 0 \le t \le t_0$$

where Y is the survival percentage at time t, k is the specific death rate (day^{-1}), t_0 is the delay time (days), and S is the estimated asymptotic survival level (%) at the end of the bioassay. The best fits were obtained by nonlinear least-squares regression using the Solver function in Microsoft Excel (version 365). The median lethal time (LT_{50}) was further estimated from cumulative mortality data using probit analysis.

2.7. Statistical Analysis

The data were analyzed using R statistical software (version 4.4.1) [74]. The colony diameter of strains grown on 1/2 PDA plates and conidial yield of strains grown on 1/2 PDA plates and cultured on rice were analyzed using a generalized linear model (GLM) [75] with a Gamma distribution and a log link to identify significant differences among treatments, followed by post hoc comparisons using Tukey's HSD test (multcomp package). For the virulence assay of *B. bassiana* conidia produced on 1/2 PDA and rice against *C. formicarius*, the three parameters derived from the mortality decline model t_0, k, and S were analyzed using a GLM with a Gamma distribution and a log link to identify significant differences among treatments, followed by post hoc comparisons using Tukey's HSD test (multcomp package). The median lethal time (LT_{50}) was estimated along with 95% confidence intervals using Probit analysis (ecotox package) [76] based on the cumulative mortality data over 10 days. The mortality rate at day 10 was analyzed using a GLM with a binomial distribution and a log link to identify significant differences among treatments, followed by post hoc comparisons using Tukey's HSD test (multcomp package).

3. Results

3.1. Morphological Characteristics and Phylogenetic Analysis

Based on the descriptions in [77] identification key for EPF, the morphological structures of the experimental strains in this study were compared with descriptions and images of different fungal species. The results confirmed that all 11 strains shared characteristics consistent with the genus *Beauveria*. The conidiogenous cells of these strains were produced either laterally or terminally on the hyphae, with a swollen base forming a flask-shaped or globose structure, and an extended, slender, and denticulate apex. Single conidia were formed at each denticulate point (Figure 1). However, comparison of the conidiogenous cells with the descriptions of different *Beauveria* species published by [64] did not allow accurate classification of these strains into specific species. Therefore, phylogenetic analysis was performed following the methods of [64], using sequence data from four gene loci (Bloc, *RPB1*, *RPB2*, *TEF*) of 41 *Beauveria* strains (Appendix A Table A1). After removing incomplete gaps in the sequence alignment, the final dataset comprised a total sequence length of 7406 bp (Bloc: 1556 bp, *RPB1*: 2820 bp, *RPB2*: 2107 bp, *TEF*: 923 bp). The phylogenetic trees constructed using ML and BI analyses grouped the 41 strains into 10 clades (Figure 2), with all 11 strains in this study clustering within the *B. bassiana* clade.

Based on the combined results of morphological and molecular identification, all 11 strains were confirmed to belong to *B. bassiana*. Additionally, according to the phylogenetic tree and comparisons of strain collection sites and hosts, strains TyEf0011 and TyEf0059, both isolated from *C. formicarius* and collected in Taoyuan, exhibited the closest genetic relationship. The other strains showed no apparent correlation with specific collection sites or hosts.

3.2. Colony Growth and Sporulation of Beauveria Strains

To screen *Beauveria* strains with the fastest growth rate and highest sporulation, 1/2 PDA medium was used for plate cultivation under laboratory conditions. After 7 days of inoculation, the colonies of all strains were white and fluffy (Figure 3). The colony diameter of strain TyEf0055 was 2.95 ± 0.04 cm, significantly larger than the other strains ($\chi^2_{44} = 0.04$, $p = 2.22 \times 10^{-16}$) (Figure 4). The second largest colony was TyEf0012, with a diameter of 2.76 ± 0.08 cm, while TyEf0058 exhibited the smallest diameter at only 1.26 ± 0.03 cm. TyEf0058 also had the lowest sporulation, producing only $3.25 \times 10^4 \pm 2.68 \times 10^4$ conidia/mL, which was significantly lower than the other strains ($\chi^2_{44} = 8.67$, $p = 2.22 \times 10^{-16}$). At 14 days post-inoculation, the colony diameter of TyEf0055 increased to 6.52 ± 0.09 cm, making it the

fastest-growing strain, with a diameter significantly larger than the other strains ($\chi^2_{44} = 0.05$, $p = 2.22 \times 10^{-16}$). The next fastest-growing strains were TyEf0060 and TyEf0012, with diameters of 5.88 ± 0.05 cm and 5.86 ± 0.05 cm, respectively. The slowest-growing strain was TyEf0003, with a diameter of only 2.09 ± 0.06 cm. By this time, all strains had reached sporulation levels of 1×10^7 conidia/mL.

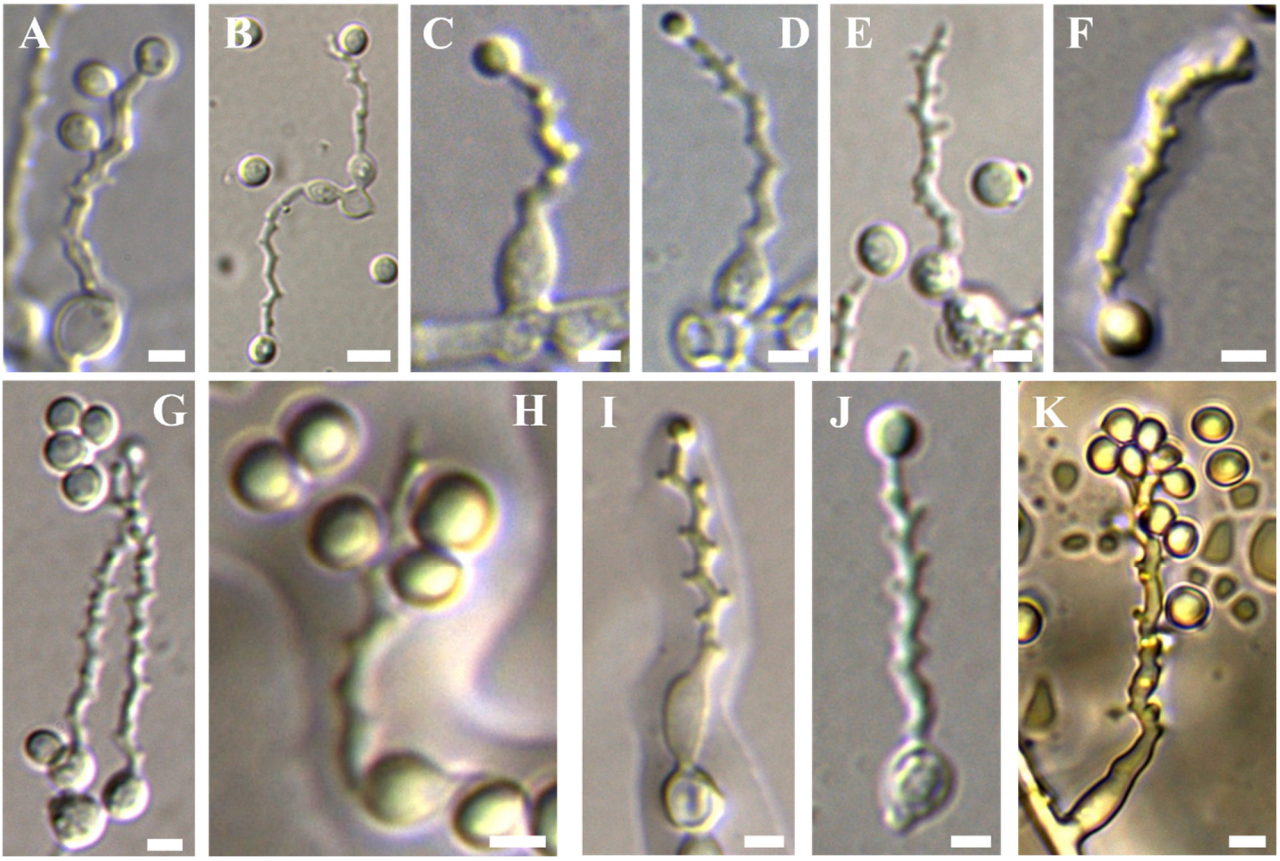


Figure 1. Conidiogenous cells of *Beauveria* strains. (**A**): TyEf0003. (**B**): TyEf0011. (**C**): TyEf0012. (**D**): TyEf0013. (**E**): TyEf0015. (**F**): TyEf0053. (**G**): TyEf0054. (**H**): TyEf0055. (**I**): TyEf0058. (**J**): TyEf0059. (**K**): TyEf0060. Bar scales: (**A**) = 2 μm. (**B**) = 5 μm. (**C**–**K**) = 2 μm.

3.3. Screening of B. bassiana Strains for High Sporulation in Solid-State Fermentation

To screen *B. bassiana* strains with high sporulation under solid-state fermentation, rice was used as the substrate. Samples were collected on the 10th day post-inoculation to measure sporulation. The results showed that strains TyEf0012, TyEf0013, TyEf0015, TyEf0053, TyEf0054, and TyEf0058 showed sporulation levels greater than 1×10^9 conidia/g, significantly higher than the other strains ($\chi^2_{48} = 34.94$, $p = 2.22 \times 10^{-16}$) (Figure 5). TyEf0053 showed the highest sporulation, reaching $3.68 \times 10^9 \pm 2.59 \times 10^7$ conidia/g, followed by TyEf0054 and TyEf0058, with levels of $2.84 \times 10^9 \pm 1.09 \times 10^8$ conidia/g and $2.54 \times 10^9 \pm 5.77 \times 10^7$ conidia/g, respectively. The strain with the lowest sporulation was TyEf0055, which produced only $1.68 \times 10^7 \pm 3.2 \times 10^6$ conidia/g.

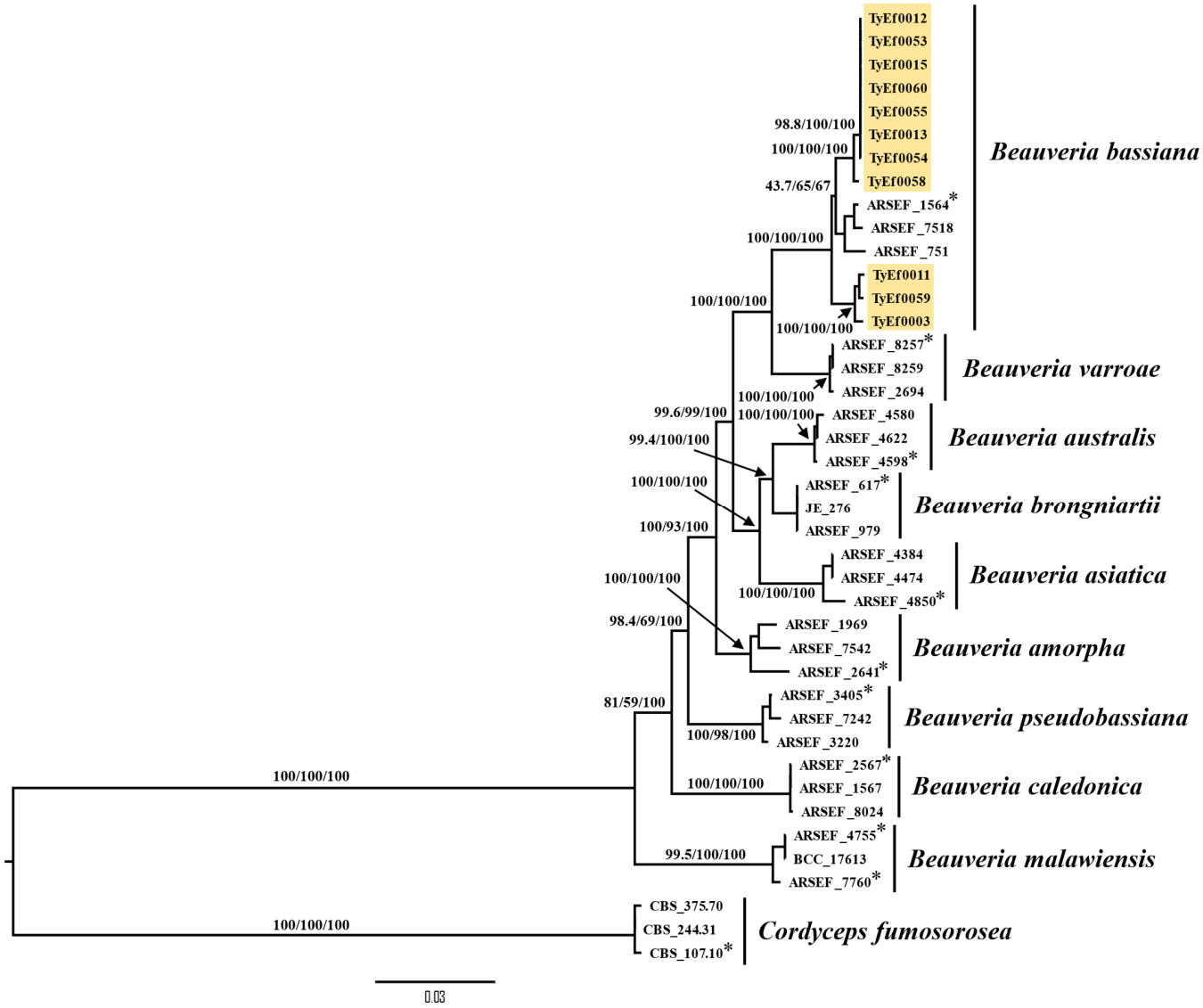


Figure 2. The phylogenetic tree based on ML analysis of the Bloc, *RPB1*, *RPB2*, and *TEF* gene loci of our strains and reference strains to infer the phylogenetic relationships among species. Node bootstrap values represent SH-aLR, Ufboot, and BB proportions. Arrows indicate branches with bootstrap support values. Ex-type strains are marked with asterisks (*), and strains from this study are highlighted in yellow.

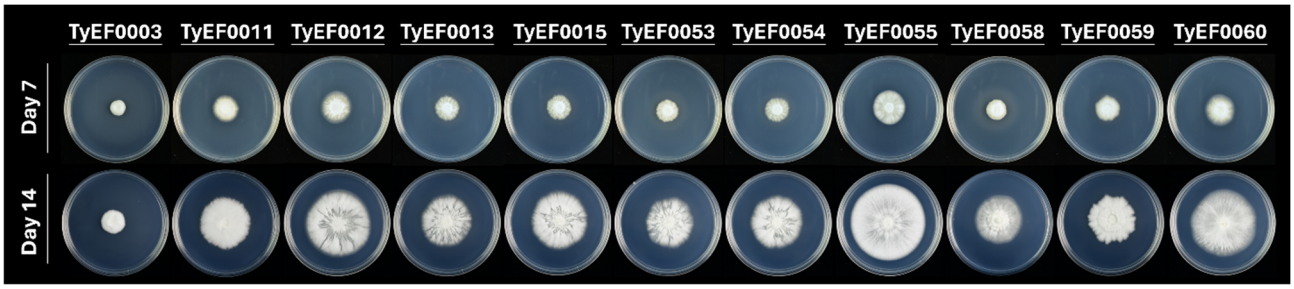


Figure 3. Top view of colonies of *B. bassiana* strains grown on ½ PDA on DAI 7 and 14.

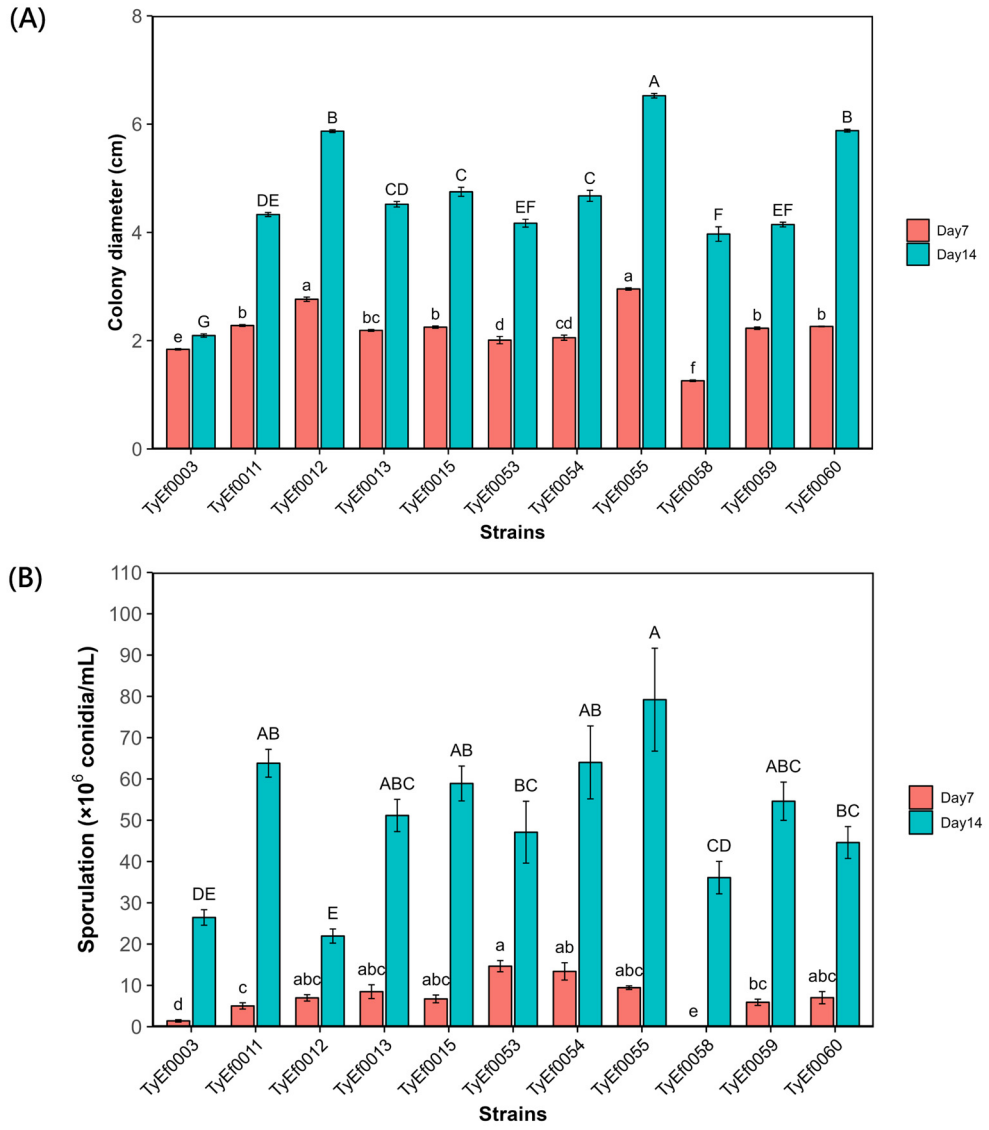


Figure 4. Colony diameter (**A**) and conidial yield (**B**) of *B. bassiana* strains cultured on ½-strength PDA for Days 7 and 14. Values are presented as mean ± SE. Data were analyzed using a GLM with a Gamma distribution and log link. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test.

3.4. Virulence of B. bassiana Strains Against C. formicarius

To screen *B. bassiana* strains with high virulence against the sweet potato weevil *Cylas formicarius*, a conidial suspension at a single concentration of 1×10^7 conidia/mL was used for inoculation. During the bioassay, Petri dishes were kept uncovered to ensure adequate air circulation, and the relative humidity was maintained at 70%. Under these conditions, the virulence of conidia produced on 1/2 PDA against *C. formicarius* is summarized in Table 2. The onset of mortality (t_0) estimated from the mortality-decline model ranged from 3.74 to 4.89 days, indicating that mortality began approximately 3 to 4 days after inoculation. Strains TyEf0058 and TyEf0055 showed the shortest t_0 values, 3.73 ± 0.23 days and 3.74 ± 0.07 days, which were significantly lower than TyEf0015 with 5.19 ± 0.37 days ($\chi^2_{44} = 0.964$, $p = 0.0063708$), indicating that these two strains triggered the earliest host mortality. Although TyEf0058 had the shortest t_0, its specific death rate k was 0.41 ± 0.11 day^{-1}, similar to TyEf0011 with 0.33 ± 0.01 day^{-1}, and both were significantly lower than TyEf0053 ($\chi^2_{44} = 14.652$, $p = 0.014964$). The slower mortality rate resulted in an LT_{50} of 7.7 days and a day-10 mortality of 61.33 ± 6.46%. In contrast, TyEf0055 had a higher k value

of 0.84 ± 0.15 day^{-1}, an LT_{50} of 5.54 days, and a day-10 mortality of 88.0 ± 3.88%. TyEf0054 showed a *k* value of 0.93 ± 0.17 day^{-1}, an LT_{50} of 5.85 days, and a day-10 mortality of 85.33 ± 3.26%. These results indicate that both TyEf0054 and TyEf0055 exhibited high virulence against *C. formicarius*. By contrast, TyEf0011 and TyEf0060 showed relatively lower virulence, with day-10 mortality below 60%. TyEf0060 had a t_0 of 3.96 ± 0.39 days, a *k* value of 0.77 ± 0.18 day^{-1}, and an LT_{50} of 7.97 days, showing that more than half of the insects died only after 8 days, with little increase between days 9 and 10. TyEf0011 had a t_0 of 4.70 ± 0.35 days, the lowest *k* value of 0.33 ± 0.01 day^{-1}, and the longest LT_{50} of 9.35 days, resulting in a day-10 mortality of 53.33 ± 4.71%. These findings indicate that TyEf0011 caused delayed infection and slow mortality progression in *C. formicarius*.

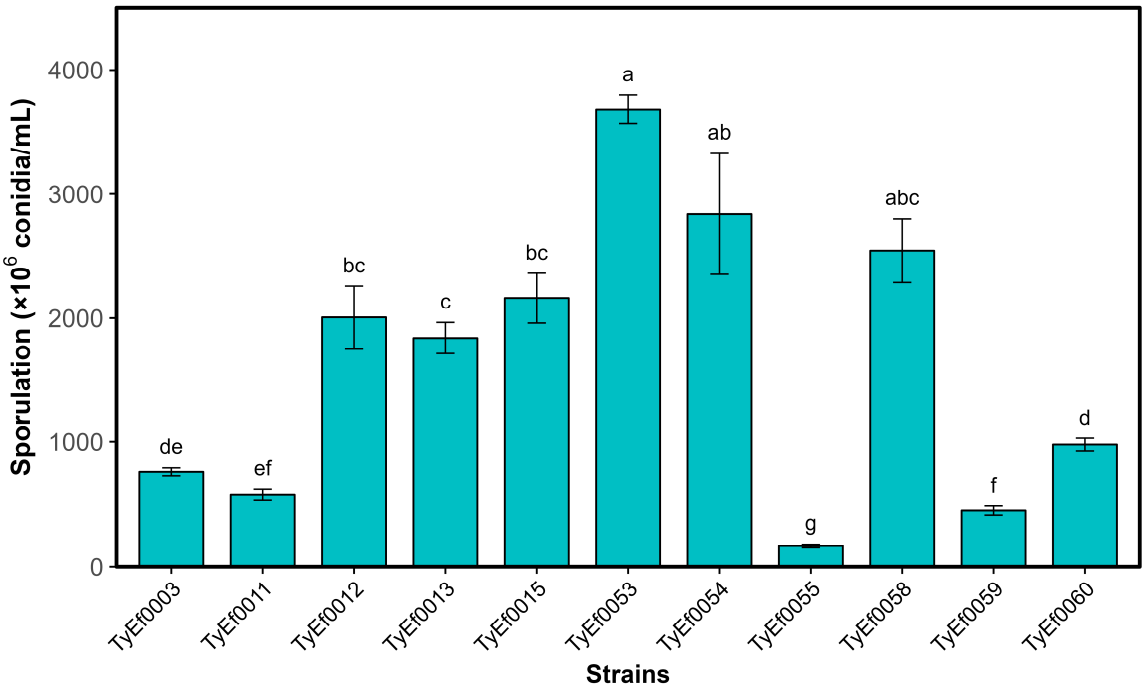


Figure 5. Conidial yield on rice at day 10 after inoculation with *B. bassiana*. Values are presented as mean ± SD. Data analyzed with GLM using Gamma distribution and log link function. Different letters indicate significant differences ($p < 0.05$) by Tukey's test.

The virulence of *B. bassiana* conidia produced on rice medium against the sweet potato weevil *C. formicarius* is summarized in Table 3. The onset of mortality (t_0) estimated from the mortality-decline model ranged from 3.51 to 4.03 days, indicating that mortality began approximately 3 to 4 days after inoculation. No significant difference in t_0 was detected among strains ($\chi^2_{24} = 0.187$, $p = 0.12058$). The specific death rate *k* ranged from 0.51 to 0.89 day^{-1}. TyEf0058 had the lowest *k* value of 0.51 ± 0.05 day^{-1}, indicating a slower mortality process that resulted in an LT_{50} of 6.82 days and a day-10 mortality of 69.33 ± 7.18%. In contrast, TyEf0015 showed the highest virulence, with a *k* value of 0.74 ± 0.11 day^{-1}, an LT_{50} of 5.49 days, and a day-10 mortality of 88.53 ± 7.43%. TyEf0054 had a t_0 of 3.55 ± 0.11 days, a *k* value of 0.64 ± 0.12 day^{-1}, an LT_{50} of 5.50 days, and a day-10 mortality of 88.44 ± 4.22%. No significant difference in virulence was observed among strains ($\chi^2_{24} = 54.577$, $p = 0.30944$). Overall, conidia produced on rice medium caused mortality within 3 to 4 days after inoculation, and all strains except TyEf0058 achieved over 80% mortality by day 10. These results indicate that rice is a suitable substrate for conidial production, yielding spores with consistently high virulence against *C. formicarius*.

Table 2. Median lethal time (LT_{50}) and decay parameters (t_0, k) estimated from the mortality-decline model of *B. bassiana* strains inoculated with *C. formicarius* using conidia produced on 1/2 PDA.

Strain	LT_{50} [1] (95% FL)	Parameter [2]		Mortality at Day 10 (%) [3]
		t_0 (Days)	k (Day^{-1})	
TyEf0003	6.23 (5.83–6.64)	4.0 ± 0.1 ab	0.71 ± 0.13 ab	79.99 ± 7.3 abc
TyEf0011	9.35 (8.55–10.61)	4.7 ± 0.35 ab	0.33 ± 0.01 b	53.33 ± 4.71 d
TyEf0012	7.66 (7.21–8.2)	4.47 ± 0.26 ab	0.57 ± 0.18 ab	65.33 ± 7.42 abcd
TyEf0013	6.29 (5.19–7.71)	3.89 ± 0.49 ab	0.62 ± 0.25 ab	77.33 ± 3.39 abc
TyEf0015	7.71 (7.33–8.15)	5.19 ± 0.37 a	0.74 ± 0.19 ab	68.0 ± 7.11 abcd
TyEf0053	6.56 (6.15–7.01)	4.23 ± 0.15 ab	1.49 ± 0.64 a	71.99 ± 6.46 abcd
TyEf0054	5.85 (5.54–6.16)	3.99 ± 0 ab	0.93 ± 0.17 ab	85.33 ± 3.26 ab
TyEf0055	5.54 (5.23–5.86)	3.74 ± 0.07 b	0.84 ± 0.15 ab	88.0 ± 3.88 a
TyEf0058	7.7 (7.23–8.27)	3.73 ± 0.23 b	0.41 ± 0.11 b	61.33 ± 6.46 bcd
TyEf0059	7.08 (6.61–7.62)	4.41 ± 0.17 ab	0.6 ± 0.09 ab	73.33 ± 8.16 abcd
TyEf0060	7.97 (7.45–8.63)	3.96 ± 0.39 ab	0.77 ± 0.18 ab	57.33 ± 6.53 cd

[1] LT_{50}: Medium lethal time. FL: Fiducial limits. [2] t_0: time of death onset; k: specific mortality rate. Values are presented as mean ± SE (Standard Error, $n = 5$). Data were analyzed using GLM with a Gamma distribution and log link. Different letters indicate significant differences ($p < 0.05$) by Tukey's test. [3] Values are presented as mean ± SE (Standard Error, $n = 5$). Data analyzed with GLM with a binomial distribution and log link. Different letters indicate significant differences ($p < 0.05$) by Tukey's test.

Table 3. Median lethal time (LT_{50}) and decay parameters (t_0, k) estimated from the mortality-decline model of *B. bassiana* strains inoculated with *C. formicarius* using conidia produced on rice.

Strain	LT_{50} [1] (95% FL)	Parameter [2]		Mortality at Day 10 (%) [3]
		t_0 (Days)	k (Day^{-1})	
TyEf0012	5.53 (5.26–5.79)	3.62 ± 0.23 a	0.61 ± 0.05 a	86.66 ± 5.57 a
TyEf0013	5.66 (5.4–5.92)	3.84 ± 0.11 a	0.68 ± 0.09 a	86.66 ± 5.96 a
TyEf0015	5.49 (5.13–5.85)	3.75 ± 0.17 a	0.74 ± 0.11 a	88 ± 5.73 a
TyEf0053	5.96 (5.64–6.28)	4.03 ± 0.07 a	0.89 ± 0.16 a	82.66 ± 4.52 a
TyEf0054	5.5 (5.23–5.77)	3.55 ± 0.11 a	0.64 ± 0.12 a	88 ± 4.42 a
TyEf0058	6.82 (6.45–7.25)	3.51 ± 0.1 a	0.51 ± 0.05 a	69.33 ± 7.18 a

[1] LT_{50}: Medium lethal time. FL: Fiducial limits. [2] t_0: time of death onset; k: specific mortality rate. Values are presented as mean ± SE (Standard Error, $n = 5$). Data were analyzed using GLM with a Gamma distribution and log link. Different letters indicate significant differences ($p < 0.05$) by Tukey's test. [3] Values are presented as mean ± SE (Standard Error, $n = 5$). Data analyzed with GLM with a binomial distribution and log link. Different letters indicate significant differences ($p < 0.05$) by Tukey's test.

4. Discussion

In this study, 11 *B. bassiana* strains were identified through morphological and molecular analyses. By combining the results from plate cultivation, solid-state fermentation, and virulence assays, several strains were identified as promising candidates for the biological control of *C. formicarius*. Morphologically, observation of conidiogenous cells only allowed classification to the genus *Beauveria*, since the conidiogenous cell structures of *B. bassiana*, *B. brongniartii*, *B. kipukae*, *B. pseudobassiana*, *B. varroae*, and *B. sungii* are highly similar [64]. Therefore, phylogenetic analysis was performed based on the Bloc, *RPB1*, *RPB2*, and *TEF* loci, and the reconstructed phylogenetic tree confirmed that all strains belonged to *B. bassiana*. Among them, strains TyEf0011, TyEf0059, and TyEf0003 were genetically close, and all showed low conidial yields on rice-based substrates. TyEf0011 and TyEf0059 were both isolated from *C. formicarius* collected in the same region, exhibiting similar colony diameters and sporulation levels on 1/2 PDA medium. However, their virulence against *C. formicarius* was not the highest, with LT_{50} values of 9.35 and 7.08 days, respectively, although TyEf0059 showed a slightly higher specific mortality rate (k) than TyEf0011. Although strains within the same phylogenetic complex may share similar host ranges, their

virulence can still vary considerably among isolates, necessitating experimental bioassays to verify virulence toward a specific host [78,79].

During the screening of spore production of *B. bassiana* strains on 1/2 PDA medium and rice-based solid-state fermentation, strains TyEf0011, TyEf0055, and TyEf0059 exhibited the largest colony diameters and highest sporulation levels on PDA. However, under rice-based fermentation, these strains produced relatively low conidial yields and showed severe clumping. In contrast, strains TyEf0012, TyEf0013, TyEf0015, TyEf0053, TyEf0054, and TyEf0058 exhibited high conidial yields on rice substrates, with uniform and dense conidial layers covering the surface. These results indicate that plate culture alone is insufficient to evaluate high-sporulating strains, and solid-state fermentation should be included as an essential criterion for strain assessment. Optimizing parameters such as aeration rate, temperature, moisture content, and carbon-to-nitrogen ratio can further maximize conidial yield [80]. Notably, the design of the fermentation system also affects sporulation performance. For example, in *M. robertsii* strain Mr Xoch8.1, the highest conidial yield was achieved in a packed-column bioreactor with forced aeration [81], whereas glass column reactors without aeration produced higher yields [82]. These findings suggest that substrate bed depth and aeration mechanisms within bioreactors play critical roles in determining conidial productivity. In addition, future studies may consider supplementing the solid substrate with specific nutrients or additives, such as yeast extract or protein sources, to further enhance sporulation efficiency [83].

In the virulence assay, a custom-designed immersion cage was employed to simultaneously immerse 15 insects, ensuring uniform exposure to the conidial suspension. This setup allowed rapid infiltration and drainage, improving both infection consistency and operational efficiency. Strain TyEf0054 exhibited the highest pathogenicity against *C. formicarius*, with an LT_{50} of 5.85 days. Notably, conidia produced from rice-based fermentation maintained similar virulence, with an LT_{50} of 5.5 days and a final mortality rate of 88%, indicating that the pathogenicity of TyEf0054 was unaffected by the cultivation 1/2 PDA or rice. In addition, conidia of TyEf0012, TyEf0013, TyEf0015, and TyEf0053 produced on rice substrates showed higher virulence than those produced on 1/2 PDA, with LT_{50} values shortened by 1–2 days and mortality rates increased to approximately 80%. Strain TyEf0058 exhibited no significant difference between the two cultivation conditions. TyEf0054 exhibited consistently high virulence, suggesting that this strain maintains stable pathogenicity regardless of cultivation substrate. Further studies may explore the potential of supplementing rice substrates with yeast extract to enhance the virulence of *B. bassiana* [84]. In addition, variations in nutrient sources have been shown to influence the pathogenic potential of strains, particularly under low-concentration conditions [85], while differences in fermentation mode and substrate composition can influence both the virulence of strains and the composition of their secondary metabolites [86].

After the inoculation of *C. formicarius* with *B. bassiana*, mortality began to appear on the 4th day. A similar phenomenon was observed in the studies by [52,53], where mortality sharply increased 72–96 h post-inoculation. Moribund insects displayed stiffened limbs, fully extended legs, and minimal antennal movement upon stimulation. When touched, their legs exhibited twitching behavior, followed by death the next day. This reaction is analogous to the symptoms of central nervous system abnormalities caused by organophosphates and carbamate insecticides, which induce excessive neural excitation and convulsions [87].

The secondary metabolites of *B. bassiana* have been shown to reduce acetylcholinester ase (AChE) activity in the *Eurygaster integriceps*, leading to the accumulation of acetylcholine (ACh) in the synaptic cleft. This accumulation causes prolonged neural excitation, resulting in convulsions and death [88]. However, whether the strains in this study sim-

ilarly reduce AChE activity in *C. formicarius* requires further investigation. Infected *C. formicarius* cadavers did not immediately exhibit external fungal growth, which may be due to the experimental humidity being controlled at 70%. When the cadavers were placed in a humid environment, fungal hyphae began to emerge from intersegmental joints by the second day (Figure 6). By the 4th day under humid conditions, powdery white conidia of *B. bassiana* covered the cadavers, forming a layer of spores that could serve as a new source of infection.

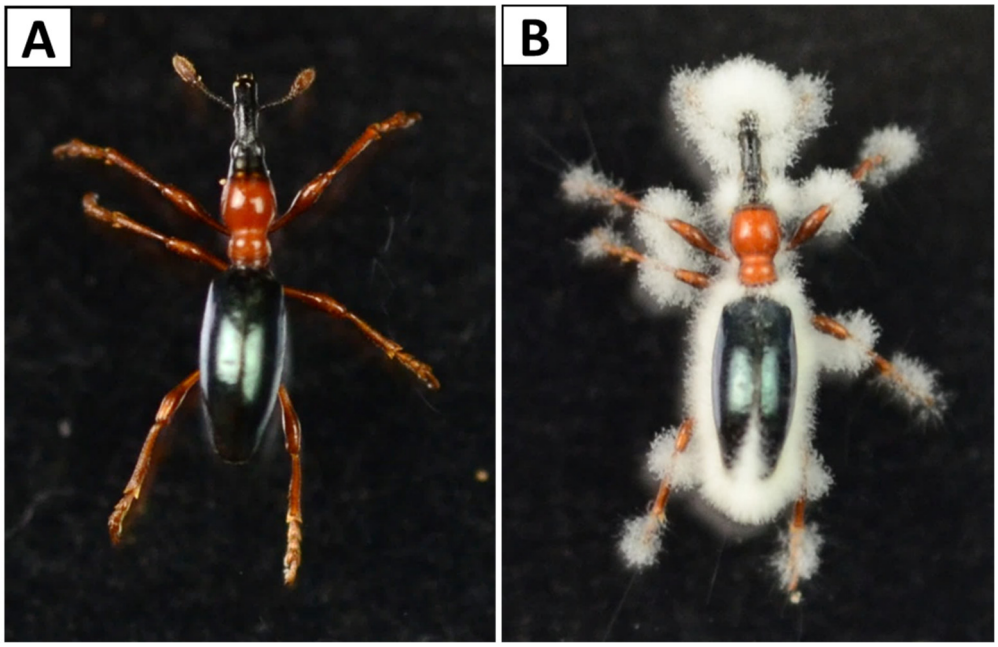


Figure 6. After *C. formicarius* was infected by *B. bassiana*, the body was stiff at the initial stage of death (**A**), and hyphae later broke through the body surface at the joints, forming conidia on the surface (**B**).

In summary, this study provides a method for the rapid screening of *B. bassiana* strains with potential for solid-state fermentation and confirms that TyEf0054 is a promising commercial strain with both high sporulation and high virulence against *C. formicarius*. Prior to field application, further research is needed to develop large-scale production techniques for *B. bassiana*. Additionally, under the concept of integrated pest management (IPM), combining *B. bassiana* with cultivation practices and pheromone trapping systems could facilitate horizontal transmission of the fungus in the field, thereby enhancing control efficiency.

5. Conclusions

This study demonstrated that *B. bassiana* strains exhibit variable virulence and sporulation performance depending on the cultivation substrate. Among the tested isolates, the TyEf0054 strain showed consistently high pathogenicity and superior conidial productivity in rice-based solid-state fermentation. These findings highlight the importance of integrating both virulence and sporulation capacity as selection criteria when identifying potential biocontrol strains. EPF are widely used as microbial biocontrol agents in many countries. Based on the combined results of the bioassay and solid-state fermentation, the most promising *B. bassiana* strain was TyEf0054. The TyEf0054 strain represents a potential microbial agent for the control of *C. formicarius*. Further field evaluations are required to determine the efficacy of TyEf0054 against *C. formicarius*, including optimization of application strategies and assessment of its ability to reduce sweet potato tuber damage.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Information on strains, hosts, and GenBank accession numbers used in phylogenetic analysis.

Species	Strain	Host/Substrate	TEF	RPB1	RPB2	Bloc	References
Beauveria amorpha	ARSEF 2641 *	Hymenoptera: Formicidae	AY531917	HQ880880	HQ880952	HQ880739	[64]
B. amorpha	ARSEF 1969	Coleoptera: Curculionidae	AY531907	HQ880879	HQ880950	HQ880738	[64]
B. amorpha	ARSEF 7542	Hymenoptera: Formicidae	HQ881007	HQ880877	HQ880949	HQ880736	[64]
Beauveria asiatica	ARSEF 4850 *	Coleoptera: Cerambycidae	AY531937	HQ880859	HQ880931	HQ880718	[89]
B. asiatica	ARSEF 4384	Coleoptera: Scarabaeidae	AY531935	HQ880857	HQ880929	HQ880716	[64]
B. asiatica	ARSEF 4474	Coleoptera: Scarabaeidae	AY531936	HQ880858	HQ880930	HQ880717	[89]
Beauveria australis	ARSEF 4598 *	Soil	HQ880995	HQ880861	HQ880933	HQ880720	[64]
B. australis	ARSEF 4580	Orthoptera: Acridiidae	HQ880994	HQ880860	HQ880932	HQ880719	[64]
B. australis	ARSEF 4622	Orthoptera: Acridiidae	HQ880996	HQ880862	HQ880934	HQ880721	[64]
B. bassiana	ARSEF 1564 *	Lepidoptera: Arctiidae	HQ880974	HQ880833	HQ880905	HQ880692	[64]
B. bassiana	ARSEF 751	Coleoptera: Chrysomelidae	AY531954	HQ880835	HQ880907	HQ880694	[64]
B. bassiana	ARSEF 7518	Hymenoptera: Pamphiliidae	HQ880975	HQ880834	HQ880906	HQ880693	[64]
B. bassiana	TyEf0003	*Lissorhoptrus oryzophilus*	LC812023	LC812001	LC812012	LC811979	This study
B. bassiana	TyEf0011	*C. formicarius*	LC812024	LC812002	LC812013	LC811980	This study
B. bassiana	TyEf0012	*Blosyrus herthus*	LC812025	LC812003	LC812014	LC811981	This study
B. bassiana	TyEf0013	*Blosyrus herthus*	LC812026	LC812004	LC812015	LC811982	This study
B. bassiana	TyEf0015	*Blosyrus herthus*	LC812027	LC812005	LC812016	LC811983	This study
B. bassiana	TyEf0053	*Colasposoma auripenne*	LC812028	LC812006	LC812017	LC811984	This study
B. bassiana	TyEf0054	*Colasposoma auripenne*	LC812029	LC812007	LC812018	LC811985	This study
B. bassiana	TyEf0055	*Colasposoma auripenne*	LC812030	LC812008	LC812019	LC811986	This study
B. bassiana	TyEf0058	*Nisotra* sp.	LC812031	LC812009	LC812020	LC811987	This study
B. bassiana	TyEf0059	*C. formicarius*	LC812032	LC812010	LC812021	LC811988	This study
B. bassiana	TyEf0060	Hemiptera	LC812033	LC812011	LC812022	LC811989	This study
Beauveria brongniartii	ARSEF 617 *	Coleoptera: Scarabaeidae	HQ880991	HQ880854	HQ880926	HQ880713	[64]
B. brongniartii	ARSEF 979	Coleoptera: Scarabaeidae	HQ880992	HQ880855	HQ880927	HQ880714	[64]
B. brongniartii	JE 276	Coleoptera: Scarabaeidae	HQ880993	HQ880856	HQ880928	HQ880715	[64]
Beauveria caledonica	ARSEF 2567 *	Soil	AY531915	HQ880889	HQ880961	HQ880748	[64]
B. caledonica	ARSEF 1567	Coleoptera: Scolytidae	AY531894	HQ880888	HQ880960	HQ880747	[64]
B. caledonica	ARSEF 8024	Coleoptera: Scarabaeidae	HQ881012	HQ880890	HQ880962	HQ880749	[64]

Table A1. *Cont.*

Species	Strain	Host/Substrate	TEF	RPB1	RPB2	Bloc	References
Beauveria malawiensis	ARSEF 4755 *	Soil	HQ881015	HQ880895	HQ880967	HQ880754	[64]
B. malawiensis	ARSEF 7760 *	Coleoptera: Cerambycidae	DQ376246	HQ880897	HQ880969	HQ880756	[64]
B. malawiensis	BCC 17613	NA	HQ881016	HQ880896	HQ880968	HQ880755	[64]
Beauveria pseudobassiana	ARSEF 3405 *	Lepidoptera: Tortricidae	AY531931	HQ880864	HQ880936	HQ880723	[64]
B. pseudobassiana	ARSEF 3220	Lepidoptera: Tortricidae	AY531928	HQ880863	HQ880935	HQ880722	[64]
B. pseudobassiana	ARSEF 7242	Hymenoptera	HQ880997	HQ880865	HQ880937	HQ880724	[64]
Beauveria varroae	ARSEF 8257 *	Coleoptera: Curculionidae	HQ881002	HQ880872	HQ880944	HQ880731	[64]
B. varroae	ARSEF 2694	Coleoptera: Curculionidae	HQ881004	HQ880874	HQ880946	HQ880733	[64]
B. varroae	ARSEF 8259	Acari: Varroidae	HQ881003	HQ880873	HQ880945	HQ880732	[64]
Cordyceps fumosorosea	CBS 107.10 *	-	MF416502	MF416659	MF416453	-	[90]
C. fumosorosea	CBS 375.70	-	MF416501	MF416658	MF416452	-	[90]
C. fumosorosea	CBS 244.31	-	MF416503	MF416660	MF416454	-	[90]

* Ex-type material.

Table A2. Cumulative mortality of *C. formicarius* inoculated with *B. bassiana* over ten days.

Strain	Mortality (%)															
	DAI [1] 1	DAI 2	DAI 3	DAI 4	DAI 5		DAI 6		DAI 7		DAI 8		DAI 9		DAI 10	
CK	0 ± 0 [2]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	d	0 ± 0	e	0 ± 0	f	0 ± 0	d	0 ± 0	e	0 ± 0	e
TyEf0003	0 ± 0	0 ± 0	0 ± 0	2.66 ± 1.63	41.33 ± 5.33	abc	57.33 ± 8.05	abc	70.66 ± 8.58	abc	72 ± 8.53	ab	78.66 ± 7.42	abc	79.99 ± 7.3	abc
TyEf0011	0 ± 0	1.33 ± 1.33	1.33 ± 1.33	4 ± 1.63	5.33 ± 2.49	d	23.99 ± 4.98	de	29.33 ± 4.52	e	42.66 ± 5.41	c	46.66 ± 6.66	d	53.33 ± 4.71	d
TyEf0012	0 ± 0	0 ± 0	0 ± 0	6.66 ± 3.65	13.33 ± 5.96	d	38.66 ± 7.99	cd	46.66 ± 6.99	cde	57.33 ± 7.48	abc	61.33 ± 8	abcd	65.33 ± 7.42	abcd
TyEf0013	1.33 ± 1.33	2.66 ± 1.63	2.66 ± 1.63	14.66 ± 6.11	25.33 ± 7.71	bcd	50.66 ± 5.81	abc	63.99 ± 5.41	abcd	73.33 ± 4.71	ab	77.33 ± 3.39	abc	77.33 ± 3.39	abc
TyEf0015	0 ± 0	0 ± 0	1.33 ± 1.33	6.66 ± 3.65	9.33 ± 3.39	d	30.66 ± 4.98	cd	43.99 ± 6.53	de	59.99 ± 6.32	abc	62.66 ± 6.18	abcd	68 ± 7.11	abcd
TyEf0053	0 ± 0	0 ± 0	0 ± 0	0 ± 0	41.33 ± 6.46	abc	57.33 ± 5.81	abc	64 ± 6.86	abcd	68 ± 6.46	abc	70.66 ± 5.41	abcd	71.99 ± 6.46	abcd
TyEf0054	0 ± 0	0 ± 0	0 ± 0	0 ± 0	51.99 ± 6.11	ab	66.66 ± 3.65	ab	73.33 ± 4.21	ab	79.99 ± 4.21	a	83.99 ± 3.99	a	85.33 ± 3.26	ab
TyEf0055	0 ± 0	0 ± 0	0 ± 0	13.33 ± 2.1	56 ± 6.86	a	70.66 ± 6.18	a	74.66 ± 5.33	a	80 ± 5.96	a	84 ± 4.98	ab	88 ± 3.88	a
TyEf0058	0 ± 0	0 ± 0	1.33 ± 1.33	9.33 ± 1.63	26.66 ± 4.71	bcd	39.99 ± 7.88	bcd	47.99 ± 8.27	bcde	51.99 ± 5.33	bc	58.66 ± 7.42	bcd	61.33 ± 6.46	bcd
TyEf0059	0 ± 0	0 ± 0	0 ± 0	2.66 ± 1.63	22.66 ± 11.1	d	43.99 ± 8.05	bcd	57.33 ± 7.77	abcd	62.66 ± 10.5	abc	66.66 ± 9.88	abcd	73.33 ± 8.16	abcd
TyEf0060	0 ± 0	0 ± 0	1.33 ± 1.33	7.99 ± 3.88	25.33 ± 7.71	bcd	43.99 ± 4.98	abcd	45.33 ± 5.73	cde	50.66 ± 4.52	bc	53.33 ± 6.32	cd	57.33 ± 6.53	cd
GLM	$\chi 2_{48} < 0.01$, $p = 1$	$\chi 2_{48} < 0.01$, $p = 1$	$\chi 2_{48} < 0.01$, $p = 1$	$\chi 2_{48} = 13.12$, $p = 0.51533$	$\chi 2_{48} = 65.32$, $p < 0.001$		$\chi 2_{48} = 60.35$, $p = 2.22 \times 10^{-16}$		$\chi 2_{48} = 64.81$, $p = 2.22 \times 10^{-16}$		$\chi 2_{48} = 65.43$, $p = 2.22 \times 10^{-16}$		$\chi 2_{48} = 66.11$, $p = 2.22 \times 10^{-16}$		$\chi 2_{48} = 60.72$, $p = 2.22 \times 10^{-16}$	

[1] Day after inoculation. [2] Values are presented as Mean ± SE (Standard Error, n = 5). Data analyzed with GLM with a binomial distribution and log link. Different letters indicate significant differences ($p < 0.05$) based on Tukey's test.

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