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Full Length Research Paper

Virulence of entomopathogenic fungi to adults and eggs of *Nilaparvata lugens* Stal (Homopera: Delphacidae)

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Brown plant hopper (BPH), *Nilaparvata lugens* is a devastating insect pest of rice. Effective control measures are desperately needed. Entomopathogenic fungi, such as *Beauveria* and *Metarhizium*, have shown great potential for the management of some sucking pests. In this study, to explore alternative strategy for sustainable control of the pest population, 12 isolates of *Beauveria* and *Metarhizium* from sucking pests were bioassayed under the concentrated standard spray of 1 × 10^8 conidia/ml in laboratory. The cumulative mortalities of adult ranged from 17.2 to 82.1%, 10 days after inoculation. The virulence among all tested isolates exhibited significant differences. The two most virulent isolates, *M. flavoviride* (Mf82) and *M. anisopliae* (Ma20) with cumulative mortalities of 82.1 and 65.4%, respectively, were selected to detect the virulence to gravid female, female and male of BPH with suspension of 1 × 10^8 conidia/ml. The susceptible sequence was gravid female > female > male. Isolates of *Beauveria* and *Metarhizium* could infect eggs of BPH. The infected eggs shrunk in shape, then turned brown and eventually had outgrowths of the sprayed fungus when maintained under high moist conditions. The virulence of Mf82 to the eggs was the greatest, whose cumulative mortality was 60.8%, 10 days after inoculation. The most virulent isolate selected in this study, *M. flavoviride* (Mf82) is a promising candidate for microbial control of BPH.

Key words: *Nilaparvata lugens*, *Beauveria*, *Metarhizium*, virulence, biocontrol agents, eggs.

INTRODUCTION

The brown plant hopper (BPH) *Nilaparvata lugens* Stål (Homopera: Delphacidae), one of major rice pests throughout Asia, is an important vector of rice viruses such as rice grassy stunt virus (RGSV) and ragged stunt (RS) (Nault and Ammar, 1989; Matsumura, 2001). It is well- known that the strategy for controlling BPH has relied on chemical insecticides for a long time (Chung et al., 1982; Liu et al., 2003). However, the large use of these compounds has caused BPH to develop resistance and detrimental impact on natural enemies (Tanaka et al., 2000; Preetha et al., 2010). The high levels of imidacloprid resistance are suspected of being causative of severe BPH outbreaks during 2005 to 2007 (Jin et al., 2008), and natural parasitoids and predators in paddies are more sensitive to most chemical insecticides than the plant hoppers (Croft and Brown, 1975; Croft, 1990). So there is an urgent need to find alternative measures that may be friendly to environment.

A number of entomopathogenic fungi are already commercially available, which offer an environmentally safe and economically viable alternative to chemical control (Peveling and Demba, 1997; Faria and Wraight, 2007). At present, *Beauveria bassiana* Vuillemin, *Metarhizium anisopliae* Sorokin, *Metarhizium flavoviride* Gams and Rozsypal, which were studied intensively have potential to develop biological control for sucking pests, such as aphids (Vandenberg et al., 2001; Shan and Feng,

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Figure 1. Experimental apparatus for culture of rice plant in which *Nilaprvate lugens* are treated by spraying test fungal isolate (A). Healthy adult of *N.lugens* (B). Adult of *N.lugens* (96 h) after death caused by *Metarhizium flavoviride* isolate Mf82 (C). Bar: 500 µm.

2010; Wraight et al., 1998). Pathogenicity of the entomopathogenic fungi Paecilomyces spp. and *Beauveria bassiana* against the silverleaf whitefly, *Bemisia argentifolii*. J. Invertebr. Pathol. 71: 217 to 226. leaf hoppers (Chu and Hirashima, 1981; Feng et al., 2004; Pu et al., 2005), and plant hopper (Rombach et al., 1986a, b; Aguda et al., 1987; Holdom et al., 1988). It is reported that these entomopathogenic fungi appeared to be the most efficient against plant hoppers because of the ease of their mass production, storage, virulence, and application (Toledo et al., 2010). Virulence is the most important indicator of measuring potential of fungi against pests and the basis of choosing high virulent fungi in laboratory bioassays. In the past years, entomopathogenic fungi have been tested against 3rd instar nymph of BPH (Roberts and Leger, 2004; Jin et al., 2008), but no research has been done against the adult. Although the eggs of *Tetranychus cinnabarinus* can be infected by entomopathogenic fungi (Shi and Feng, 2004), there are no studies referring to the virulence of fungi to BPH eggs. Therefore, more bioassays must be conducted to obtain fungal isolate with higher efficacy for BPH. The paddy field ecosystem, dependent on routine irrigation, may provide the high moisture that is required for the successful use of fungal biocontrol agents (Bateman et al., 1993; Feng et al., 1994).

In this paper, we evaluated the virulence of 12 isolates of *M. anisopliae*, *M. flavoviride*, *B. brongniartii* and *B. bassiana* to BPH adults, and the most potential candidates from these were subsequently evaluated for their virulence to male, female, gravid female and eggs of BPH.

MATERIALS AND METHODS

Plant culture

Ozja sativa cv. Nongfengyou, a rice cultivar susceptible to BPH was prepared: 10-day-old rice seedlings in petri dishes (15 cm diameter) were transplanted in plastic pots (20 cm diameter, 20 cm high) which were filled with nutrition solution and cultured for another 10 days at 25 to 30°C and 14:10 h light: dark photoperiod in the greenhouse at School of Plant Protection, Anhui Agricultural University (Hefei, China).

Brown plant hopper: Source and culture

Adults of BPH (Figure 1B), originally collected from the rice fields in Anhui Agricultural University (No.130 West Changjiang Rd, Hefei, Anhui, China), were reared on the rice seedlings described earlier. The gravid adults were removed onto the high-density rice

Table 1. List of fungal species screened against adults of *Nilaparvata lugens*, original host of Homoptera and geographic origin.

Isolate code*	Original host insect	Geographic origin
Bb89	*Nilaparvata lugens* (Delphacidae)	Guniujiang, Anhui.
Bb97	*Elaphiceps cervus* (Membraciade)	Xuancheng, Anhui.
Bb1128	*Amrasca biguttula* (Cicadellidae)	Nanjing, Jiangsu.
Bb2302	*Cryptotympana atrata* (Cicadidae)	Yuexi, Anhui.
Bbr63	*Nilaparvata lugens* (Delphacidae)	Kaili, Guizhou.
Bbr75	*Aphis gosspii* (Aphididae)	Hefei, Anhui.
Ma12	*Amrasca biguttula* (Cicadellidae)	Nanjing, Jiangsu.
Ma20	*Nilaparvata lugens* (Delphacidae)	Hangzhou, Zhejiang.
Ma39	*Callitettix versicolor* (Cercopidae)	Wuhan, Hubei.
Ma53	*Cosmoscartabispecularis* (Cercopidae)	Changsha, Hunan.
Mf19	*Myzus persicae* (Aphididae)	Fenghua, Zhejiang.
Mf82	*Nilaparvata lugens* (Delphacidae)	Chuzhou, Anhui.

* Isolate code using research center of entomopathogenic fugal number.

seedlings to let them lay eggs, then those eggs grew into adults at 25 to 30°C and 14:10 h light: dark. After reproducing two generations, 10-day-old adults were randomly collected from the rice seedlings for bioassay.

Entomopathogenic fungi isolates: source and culture

The fungal isolates of *M. anisopliae*, *M. flavoviride*, *B. brongniartii* and *B. bassiana*, used in the experiment, provided by Biocontrol Reserch Lab, Anhui Agricultural University (Hefei, China), were originally obtained from different hosts (Table 1). In order to confirm virulence of the isolates was not attenuated, original host insects were inoculated to these isolates and the fungi re-isolated on Sabouraud Dextrose Agar Yeast (SDAY) medium. The conidia colonies were inoculated to SDAY medium in petri dishes (9 cm diameter) and maintained at 25 ± 1°C in darkness for 15 days. After harvest, conidia were dried to a water content of 5% at ambient temperature on a vacuum drier (VirTis Company, Gardiner, NY). Dry conidia were preserved at 4°C in darkness for use as soon as possible in the following experiments, warranting ≥ 90% viability.

Adults

Conidia of each fungal isolate were transferred into corresponding test tubes (3 cm diameter, 20 cm length) that contained 10 ml 0.05 % (v/v) Tween-80. The suspension was homogenized for 10 min in vortex and then filtered through sterile gauze. The spore concentration was adjusted to 1×10^8 conidia/ml that was counted with a Neubauer hemacytometer according to Lane et al. (1988).

For each fungal isolate，60 adults (approximately 10-day-old) were aspirated into test tubes (3 cm diameter, 20 cm length) by sucking trap from the rice seedlings. Afterwards, they were removed in groups of 20 individuals to transparent bell glass (6 cm diameter, 20 cm high) with a bottle cap which had small holes for air circulation to avoid adults escaping. Then, 150 ml glass flasks which were filled with 100 ml nutrition solution contained rice seedlings with their roots that were placed in the nutrition solution through thin holes foam boards (Figure 1A). The suspensions of 1×10^8 conidia/ml prepared previously were sprayed onto adults and rice seedlings by a hand-held Micro Ultra sprayer (Micron Sprayers Limited, Herefordshire, UK). For each fungal isolate, a blank control was sprayed with 0.05% (v/v) Tween-80. All treatments were maintained at 25 ± 1°C, 14:10 h light: dark photoperiod and 80% RH. Rice seedlings need to be replaced every 3 days. Mortalities of adults were checked every day up to 10 days. Following the method of Lacey and Brooks (1997), the dead adults were transferred every day into 70% ethanol for 10 s, washed in sterile distilled water, then treated with 0.5% sodium hypochlorite for 30 s, and washed again in sterile distilled water. After that, the specimens were placed in Petri dishes (9 cm diameter) plus filter paper which was moistened with sterile distilled water and maintained at 25 ± 1°C for 3 to 5 days. Only those dead adults showing external mycelial growth were considered to have died from fungal infection.

Gravid female, female or male

Mf82 and Ma20 were chosen due to their highest virulence according to the results above. Gravid female, female and male of BPH were sprayed at the suspensions of 1×10^8 conidia/ml by a hand-held Micro Ultra sprayer respectively. The aqueous solution of 0.05% (v/v) Tween-80 was used as the control. Four replications (20 BPHs each) were included in each treatment. The mortalities of BPH were observed as earlier mentioned.

Eggs

Fifty gravid females were removed to rice seedlings without BPH in order to let them lay. The rice seedlings with 1-day-old eggs were collected and transferred into Petri dishes (15 cm diameter) containing nutrition solution. The suspensions (1×10^8 conidia/ml) of each fungal isolate were respectively sprayed to eggs and rice seedlings using the methods described above. In bioassay of each isolate, a spray of 0.05% Tween-80 was also included as a blank control. All treatments were replicated four times (10 eggs/replicate). After all treatments, all eggs on rice seedlings in Petri dishes were covered with lids and maintained in an incubators at 25 ± 1°C under a photophase of 14:10 L:D and RH ≥ 95%. Daily examination for hatched eggs was made until no more eggs hatched for three consecutive days in all treatments. The unhatched eggs, together with the rice seedlings, were individually examined under a dissecting microscope at 100 × magnification for verification of fungal infection. Those unhatched eggs with external mycelial growth were recorded as dead eggs killed by the fungal pathogen.

Table 2. Bioassay results for adults of *Nilaparvata lugens* representing isolates of *Metarhizium* and *Beauveria*.

Isolate code	Mortality (%) days after inoculation			Median lethal time (d) LT_{50}
	4	7	10	
Bb89	7.2 ± 0.9^{def}	20.2 ± 2.1^{ef}	35.1 ± 2.7^{e}	12.1
Bb97	4.4 ± 1.9^{f}	10.1 ± 2.7^{hi}	20.2 ± 8.2^{gh}	14.5
Bb1128	6.1 ± 3.4^{ef}	13.1 ± 2.7^{gh}	22.8 ± 3.7^{fg}	14.1
Bb2302	3.9 ± 0.9^{f}	6.5 ± 2.1^{i}	17.2 ± 1.0^{h}	17.2
Bbr63	7.7 ± 0.9^{def}	24.3 ± 2.7^{e}	42.8 ± 3.1^{d}	11.1
Bbr75	6.1 ± 1.9^{ef}	17.2 ± 1.0^{fg}	27.9 ± 2.7^{f}	12.9
Ma12	10.6 ± 2.5^{de}	24.3 ± 2.7^{e}	33.9 ± 4.7^{e}	10.4
Ma20	17.8 ± 3.5^{b}	51.8 ± 3.6^{b}	65.4 ± 5.6^{b}	6.7
Ma39	10.6 ± 2.5^{de}	33.9 ± 3.6^{d}	41.6 ± 2.7^{d}	8.6
Ma53	12.2 ± 2.5^{d}	35.7 ± 1.8^{d}	42.8 ± 3.6^{d}	9.2
Mf19	23.3 ± 4.4^{c}	41.6 ± 2.1^{c}	49.3 ± 2.7^{c}	7.9
Mf82	34.4 ± 4.2^{a}	67.3 ± 2.7^{a}	82.1 ± 1.8^{a}	4.9
Control	0.0	6.7	6.7	--

Mean (±SD) was estimated from three replicate bioassays, mean with same letter in the same column are not significantly different (P > 0.05), by ANOVA followed by Duncan's test.

Date analysis

The mortalities (%) of BPH in treatments (M_2) were corrected by the mortality (%) in blank controls (M_1) according to Abbortt's formula, $Mc = (M_2 - M_1) / (100-M_1)$. All corrected mortalities (%) date were subjected to an arc-sine transformation prior to analysis of variance (ANOVA) to detected differences in mortalities of BPH. Mean separation was by a Duncan's multiple range test (P = 0.05). The mortality of BPH adults also was subjected to Probit analysis, generating a time-mortality relationship for the estimates of LT_{50} for the isolates tested. Statistical analyses were performed using the DPS Program (Tang and Feng, 2007).

RESULTS AND DISCUSSION

Mortalities of *Nilaparvata lugens* adults attributed to 12 isolates

The final corrected mortalities of BPH differed significantly among the 12 isolates of fungi ($F_{11, 24}$ = 173.24, P < 0.01) 10 days after treatment. Only two isolates which were originally derived from BPH, Mf82 and Ma20, killed more than 50% of BPH adults, Mf82 of which was the highest, caused a cumulative corrected mortality of 82.1% (Table 2).

The relationship between virulence and hosts is observed in this study. Mf82 originally isolated from BPH exhibited the highest mortality, as well as fastest speed of killing target insects. Mf19 was derived from *Myzus persicae* and its virulence was lower than that of Mf82. It is generally believed that fungus isolates which are obtained from target insects or a closely related species are more pathogenic (Soares et al., 1983; Jackson et al., 1985). However, Feng (2003) and Toledo et al. (2007) have reported that there is no direct relationship between virulence and isolates' original hosts. The result observed in this study provides an evidence for the former standpoint.

To the best of our knowledge, the work of seeking efficient entomopathogenic fungi to *N. lugens* was also made by Jin et al. (2008). They evaluated the virulence of 35 isolates of *Metarhizium* against *N. lugens* nymphs, finding pathogenicity of different isolates of *M. flavoviride* to *N. lugens* nymph was poor, and the highest cumulative mortalities was only 41.7% at a dose of 1×10^8 conidia/ml. However, in this current study, we fund that the pathogenicity of one isolate (Mf82) of *M. flavoviride* to *N. lugens* adults was preferable, with cumulative mortality of 82.1% at the same conditions. Meanwhile, our study also showed Mf82 could kill more than 50% of the nymphs (it will be written in another paper). So Mf82 was a potential candidate for microbial control of *N. lugens*.

LT_{50} can reflect the virulence of isolate to target pest. The LT_{50} of Mf82 was the shortest, in 4.9 days (95% CI: 4.57~5.41) (Table 2). Those entomopathogenic fungi isolates (LT_{50}>14 days) were considered to be nonpathogenic (Samuels et al., 1989). In this study, LT_{50} values from 4.9 days (highly pathogenic) to >14 days (nonpathogenic).

Effect of *Metarhizium flavoviride* 82 and *Metarhizium anisopliae* 20 on male, female and gravid female of BPH

The time-mortality tendency of the two isolates, Mf82 and Ma20 to gravid female, female and male of BPH after treatments are shown in Figure 2. Gravid female had the strongest susceptibility to the two isolates. The two

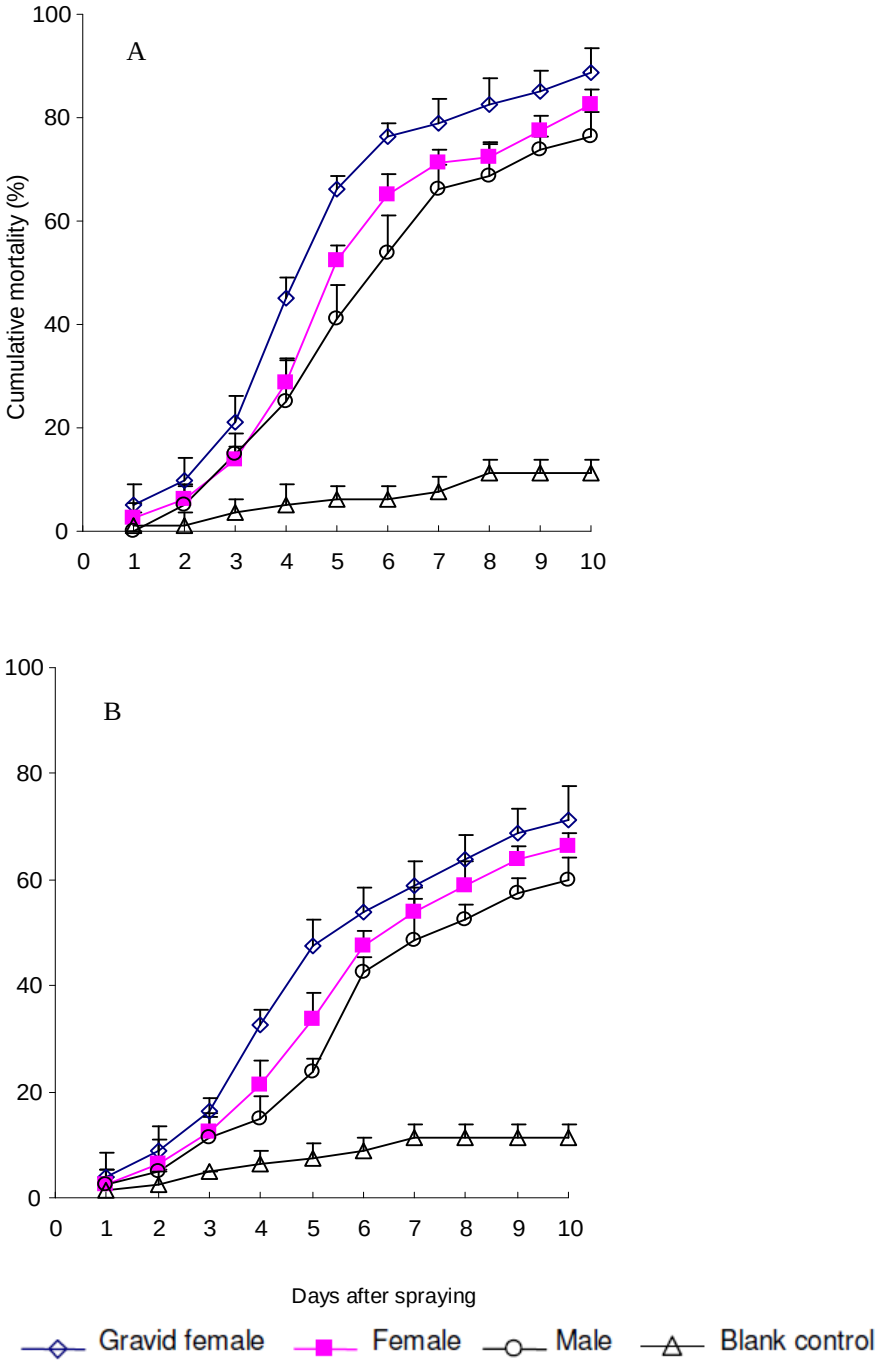

Figure 2. Cumulative mortalities of *Nilaparvata lugens* adults of male, female and gravid female after exposure to fungal sprays (A: *Metarhizium flavoviride* 82; B: *Metarhizium anisopliae* 20). Error bars represent the standard deviation (SD) for the means of four replicates.

isolates had the fastest killing speed of gravid female according to the LT_{50}, achieving 4.3 (3.7 ~ 4.9) and 5.7 (4.8 ~ 7.1) days, respectively (Table 3). The sequence of susceptibility was gravid female > female >male. The cumulative mortalities of gravid female, female and male increased rapidly from day 4 to day 6 after spraying and then smoothly from day 7 to day 10. The cumulative mortalities of the two isolates to gravid female were 88.7 and 71.3%, respectively in 10 days, which were significantly higher than female and male. The control treatment showed a very low level of mortality, up to 11.2% in 10 days.

Table 3. Cumulative mortality and median lethal time (LT50) caused by *Metarhizium flavoviride* (Mf82) and *Metarhizium anisopliae* (Ma20).

Treatment	Cumulative mortality (%) (Mean ± SD)				Median lethal time (d) LT_{50}	
	Mf82		Ma20			
	5 days	10 days	5 days	10 days	Mf82	Ma20
Gravid female	66.3 ± 2.5^{a}	88.7 ± 4.7^{a}	47.5 ± 5.0^{a}	71.3 ± 6.3^{a}	4.3	5.7
Female	52.5 ± 2.9^{b}	82.5 ± 2.9^{b}	33.8 ± 4.7^{b}	66.2 ± 2.5^{b}	4.7	6.3
Male	41.3 ± 6.2^{c}	76.3 ± 4.7^{bc}	23.7 ± 2.5^{c}	60.0 ± 4.1^{bc}	5.6	7.2
Control	6.3 ± 2.5^{d}	11.2 ± 2.5^{b}	6.3 ± 2.5^{d}	11.2 ± 2.5^{c}	--	--

Mean with same letter in the same column are not significantly different (P>0.05), by ANOVA followed by Duncan's test.

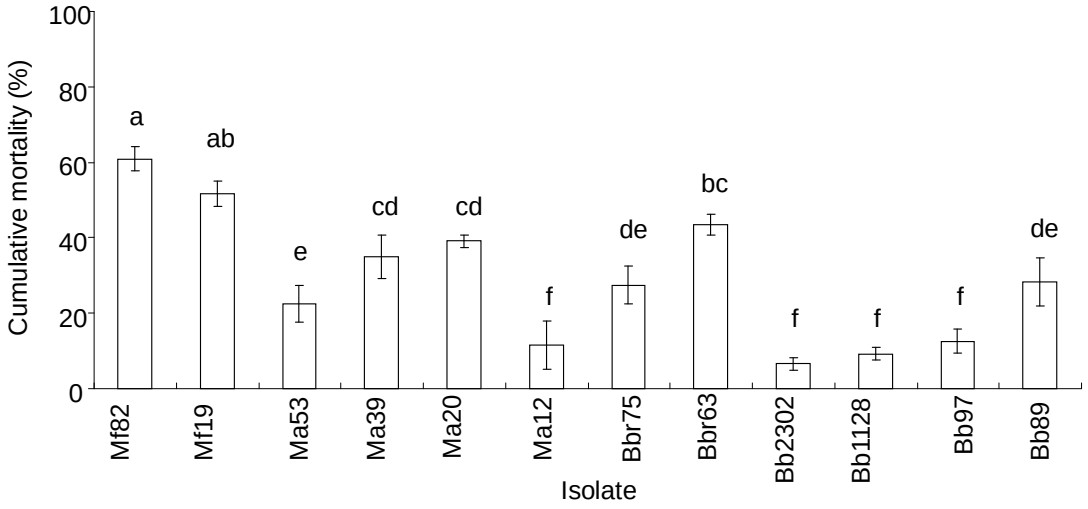

Figure 3. Comparison of *Nilaparvata lugens* eggs on day 10 after treatment with conidial suspension (1 x 10^{8} conidia/ml) of *Metarhizium* and *Beauveria* isolates. Error bars represent the standard deviation (SD) for the means of four replicates. Mean with same letter are not significantly different (P > 0.05), by ANOVA followed by Duncan's test.

In this study, we found that gravid female was more susceptible to Mf82 infection than the female and male. Homoplastically, Toledo et al. (2007) and Tafoya et al. (2004) assayed the virulence of different fungal species to different species of insects respectively, and found that proportionally more females than males were infected. The possible reason was that gravid female had larger abdominal surface area and more fat than male. By germinated conidia of *B. bassiana* and *M. anisopliae* penetrating the body surface of the planthopper *Peregrinus maidis*, the result showed that body fat was the most affected tissue (Toledo et al., 2010).

Effect of tested isolates on BPH eggs

The Isolates of *Beauveria* and *Metarhizium* could infect eggs of BPH, but the virulence of them differed significantly ($F_{11, 36}$ = 36.2, P < 0.01), the virulence of the two isolates *of M. flavoviride* to eggs was higher than the isolates of other fungi. The isolate Mf82 was most virulent to the eggs in all treated isolates, causing a cumulative mortality of 60.8% corrected with the control mortality (4.3%) 10 days after treatment (Figure 3).

The eggs laid on rice leaf sheath showed transparent and fresh (Figure 4A). Within first few days after treatment, eggs that subsequently became infected by the sprayed fungal pathogen had little change in color. But at 10 day after treatment, the infected eggs turned brown and completely shrunk, and a large number of conidia on the cuticle of eggs for Mf82. By contrast, the eggs in blank control hardly shrunk and never had fungal outgrowths on the surface at last (Figure 4). However, it is difficult to judge whether treated eggs were dead or not because of no movement of them, so by counting the hatchability and then converting it to mortality to assess the infection effects of isolate suspension on eggs of BPH accurately.

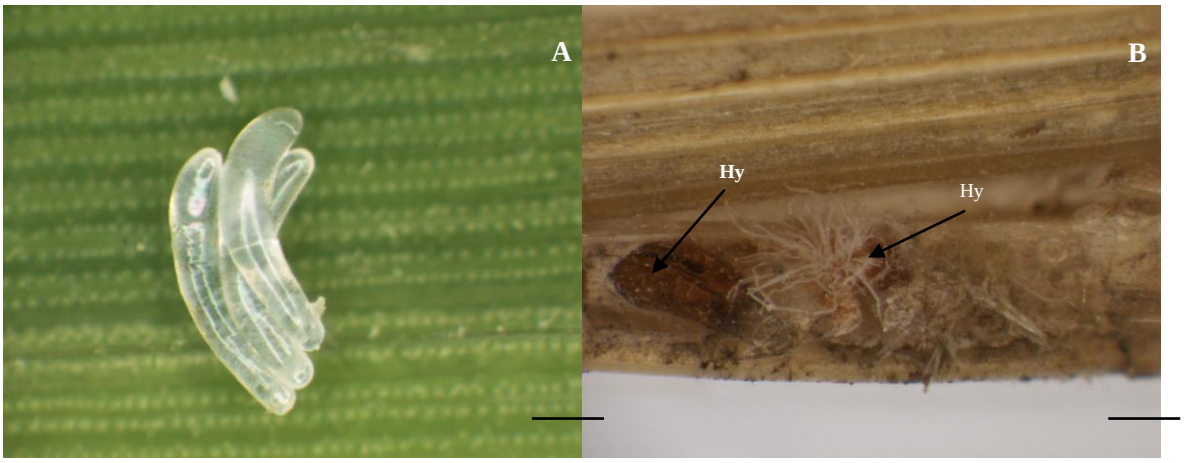

Figure 4. Infection of *Metarhizium flavoviride* (Mf82) to eggs of *Nilaparvata lugens* laid in the leaf sheath of rice. Fresh, transparent eggs (A). *N. lugens* eggs killed by *M. flavoviride* and the detail of their fungal outgrowths (B). Arrows indicate typical feature for the fungal species involved. Hy: hypha, Co: conidia. Bar: 200 μm.

Conclusion

The outcome of this study indicated that the isolate (Mf82) of *M. flavoviride* caused the highest mortality (82.1%) and the lowest LT_{50} (4.9 d) to the adults and eggs of BPH and showed significant differences from other isolates. Furthermore, Gravid female was more susceptible to Mf82 infection than the female and male. The isolates of *Beauveria* and *Metarhizium* could infect eggs of BPH, and the virulence of Mf82 to the eggs was the greatest, up to 60.8% cumulative mortality for 10 day. Thus, the isolate Mf82 obtained in this study provides a promising candidate for further development as environmentally friendly biocontrol agents for BPH.

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