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Full Length Research Paper

Efficiency of entomopathogenic fungi in the control of eggs of the brown planthopper *Nilaparvata lugens* Stål (Homopera: Delphacidae)

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The brown planthopper, *Nilaparvata lugens* Stål (Homopera: Delphacidae), is an important pest of rice. Although current research indicates that the fungus can infect the adult and nymph, there is no information on the efficacy of entomopathogenic fungi against the egg. In this study, the virulence of two isolates of *Metarhizium anisopliae*, and six isolates of *Beauveria* to *N. lugens* egg were tested in the laboratory. Different stage eggs were sprayed with a standard concentration of 1.0×10^8 conidia/ml. During a 12 day observation period after spray, the infected eggs shrunk in shape, then turned orange-brown for *Beauveria*, and eventually had outgrowths of the sprayed fungus when maintained under the condition of RH ≥ 95%. The 1 day old of *N. lugens* egg was the most sensitive to the *Beauveria* infection followed by the 2, and 3 day old. Only two *B. brongniartii* Bbr03, and Bbr09 isolates killed >50% of the eggs. Both isolates were further bioassayed against the eggs with sprays of 1×10^6, 1×10^7, and 1×10^8 conidia/ml with 4 replicates at each concentration. Based on the LC_{50} estimates determined by the concentration–mortality relationships of two isolates from probit analysis, *B. brongniartii* Bbr09 with an LC_{50} of 1.40×10^7 conidia/ml was highly infectious to *N. lugens* eggs, followed by *B. brongniartii* Bbr03 (2.97×10^7 conidia/ml). The results confirmed the ovicidal activity of the two fungal species and suggested the feasibility to search for more ovicidal isolates from fungal species that may serve as biocontrol agents against *N. lugens*.

Key words: *Beauveria bassiana*, *Beauveria brongniartii*, *Metarhizium anisopliae*, *Nilaparvata lugens*, ovicidal activity, microbial control.

INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* Stål, is a major sap-sucking pest of rice, *Oryza sativa* L, inducing symptoms commonly referred to hopperburn and causing substantial yield loss in most rice-producing countries (Heong et al., 1992; Backus et al., 2005; Song and Feng, 2011). Heretofore, neither was the biology or ecology clearly understood, nor was there an effective method of controlling this sucking pest other than using chemicals (Jin et al., 2008; Zhang et al., 2010). To date, it is well-known this pest has developed high resistance to a variety of chemical insecticides including neonicotinoid compounds, such as imidacloprid (Liu et al., 2006). In addition, a versatile neonicotinoid insecticide, imidacloprid, against sucking insects in the past decade may have increased outbreaks of brown planthoppers because it may act as a chemical for BPH fertility (Chung et al., 1982; Liu et al., 2003; Wang and Wang 2007). These problems therefore urge to search for alternatives to chemical control which are effective and safe to the environment.

In recent years, many trials using entomopathogens to control insect pests have been carried out (Milner, 1997; Faria and Wraight, 2001). Entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuellemin and *Metarhizium anisopliae* (Mets-chnikoff) Sorokin are

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Table 1. Fungal species screened against eggs of *Nilaparvata lugens*, original host and geographic origin.

Isolate code*	Original host insect	Geographic origin
Bb43	*Elaphiceps cervus* (Homoptera: Membraciade)	Suiyang,Guizhou
Bb97	*Pieris rapae* (Lepidoptera: Pieridae)	Xuancheng, Anhui
Bb119	*Lycorma delicatula* (Homoptera: Fulgoridae)	Nanjing, Jiangsu
Bb347	*Elaphiceps cervus* (Homoptera: Membraciade)	Hekou, Yunnan
Bbr03	*Oxya chinensis* (Orthoptera: Catantopidae)	Yanqing, Beijing
Bbr09	*Anomala corpulenta* (Coleoptera: Rutelidae)	Hefei, Anhui
Ma16	*Holotrichia oblita* (Coleoptera: Melolonthidae)	Hefei, Jiangsu
Ma22	*Scotinophara lurida* (Hemiptera: Pentatomidae)	Hangzhou, Zhejiang

* Isolate code using research center of entomopathogenic fugal number.

important biocontrol agents and have been formulated for application in insect pest management systems (Rombach et al., 1986; Vandenberg et al., 2001; Feng et al., 2004). These fungi may have potential for use in BPH control though they infect BPH infrequently in the field (Aguda et al., 1987; Samuels, et al., 1989; Toledo et al., 2008). However, research reports on the use of *Beauveria* and *Metarhizium* against BPH are limited. Previously, selection of global *Metarhizium* isolates for the control of the rice pest *N. lugens* in the greenhouse has been carried out (Jin et al., 2008). Effort also has been made to evaluate susceptibility of the planthopper pests *Peregrinus maidis* and *Delphacodes kuscheli* (Toledo et al., 2007) to numerous isolates of *B. bassiana*, *M. anisopliae*, and other fungal species under laboratory conditions. Recently, some studies were aimed at developing a laboratory bioassy system for efficient assessment of fungal biocontrol agents against pest eggs (Tounou et al., 2003; Ekesi et al. 2002; Shi and Feng, 2004; Ondiaka et al., 2008). However, it has not been reported on a possibility of such fungal pathogens to kill *N. lugens* eggs.

In this study, we evaluated the pathogenicity of different isolates of *B. bassiana*, *B. brongniartii*, and *M. anisopliae* to eggs of *N. lugens* in the laboratory to search for isolates of high ovicidal activity to serve as biocontrol agent of *N. lugens*. Two isolates of *B. bassiana* were further evaluated for their lethal effects on *N. lugens* eggs at different concentrations of conidia.

MATERIALS AND METHODS

Preparation of brown planthopper eggs

An experimental *N. lugens* colony was maintained on rice seeding in a growth chamber at 28±1°C and 12:12 L: D. To obtain *N. lugens* eggs of uniform age, 20 gravid female adults were arbitrarily taken from the plants of rice and transferred onto rice seeding with their roots surrounded by cotton and immersed in distilled water in a Petri dish (15 cm diameter), and allowed to freely lay eggs for 24 h. Subsequently, the females were removed, leaving a number of eggs/leaf for bioassays. The detached leaf system was maintained over 15 days so as to allow normal hatching of *N. lugens* eggs.

Sources of fungi

The fungal isolates of *M. anisopliae*, *B. bassiana* and *B. brongniartii*, used in the experiment, provided by Biocontrol Reserch Laboratory, Anhui Agricultural University (Hefei, China), were originally obtained from different hosts (Table 1). These isolates were preserved at -70°C prior to use. The conidia colonies were inoculated to SDAY (dextrose 40 g, peptone 10 g, yeast extract 10 g, and agar 20 g in 1000 ml H_2O) medium in petri dishes (9 cm diameter) and maintained at 25 ± 1°C in darkness for 15 d. Conidia were harvested from these surface cultures directly by scraping and dried through ventilation on a vacuum drier (VirTis Company, Gardiner, NY) at <35°C for 48 h. Dry conidia were preserved at 4°C in darkness for use as soon as possible in the following experiments, warranting ≥ 90% viability. Inoculate were suspended in sterile distilled water by shaking in 10 ml flasks containing 5 to 6 dozen glass beads (3 mm diameter). No surfactant (0.02% Tween-80) was added, since 5 min of agitation at 700 oscillations on a mechanical shaker to produce homogeneous suspensions of viable single conidium. The suspensions were then adjusted to defined concentrations according to the haemacytometer counts.

Bioassay procedures

Two different series of bioassays were performed. The first series included all 8 isolates of 3 fungi species (*B. bassiana*, *B. brongniartii*, and *M. anisopliae*). For each of the isolates, a high concentration of spore suspension (1×10^8 conidia/ml) was prepared in 0.02% Tween-80. The spore suspension was sprayed onto different age eggs (0.5, 1.5, and 2.5-day old) of the BPH among the rice leaves with a hand-held Micro Ulva sprayer (Micron Sprayers Limited, Herefordshire, UK). The sprayer was held 0.25 m above the bottom of Petri dishes (15 cm diameter). After 20 s spraying followed by a 5 min deposition period, the rice seedlings in Petri dishes were covered with lids and maintained in incubators at 25±1°C under a photophase of 14:10 L:D and RH ≥ 95%. The bioassay of each fungal isolate, including a blank control treatment (sprayed with 0.02% Tween-80), was repeated 4 times (25-45 eggs/ replicate in each bioassay). Daily examination for hatched eggs was made until no more eggs hatched for 3 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. All 8 isolates were bioassayed during a 2-month period.

The second series of bioassays included only those isolates that caused greater than 50% BPH egg mortality in the first series of experiment. Bioassays were carried out by using 3 concentrations

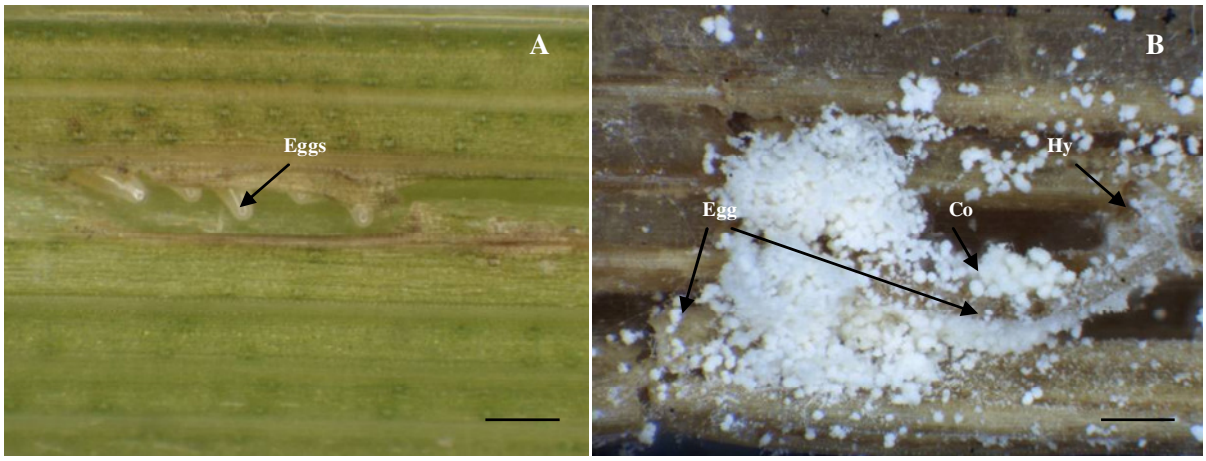

Figure 1. Infection of *Beauveria brongniartii* (Bbr09) to eggs of *Niloparvata lugens* lay in the leaf sheath of rice. (A) Fresh, transparent eggs. (B) Eggs killed by *B. brongniartii* and the detail of their fungal outgrowths. Arrows indicate typical feature for *B. brongniartii*. Hy: Hypha, Co: Conidia. Bar: 200 μm.

of dry conidia prepared as above were suspended in distilled water containing 0.02% Tween-80 and standardized at 1×10^6, 1×10^7, and 1×10^8 conidia/ml for the selected isolates (Bbr03 and Bbr09), respectively. In bioassay of each isolate, a spray of 0.02% Tween-80 was also included as a blank control (CK). Infection tests were conducted by direct spraying onto fresh eggs (1 day old) with 10 ml conidia suspensions in the tower apparatus. Each treatment was consisted of 25-45 newly fresh eggs. The bioassays were repeated 4 times. Daily examination for hatched eggs was made using the methods described above.

Data analysis

Percent BPH eggs mortalities (M_1) observed in the first bioassays were corrected with those in blank controls (M_2) using Abbott's formula $M_C = (M_1 - M_2)/(100 - M_2)$. The corrected mortalities transformed as $\sin^{-1}\sqrt{M_C}$ was subjected to analysis of variance (ANOVA) between the tested isolates. Data from the second bioassays were corrected using Abbott's formula on a basis of natural background mortality of the eggs observed in the blank control and subjected to probit analysis, generating a concentration–mortality relationship for the estimates of LC_{50} and associated 95% confidence limits for each of the fungal isolates tested. All analyses were performed using updated DPS software (Tang and Feng, 2007).

RESULTS

Infection of fungal pathogens to *Nilaparvata lugens* eggs

The healthy *N. lugens* eggs laid in rice leaf sheath showed undamaged, translucent, glossy, and fresh (Figure 1 A). After three days' exposure to a concentration of 1×10^8 conidia/ml at 25 ± 1°C under a photoperiod of 12 L:12 D. Subsequently, eggs that became infected by fungal conidia had little change in color, but hyphae outgrew inside eggs when observed under a dissecting microscope. If continued to be observed, banana-shaped, transparent and glossy eggs turned orange-brown for *Beauveria*. Eggs completely shrunk, turned brown and a large number of conidia were produced for *Beauveria* isolates at last. By contrast, eggs of blank control hardly shrunk and never had fungal outgrows (Figure 1A and B).

Virulence of *Beauveria* and *Metarhizium* to *Nilaparvata lugens* eggs

Virulence of *Beauveria* and *Metarhizium* isolates to different age eggs of BPH were shown in Table 2. When the egg age was 1 day, the mortalities corrected with the control mortalities ranged from 19.2 to 62.5% on 12 day after spray. Isolates Bbr09 and Bbr03 were the most effective, with mortality rates of 62.5 and 55.8%, respectively. When the egg age was 2 and 3 days, only one isolate, Bbr09 killed more than 50% of the eggs.

Concentration and mortality relationship

The two isolates Bbr03 and Bbr09, which killed more than 50% of BPH eggs in the first series of bioassays, were further tested with three conidial concentrations. Cumulative mortalities of eggs increased with conidial concentration (Figure 2). Figure 2 shows that *N. lugens* eggs did not hatch until 7 days after treatment in all bioassays and then hatched gradually, followed by a peak hatch rate on day 11. Hatch rates between fungal and control treatments were greatly differed on day 11 after treatment, and hatch rates of fungal treatments decreased with the increasing concentrations of conidia, whereas the hatch rates in the control were consistently the highest in two experiments (Figure 2).

Fungal sprays resulted in final mortalities of 33.8, 47.5 and 62.5% for Bbr09. The isolate Bbr03 resulted in 31.3, 43.8 and 55% mortality. By contrast, the mortalities observed in the blank controls averaged 7.5% for Bbr09 and 5.0% for Bbr03, both values being significantly lower than those caused by fungal treatment. The test for the goodness of fitting indicates no significant heterogeneity for two fungal isolates tested (small or very small χ^2 values, $P > 0.05$). Based on the estimates of the LC_{50} and associated 95% CI (Table 3), the two isolates Bbr09, and Bbr03 with the LC_{50}s of 1.4×10^7, and 3.6×10^7 conidia/ml were infectious to *N. lugens* eggs.

DISCUSSION

This is the first report on the virulence of fungal isolates to *N. lugens* eggs. As presented above, different fungal species or isolates varied in ability to infect *N. lugens* eggs. In three fungal species, *B. bassiana*, *B. brongniartii*

Table 2. Mortality of the tested *Beauveria bassiana*, *Beauveria brongniartii* and *M. anisopliae* isolates against different age eggs of *Nilaparvata lugens*.

Egg age (day)	Isolates	Number of eggs treated	Egg mortality on day 12 (%)
1	Bb43	127	29.5±7.9^{d}
	Bb97	124	46.9±8.7^{bc}
	Bb119	134	37.7±7.3^{cd}
	Bb347	140	48.6±6.9^{b}
	Bbr03	124	55.9±3.5^{ab}
	Bbr09	126	62.8±2.6^{a}
	Ma16	130	19.8±4.1e
	Ma22	125	20.0±6.9^{e}
	CK	125	8.7±2.0^{f}
	F test		$F_{8,27}$=38.75, P<0.01
2	Bb43	130	25.3±3.0^{c}
	Bb97	140	37.7±8.6^{b}
	Bb119	122	30.2±6.3^{bc}
	Bb347	138	35.8±6.0^{b}
	Bbr03	140	47.4±4.2^{a}
	Bbr09	137	50.8±7.1^{a}
	Ma16	127	16.8±4.2^{d}
	Ma22	130	17.4±5.7^{d}
	CK	127	4.9±2.2^{f}
	F test		$F_{8,27}$=33.12, P<0.01
3	Bb43	128	14.9±3.7^{d}
	Bb97	146	21.3±1.8^{c}
	Bb119	126	22.8±4.0^{bc}
	Bb347	125	20.8±1.7^{c}
	Bbr03	134	28.8±6.4^{ab}
	Bbr09	125	32.5±5.0^{a}
	Ma16	129	9.5±2.7^{e}
	Ma22	127	10.9±3.5^{de}
	CK	133	3.8±1.3^{f}
	F test		$F_{8,27}$ = 26.59, P<0.01

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

and *M. anisopliae*could could infect *N. lugens* eggs. The isolate of *B. brongniartii* Bbr09 was found to be the highest virulence to BPH eggs among the 8 fungal isolates tested.

Impact of the same isolate on the egg mortalities largely depended on the conidial concentrations sprayed. As demonstrated by the results of this study, the lethal concentrations of *B. brongniartii* Bbr09 and Bbr03 isolates sufficient to kill 50 % of the treated 1 day age eggs (LC_{50}) were 1.40×10^{7} and 2.97×10^{7} conidia/ml, respectively. *B. brongniartii* Bbr09 isolate at 1.0×10^{8} conidia/ml had shown a highest pathogenicity against BPH eggs compared with that recorded at 1.0×10^{6} and 1.0×10^{7} conidia/ml. Islam et al. (2010) reported, in congruence with the present study, that one isolate of *B. bassiana* had shown a significantly lower LC_{50} value based on dose variation in mortality. Chikwenhere and Vestergaard (2008) reported the mortality of *Neochetina bruchi* Hustache egg increased as the conidia concentrations of *B. bassiana* isolate increased. This result is in the same with the present study.

Infection of entomopathogenic fungi to *N. lugens* eggs was concerned with instar. In this study, the 1 day old age of *N. lugens* eggs was the most sensitive to *B. bassiana* and *B. brongniartii* infection when compared with the 2 and 3 day-old age. *N. lugens* could secret honeydew on oviposition mark when they lay eggs on rice plants, the water and nutrients in honeydew promoted the germination of fungal spores and further infection of fungal hyphae to *N. lugens* eggs. But with the extension of time, the honeydew would lose water and solidificate, which would influence the germination of fungal spores.

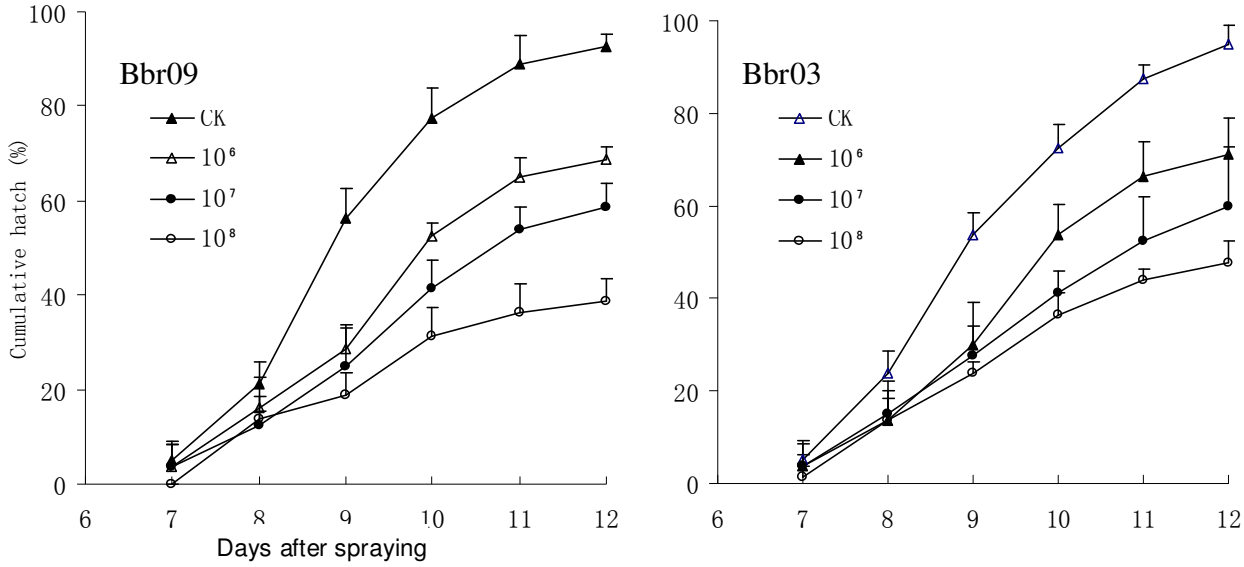

Figure 2. Trends in the hatch rates of *Nilaparvata lugens* eggs at 25℃ and 12:12 L: D after exposure to three concentrations (conidia/ml) of *Beauveria brongniartii* (Bbr) isolates. Error bars: SD.

Table 3. Outputs of probit analysis on target mortality date on day 12 due to infection with *Beauveria brongniartii* isolates against *Nilaparvata lugens* eggs.

Isolate	Intercept	Slope	χ^2	Mean lethal concentration (LC_{50}) with 95% CI (conidia/mm^2)			Correlation co-efficient
				Lower	LC_{50}	Upper	
Bbr09	4.52	0.37	0.81	1.30×10^7	1.40×10^{7a}	1.51×10^7	0.999
Bbr03	4.52	0.31	1.53	3.66×10^7	2.97×10^{7b}	4.51×10^7	0.998

In the present study, the egg mortalities caused by entomopathogenic fungi were lower than that of nymphs and adults when compared with other studies (Roberts, 1981; Abdo et al., 2008; Espinel et al., 2009; Mochi et al., 2010). First, the egg stage is generally believed to be more resistant to infection of entomopathogenic fungi than the other stages. For example, the study on the susceptibility of *Heliothis armigera* Hubner to *M. anisopliae* showed that eggs were not susceptible to fungal infection (Gopalakrishnan and Narayanan, 1989). Second, the small size of *N. lugens* eggs (1 mm in length) in the rice leaf sheath orderly made them infected not easily. Based on the data presented here, *B. brongniartii* Bbr09 was the most promising candidate for use in the management of *N. lugens*. Mass production of *B. brongniartii* is easy and cheap and does not require high input technology (Feng, 1994). Additionally, the fungus can be formulated and applied in a variety of ways, and therefore could provide a novel alternative to the use of chemical insecticides for the control of these pests. Since the number of isolates included in this study was limited for each of the 3 fungal species, a search for more ovicidal isolates of *B. brongniartii*, *B. bassiana* and *M. anisopliae* or other fungal species can be performed by means of the egg bioassay system developed in our study.

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