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ORIGINAL ARTICLE

Efficacy of Entomopathogenic Fungi Against Brown Planthopper *Nilaparvata Lugens* (Stål) (Homoptera: Delphacidae) Under Controlled Conditions

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Abstract

The current study was conducted to evaluate the efficacy of entomopathogenic fungi (EPF) as an alternative strategy for the sustainable control of *Nilaparvata lugens*. Three species of EPF, *Beauveria bassiana, Metarhizium anisopliae* and *Lecanicillium lecanii,* were tested against *N. lugens* with two suspensions of each tested EPF applied in different treatments. The observed mortality of *N. lugens* adults during the overall exposure period for the lowest and highest concentrations of each EPF ranged from 0–100%. At both highest and lowest concentrations of EPF, when sprayed on adult, the mortality of *N. lugens* was higher as compared to when sprayed on the stem pieces as food. A higher mortality rate was observed when the stem pieces were absent than when stem pieces were present. Maximum percent mortality of *N. lugens* was recorded due to the spray of highest concentration of *M. anisopliae* on only stem pieces (82.67%) and on adult *N. lugens* provided with stem pieces as food (93.33%) after 14 days of exposure interval. On the other hand, *B. bassiana* and *L. lecanii* were also responsible to cause more than 50% mortality of *N. lugens* in both treatments after 14 days of exposure interval. Results also indicated that maximum and minimum percent mycosis and sporulation from dead cadavers of *N. lugens* were recorded in both conidial suspensions of *L. lecanii* and *M. anisopliae*, respectively, in all treatments. The high efficacy levels recorded in the current study indicates that *M. anisopliae* can be effective biological control agents against *N. lugens*.

Keywords Rice stem · *Nilaparvata lugens* · *Beauveria bassiana* · *Metarhizium anisopliae* · *Lecanicillium lecanii* · Biocontrol agents · Virulence

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Wirksamkeit entomopathogener Pilze zur Bekämpfung der Braunrückigen Reiszikade *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) unter kontrollierten Bedingungen

Zusammenfassung

Die aktuelle Studie wurde durchgeführt, um die Wirksamkeit entomopathogener Pilze (EPF) als alternative Strategie zur nachhaltigen Bekämpfung von *Nilaparvata lugens* zu bewerten. Drei EPF-Arten, *Beauveria bassiana, Metarhizium anisopliae* und *Lecanicillium lecanii*, wurden gegen *N. Lugens* getestet, wobei zwei Suspensionen von jedem getesteten EPF in verschiedenen Behandlungen angewendet wurden. Die beobachtete Mortalität von adulten *N. lugens* während des gesamten Expositionszeitraums für die niedrigste und höchste Konzentration jedes EPF lag zwischen 0 und 100 %. Sowohl bei der höchsten als auch bei der niedrigsten EPF-Konzentration war die Mortalität von *N. lugens* beim Sprühen auf Adulte höher als beim Sprühen auf Stängelteile als Nahrung. Die Mortalität war höher, wenn Stängelteile vorhanden waren als wenn keine Stängelteile vorhanden waren. Die maximale prozentuale Mortalität von *N. lugens* wurde nach 14-tägiger Exposition erreicht durch das Sprühen mit der höchsten Konzentration von *M. anisopliae* nur auf Stängelteile (82.67 %) und auf adulte *N. lugens*, die mit Stängelteilen als Nahrung (93.33 %) versorgt wurden. Andererseits waren *B. bassiana* und *L. lecanii* auch dafür verantwortlich, dass bei beiden Behandlungen nach 14 Tagen Exposition eine Mortalität von mehr als 50 % bei *N. lugens* auftrat. Die Ergebnisse zeigten auch, dass die maximale und minimale prozentuale Mykose und Sporulation von toten Körpern von *N. lugens* in beiden konidialen Suspensionen von *L. Lecanii* und *M. anisopliae* bei allen Behandlungen registriert wurden. Die in der aktuellen Studie festgestellte hohe Wirksamkeit weist darauf hin, dass *M. anisopliae* ein wirksames biologisches Bekämpfungsmittel gegen *N. lugens* sein kann.

Schlüsselwörter Reisstängel · *Nilaparvata lugens* · *Beauveria bassiana* · *Metarhizium Anisopliae* · *Lecanicillium lecanii* · Biokontrollmittel · Virulenz

Introduction

Brown planthopper, *Nilaparvata lugens*, is a typical phloem sap feeder and one of the most serious and devastating rice pests in Asia (Normile 2008; Heong and Hardy 2009). In the case of epidemics, *N. lugens* causes losses of up to 60% (Srivastava et al. 2009; Kumar et al. 2012). In the rice blanket for Pakistan, the rice fields are prone to the attack of pests that may decrease the yield up to 7–10% annually. Sabir et al. (2019) stated that the average grain yield loss due to the attack of *N. lugens* was 10 maund/acre, amounting to 100 US-$/acre during the growing season 2017/2018.

It is difficult to monitor such pests regularly because both the nymphs and adults suck sap from the leaves and leaf sheaths of rice so that infested leaves become yellow, in addition to the reduction in plant tellering and plant height with more number of unfilled grain. Infestation with *N. lugens* also causes a decrease in chlorophyll and protein content of the leaves and the rate of photosynthesis, while the severe attack of *N. lugens* produces symptoms of 'hopper burn' (Liu et al. 2008; Horgan 2009; Vanitha et al. 2011). It also transmits viral diseases such as ragged stunt (Baehaki et al. 2017), grassy stunt (Chomchan et al. 2002) and wilted stunt (Chen et al. 1978).

Although many chemicals are recommended to control this pest (Sarao 2015), farmers are unable to control this pest effectively because of their feeding behavior at the lower part of the plant. As a result, farmers use large number of insecticides that often destroy the ecological balance of rice ecosystem and the treated insects become more resistant to insecticide application (Gorman et al. 2008; Matsumura et al. 2009). Extensive chemical use against *N. lugens* on rice can lead to serious problems, toxicity to natural enemies of brown planthopper (Wang et al. 2008), an increase in total production costs and possible long-term damage to the agro-ecosystem and human health (Rola and Pingali 1993; Huang et al. 2000).

Many entomopathogenic fungi (EPF) have been commercialized and offer environmentally safe and economically viable chemical control alternatives for many insect pests (Wraight et al. 2000; Neves et al. 2001; Scholte et al. 2005; Rizwan et al. 2019a, 2019b). Currently, *Beauveria bassiana, Metarhizium anisopliae* and *Metarhizium flavoviride*, after intensive research, have the potential to develop biological control of sucking pests such as aphids (Yeo et al. 2003; Saranya et al. 2010; Jandricic et al. 2014), leaf hoppers (Tounou et al. 2003; Pu et al. 2005; Toledo et al. 2011), and plant hopper (Li et al. 2012a, 2014; Shaikh and Mohite 2015; Mohan et al. 2016).

Although there are many reports of the use of entomopathogenic fungi in various treatments of insect pests, there is very little information on the simultaneous evaluation of their application methods against *N. lugens*. Reddy et al. (2013) reported that *M. anisopliae* and *B. bassiana* were found effective against *N. lugens* and less toxic to predators under field condition. Li et al. (2012b) also reported that isolates of *B. bassiana* were found highly infectious to *N. lugens* eggs.

The objectives of the present study were to test the insecticidal efficacy of *B. bassiana, M. anisopliae* and *L. lecanii* against *N. lugens* under different treatments under controlled conditions by using various concentrations of conidial suspension.

Materials and Methods

Plant Material

Fine rice Basmati-515 variety was used to evaluate the pathogenicity of entomopathogens (*B. bassiana, M. anisopliae* and *L. lecanii*) against *N. lugens*. Rice seedlings of 10-day-old were transplanted in plastic pots (20 × 20 cm), and grown for another 10 days under controlled conditions (25 ± 5 °C; 80% R. H. and 14:10 hrs. L:D photoperiod) for being used in bioassays.

Insect Material

Adult insects of *N. lugens* were used in the study. A stock culture of the *N. lugens* was maintained following Heinrichs and Medrano (1985) method. Adults of *N. lugens* were collected from the rice fields in Rice Research Institute, Kala Shah Kaku, Pakistan (31° 43′ 17″ N, 74° 16′ 14″ E) and released onto potted rice plants. The gravid adults were removed from these plants and released onto individual potted plants kept inside the oviposition cages (120 × 80 × 50 cm) for egg laying. These eggs developed into adult *N. lugens* at 25 ± 5 °C and 14:10 hrs. L:D photoperiod. Adults of 10-day-old were collected from the rice seedlings for bioassay. For this purpose, the adults were transferred into petri dishes.

Entomopathogenic Fungi

Three commercial formulations of the entomopathogenic fungi *Beauveria bassiana, Metarhizium anisopliae* and *Lecanicillium* (*Verticillium*) *lecanii* in talc powder were purchased from AgriLife SOM Phytopharma (India) Limited (www.agrilife.in). Two concentrations of each EPF formulation were tested against *N. lugens*: 1×10^8 and 1×10^6 CFU/ml. The concentration of fungal conidia in the conidial suspension was checked by using a hemocytometer. Potato Dextrose Agar (PDA) was used to determine the conidial germination. The conidial germination was measured, based on the counts of 200 random conidia per plate, 18 h post incubation at 25 ± 2 °C (Ayala-Zermeño et al. 2015; Rizwan et al. 2019b).

Preparation of Conidial Suspension

The conidial suspension of 1×10^6 conidia/ml of all tested EPF was prepared according to the method followed by Ali et al. (2018) by dissolving 1 g of EPF formulation in 100 ml of distilled water.

Bioassays

Three experimental treatments with the conidial suspensions of EPF were applied in bioassays as follows:

i. Pieces of rice stem were sprayed with the conidial suspensions of EPF then placed in petri dishes containing adults of *N. lugens*.
ii. Adults of *N. lugens* were sprayed with the conidial suspensions of EPF then placed in petri dishes without pieces of rice stem.
iii. Adults of *N. lugens* were sprayed with the conidial suspensions of EPF then placed in petri dishes with untreated pieces of rice stem.

The technique of treatment of rice stem pieces with the conidial suspensions of EPF was applied as follows: (i) applying 1 ml of the conidial suspension of each concentration of each EPF per replicate, (ii) spraying of the conidial suspension was carried out by the help of a Pump Pressure Sprayer (Hommold, Lahore) and then cleaned with acetone after application of each concentration. The sprayed stem pieces were then transferred into petri dishes (with 1.5 cm diameter holes in the middle), containing moistened filter paper at their bottom, to maintain the freshness and turgidity of stem pieces.

Adults of *N. lugens* were treated with the conidial suspensions of EPF according to the following technique: (i) placing the adults in cylindrical plastic jar (24 × 14 × 14 cm) with a top covered with muslin cloth for aeration and to prevent insects from escape, and held for 30 min to reduce their movement (Hluchý and Samšiňáková 1989), (ii) applying 1 ml of each suspension (one suspension for each concentration of each of the EPF) by the help of same device mentioned previously, (iii) transferring the treated adults into petri dishes by aspirator, then the dishes were sealed with teflon tape to prevent the insects from escaping and placed in incubator at 27 °C and 65% R. H. during the entire experimental period.

Assessment or Evaluation of the Treatment Efficacy

Adult mortality was determined by prodding with a camel hair brush to detect movement under stereomicroscope (Cole-Parmer 625 East Bunker Court Vernon Hills, IL60061 USA) after 1, 2, 6, 7, 10 and 14 days of exposure. Mycosis percentage was recorded from the dead cadavers of

N. lugens after 14 days of exposure interval. These cadavers were preserved in sterile petri plates, washed thrice in sterile dH_2O and surface sterilized by 0.05% sodium hypochlorite solution for 2–3 min. These cadavers were shifted to Sabouraud dextrose agar (SDA) plates for incubation at 25 ± 2 °C, 75 ± 5 RH for 7 days to detect the external white fungal growth under a stereomicroscope (Cole-Parmer 625 East Bunker Court Vernon Hills, IL, 60061, USA) (Beris et al. 2013; Rizwan et al. 2019a). Mycosed cadavers from each replication were mixed in a beaker with a drop of Tween 80 with 20 ml of dH_2O for assessing sporulation (Tefera and Pringle 2003; Rizwan et al. 2019a). The solution was carefully stirred and the number of conidia was counted by using a haemocytometer under the stereomicroscope (Rizwan et al. 2019a).

The main variables studied here were the treatment, concentration and exposure interval Interaction between these variables such as treatment × concentration, exposure interval × treatment, exposure interval × concentration and exposure interval × treatment × concentration were taken in consideration. The percent mortality was the response of the variables.

Statistical Analysis

The mortality and mycosis rate was converted into percent. Data was analyzed using Statistix software (version 8.1) (Tallahassee, FL). Three-way ANOVA was applied to factorial experiments in CRD for percent mortality, percent mycosis and sporulation from dead cadavers of *N. lugens* to understand the main and interactions of the variables. The means were compared with Tukey's HSD means separation test at $P < 0.05$ (Sokal and Rohlf 1995).

Results

Treatment Effect of *Beauveria Bassiana* Against the Adults of *Nilaparvata Lugens*

Mortality of *N. lugens* adults was significantly affected by the exposure interval ($F_{5,\ 179} = 214.41$, $P < 0.01$). Repeated measures variables, for main effects and their associate interactions, are presented in Table 1. Percent mortality of adult *N. lugens* was low for EFP sprayed on only stem pieces and sprayed on adults (with stem pieces) treatments and concentrations after 2 days exposure interval and did not exceed 10%. Percent mortality was significantly increased at 2 days exposure interval when *B. bassiana* was sprayed on adults (without stem pieces), while 6 days exposure interval when *B. bassiana* was sprayed on adults (with stem pieces) as compared with when it was sprayed on only stem pieces. All adults died in both concentration treatments at 6 days exposure interval in the absence of stem pieces. Percent mortality of adult *N. lugens* was significantly increased after 10 days exposure interval and exceeded 25% in both concentration in which the *B. bassiana* was sprayed on only stem pieces (Table 2).

Table 1 Repeated measures MANOVA parameters for main effects and associated interactions for mortality levels of adult *Nilaparvata lugens*

Fungus species	Source	*Df*	*F*	*P*
Between exposure intervals				
Beauveria bassiana	Treatment	2	310.52	<0.01
	Concentration	1	8.80	<0.01
	Treatment × Concentration	2	0.87	0.42
Metarhizium anisopliae	Treatment	2	322.33	<0.01
	Concentration	1	5.72	<0.05
	Treatment × Concentration	2	1.01	0.37
Lecanicillium lecanii	Treatment	2	373.61	<0.01
	Concentration	1	8.06	<0.01
	Treatment × Concentration	2	3.62	<0.05
Within exposure intervals				
Beauveria bassiana	Exposure interval × Treatment	10	28.82	<0.01
	Exposure interval × Concentration	2	0.41	0.85
	Exposure interval × Treatment × Concentration	10	0.55	0.85
Metarhizium anisopliae	Exposure interval × Treatment	10	27.04	<0.01
	Exposure interval × Concentration	2	0.44	0.82
	Exposure interval × Treatment × Concentration	10	0.28	0.99
Lecanicillium lecanii	Exposure interval × Treatment	10	27.40	<0.01
	Exposure interval × Concentration	2	0.88	0.50
	Exposure interval × Treatment × Concentration	10	0.39	0.95

MANOVA multivariate analysis of variance, *Error df* = 140 for all EPF, *Total df* = 179 for all EPF

Table 2 Percent mortality (Mean ± SE, $n = 5$) of *Nilaparvata lugens* adult exposed to *Beauveria bassiana* in two concentrations by three treatments at different exposure intervals

Exposure interval (days)	Concentration (Conidia/ml)	Conidial suspension of EPF sprayed on stem pieces	Conidial suspension of EPF sprayed on adults of *N. lugens*		*F*	*P*
			Without stem pieces	With stem pieces		
1	1×10^6	0.00 ± 0.00j	0.00 ± 0.00j	2.67 ± 0.73j	2.67	0.11
	1×10^8	0.00 ± 0.00j	0.00 ± 0.00j	4.00 ± 1.19j	2.25	0.15
F		–	–	0.18		
P		–	–	0.68		
2	1×10^6	0.00 ± 0.00j	42.67 ± 6.57defgh	6.67 ± 1.33ij	7.03	<0.01
	1×10^8	1.33 ± 0.60j	49.33 ± 5.86cdefg	9.33 ± 1.79ij	10.5	<0.01
F		1.00	0.11	0.29		
P		0.35	0.75	0.61		
6	1×10^6	0.00 ± 0.00j	100.00 ± 0.00a	16.00 ± 3.07hij	184	<0.01
	1×10^8	2.67 ± 0.73j	100.00 ± 0.00a	21.33 ± 5.53ghij	51.5	<0.01
F		2.67	–	0.14		
P		0.14	–	0.72		
7	1×10^6	2.67 ± 1.19j	100.00 ± 0.00a	34.67 ± 4.15fghi	79.1	<0.01
	1×10^8	5.33 ± 1.74ij	100.00 ± 0.00a	46.67 ± 4.90cdefg	50.0	<0.01
F		0.32	–	0.70		
P		0.59	–	0.43		
10	1×10^6	25.33 ± 5.28fghij	100.00 ± 0.00a	53.33 ± 4.22cdef	18.7	<0.01
	1×10^8	40.00 ± 6.53efgh	100.00 ± 0.00a	66.67 ± 4.42bcde	8.71	<0.01
F		0.61	–	0.95		
P		0.46	–	0.36		
14	1×10^6	65.33 ± 5.77bcde	100.00 ± 0.00a	76.00 ± 5.22abc	3.13	0.09
	1×10^8	72.00 ± 5.02abcd	100.00 ± 0.00a	86.67 ± 3.13ab	3.36	0.07
F		0.15	–	0.62		
P		0.71	–	0.46		

Within each column, means with different letters are significantly different; in all cases, $df = 1, 9$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

Within each row, means with different letters are significantly different; in all cases, $df = 2, 14$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

– no analysis was performed

Treatment Effect of *Metarhizium Anisopliae* Against the Adults of *Nilaparvata Lugens*

Mortality of *N. lugens* adults was significantly affected by the exposure interval ($F_{5, 179} = 172.92$, $P < 0.01$). Repeated measures variables, for main effects and their associate interactions, are presented in Table 1. Percent mortality of adult *N. lugens* was low for EFP sprayed on only stem pieces and sprayed on adults (with stem pieces) treatments and concentrations after 2 days exposure interval and did not exceed 15%. Percent mortality was significantly increased at 2 days exposure interval when *M. anisopliae* was sprayed on adults (without stem pieces), while 6 days exposure interval when *M. anisopliae* was sprayed on adults (with stem pieces) as compared with when it was sprayed on only stem pieces. All adults died in both concentration treatments at 6 days exposure interval in the absence of stem pieces. Percent mortality of adult *N. lugens* was significantly increased after 10 days exposure interval and exceeded 30% in both concentration in which the *M. anisopliae* was sprayed on only stem pieces (Table 3).

Treatment Effect of *Lecancillium Lecanii* Against the Adults of *Nilaparvata Lugens*

Mortality of *N. lugens* adults was significantly affected by the exposure interval ($F_{5, 179} = 135.51$, $P < 0.01$). Repeated measures variables, for main effects and their associate interactions, are presented in Table 1. Percent mortality of adult *N. lugens* was low for EFP sprayed on only stem pieces and sprayed on adults (with stem pieces) treatments and concentrations after 2 days exposure interval and did not exceed 7%. Percent mortality was significantly increased at 2 days exposure interval when *L. lecanii* was sprayed on adults (without stem pieces), while 6 days exposure interval when *L. lecanii* was sprayed on adults (with

Table 3 Percent mortality (Mean ± SE, $n = 5$) of *Nilaparvata lugens* adult exposed to *Metarhizium anisopliae* in two concentrations by three treatments at different exposure intervals

Exposure interval (days)	Concentration (conidia ml^{-1})	Conidial suspension of EPF sprayed on stem pieces	Conidial suspension of EPF sprayed on adults of *N. lugens*		*F*	*P*
			Without stem pieces	With stem pieces		
1	1×10^6	0.00 ± 0.00j	0.00 ± 0.00j	4.00 ± 1.19ij	2.25	0.15
	1×10^8	0.00 ± 0.00j	0.00 ± 0.00j	6.67 ± 1.63hij	3.33	0.08
F		–	–	0.35		
P		–	–	0.58		
2	1×10^6	0.00 ± 0.00j	54.67 ± 5.77def	9.33 ± 1.79hij	14.1	<0.01
	1×10^8	4.00 ± 1.19ij	66.67 ± 5.58bcde	13.33 ± 2.11hij	18.5	<0.01
F		2.25	0.45	0.42		
P		0.17	0.52	0.54		
6	1×10^6	1.33 ± 0.60j	100.00 ± 0.00a	25.33 ± 4.46ghij	78.4	<0.01
	1×10^8	8.00 ± 1.74hij	100.00 ± 0.00a	32.00 ± 5.77fghi	37.7	<0.01
F		2.63	–	0.17		
P		0.14	–	0.69		
7	1×10^6	5.33 ± 1.12ij	100.00 ± 0.00a	46.67 ± 4.52efg	62.3	<0.01
	1×10^8	10.67 ± 1.52hij	100.00 ± 0.00a	57.33 ± 4.86cdef	46.2	<0.01
F		1.60	–	0.52		
P		0.25	–	0.50		
10	1×10^6	34.67 ± 5.61fgh	100.00 ± 0.00a	69.33 ± 4.86bcde	11.6	<0.01
	1×10^8	56.00 ± 6.64def	100.00 ± 0.00a	76.00 ± 4.28abcd	4.67	<0.05
F		1.20	–	0.21		
P		0.30	–	0.66		
14	1×10^6	76.00 ± 4.77abcd	100.00 ± 0.00a	85.33 ± 4.26abc	2.15	0.16
	1×10^8	82.67 ± 4.38abcd	100.00 ± 0.00a	93.33 ± 2.31ab	1.87	0.20
F		0.21	–	0.55		
P		0.66	–	0.48		

Within each column, means with different letters are significantly different; in all cases, $df = 1, 9$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

Within each row, means with different letters are significantly different; in all cases, $df = 2, 14$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

– no analysis was performed

stem pieces) as compared with when it was sprayed on only stem pieces. All adults died in both concentration treatments at 6 days exposure interval in the absence of stem pieces. Percent mortality of adult *N. lugens* was significantly increased after 10 days exposure interval and exceeded 15% in both concentration in which the *L. lecanii* was sprayed on only stem pieces (Table 4).

Comparison of the Treatment Effects of Three Entomopathogenic Fungi Against the Adult *Nilaparvata Lugens*

The maximum percent mortality of adults of *N. lugens* caused at higher concentration of *B. bassiana, M. anisopliae* and *L. lecanii* after 14 days of exposure were 86.67, 93.33 and 80.00%, respectively, when adults were sprayed with the conidial suspension of EPF and feed on untreated pieces of rice stem. While maximum percent mortality of adults of *N. lugens* caused at same concentration of *B. bassiana, M. anisopliae* and *L. lecanii* after 14 days of exposure were 72.00, 82.67 and 54.67%, respectively when only stem pieces were sprayed with conidial suspension of EPF. *M. anisopliae* was responsible for highest mortality of *N. lugens* as compared with *B. bassiana* and *L. lecanii* (Fig. 1).

Mycosis and Sporulation from the dead Cadavers of *Nilaparvata Lugens*

The main effect of treatment methods of all entomopathogenic fungi on mycosis ($F_{2,\ 89} = 27.95$) and sporulation ($F_{2,\ 89} = 180.92$) from dead cadavers of *N. lugens* was highly significant ($P < 0.01$). Similarly the main effect of all entomopathogenic fungi on mycosis ($F_{2,\ 89} = 137.92$) and sporulation ($F_{2,\ 89} = 841.64$) was highly significant ($P < 0.01$). Likewise the main effect of different concentrations of entomopathogenic fungi on mycosis ($F_{1,\ 89} = 45.41$) and sporu-

Table 4 Percent mortality (Mean ± SE, $n = 5$) of *Nilaparvata lugens* adult exposed to *Lecanicillium lecanii* in two concentrations by three treatments at different exposure intervals

Exposure interval (days)	Concentration (conidia ml^{-1})	Conidial suspension of EPF sprayed on stem pieces	Conidial suspension of EPF sprayed on adults of *N. lugens*		*F*	*P*
			Without stem pieces	With stem pieces		
1	1×10^6	0.00 ± 0.00g	0.00 ± 0.00g	1.33 ± 0.60g	1.00	0.40
	1×10^8	0.00 ± 0.00g	0.00 ± 0.00g	2.67 ± 1.19g	1.00	0.40
F		–	–	0.20		
P		–	–	0.67		
2	1×10^6	0.00 ± 0.00g	38.67 ± 6.76cde	4.00 ± 1.19g	5.76	<0.05
	1×10^8	0.00 ± 0.00g	40.00 ± 6.18cde	6.67 ± 1.63 fg	6.74	<0.05
F		–	0.00	0.35		
P		–	0.95	0.58		
6	1×10^6	0.00 ± 0.00g	100.00 ± 0.00a	6.67 ± 1.63 fg	703	<0.01
	1×10^8	1.33 ± g	100.00 ± 0.00a	17.33 ± 5.04efg	65.3	<0.01
F		1.00	–	0.81		
P		0.35	–	0.39		
7	1×10^6	1.33 ± 0.60g	100.00 ± 0.00a	22.67 ± 3.48defg	130	<0.01
	1×10^8	2.67 ± 1.19g	100.00 ± 0.00a	38.67 ± 4.56cde	65.4	<0.01
F		0.20	–	1.56		
P		0.67	–	0.25		
10	1×10^6	16.00 ± 3.84efg	100.00 ± 0.00a	36.00 ± 4.07cdef	36.9	<0.01
	1×10^8	29.33 ± 6.57cdefg	100.00 ± 0.00a	57.33 ± 4.68bc	11.7	<0.01
F		0.61	–	2.37		
P		0.46	–	0.16		
14	1×10^6	49.33 ± 6.01 cd	100.00 ± 0.00a	58.67 ± 5.11bc	7.01	<0.01
	1×10^8	54.67 ± 6.07bc	100.00 ± 0.00a	80.00 ± 3.13ab	6.65	<0.05
F		0.08	–	2.53		
P		0.79	–	0.15		

Within each column, means with different letters are significantly different; in all cases, $df = 1, 9$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

Within each row, means with different letters are significantly different; in all cases, $df = 2, 14$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

– no analysis was performed

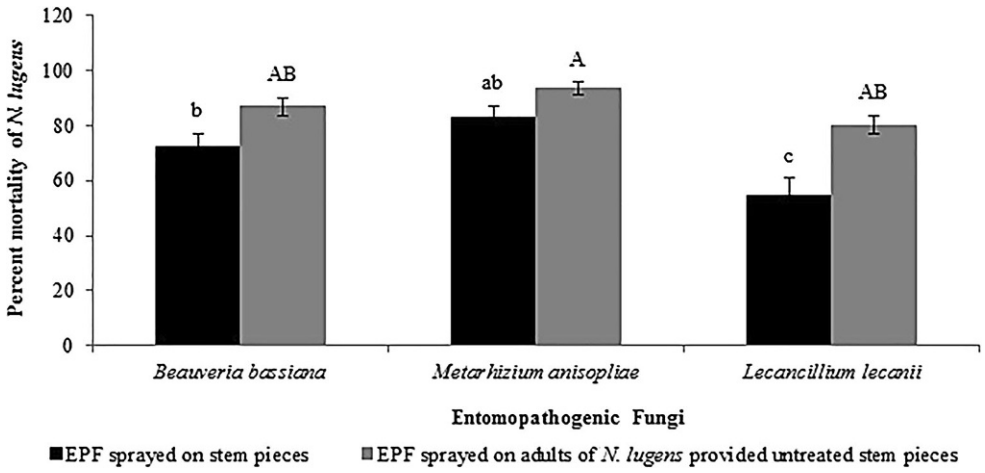

Fig. 1 Percent mortality (Mean ± SE, $n = 5$) of *Nilaparvata lugens* adult exposed to entomopathogenic fungi in higher concentration (1×10^8 conidia/ml) by two treatments after 14 days of exposure

Table 5 Repeated measures ANOVA parameters for main effects and associated interactions for mycosis and sporulation from dead cadavers of adult *Nilaparvata lugens*

Source	*Df*	Percent mycosis		Sporulation (conidia ml^{-1})	
		F	*P*	*F*	*P*
Treatment	2	27.95	<0.01	180.92	<0.01
Entomopathogenic fungi	2	137.92	<0.01	841.64	<0.01
Concentration	1	45.41	<0.01	363.52	<0.01
Treatment × Entomopathogenic fungi	4	2.79	<0.05	9.92	<0.01
Treatment × Concentration	2	0.39	0.68	0.43	0.65
Entomopathogenic fungi × Concentration	2	0.39	0.68	1.51	0.23
Treatment × Entomopathogenic fungi × Concentration	4	2.23	0.08	16.78	<0.01

Error df = 68, Total *df* = 89

lation ($F_{1,\ 89} = 363.52$) was highly significant ($P < 0.01$). Moreover, highly significant effect of interactions among the treatment methods × entomopathogenic fungi were also observed ($P < 0.05$) on mycosis ($F_{4,\ 89} = 2.79$) and sporulation ($F_{4,\ 89} = 9.92$). Non-significant effect of interactions among the treatment methods × concentration and entomopathogenic fungi × concentration was observed ($P > 0.05$) on mycosis ($F_{2,\ 89} = 0.39$ and $F_{2,\ 89} = 0.43$, respectively) and sporulation ($F_{2,\ 89} = 0.39$ and $F_{2,\ 89} = 1.51$, respectively). Non-significant and significant effect of interactions among the treatment methods × entomopathogenic fungi × concentration was observed on mycosis ($F_{4,\ 89} = 2.23$, $P > 0.05$) and sporulation ($F_{4,\ 89} = 16.78$, $P < 0.01$), respectively (Table 5).

Maximum mycosis from dead cadavers of *N. lugens* was recorded in both conidial suspension of *L. lecanii* sprayed on adults with the provision of stem pieces as food (96.0 and 88.0%), while similar trend was also observed on other two treatments, conidial suspension of *L. lecanii* sprayed on adults without the provision of stem pieces as food (89.6 and 80.8%) and conidial suspension of *L. lecanii* sprayed on stem pieces (84.8 and 76.8%). While, minimum mycosis from dead cadavers of *N. lugens* was recorded in both conidial suspension of *M. anisopliae* sprayed on adults with the provision of stem pieces as food (68.8 and 56.0%), while similar trend was also observed on other two treatments, conidial suspension of *M. anisopliae* sprayed on adults without the provision of stem pieces as food (64.8 and 64.0%) and conidial suspension of *M. anisopliae* sprayed on stem pieces (60.0 and 52.0%) (Table 6).

Similarly, maximum sporulation from dead cadavers of *N. lugens* was recorded in both conidial suspension of *L. lecanii* sprayed on adults with the provision of stem pieces as food (205.8 and 184.6 conidia ml^{-1}), while similar

Table 6 Percent mycosis (Mean ± SE, $n = 5$) from dead cadavers of *Nilaparvata lugens* adult exposed to entomopathogenic fungi in two concentrations by three treatments after 14 days of exposure interval

Entomopathogenic fungi	Concentration (conidia ml^{-1})	Conidial suspension of EPF sprayed on stem pieces	Conidial suspension of EPF sprayed on adults of *N. lugens*		*F*	*P*
			Without stem pieces	With stem pieces		
Beauveria bassiana	1×10^6	72.8 ± 4.36cdef	80.8 ± 3.17bcd	84.0 ± 3.88abc	0.45	0.65
	1×10^8	60.0 ± 3.79fgh	68.8 ± 4.21defg	80.0 ± 3.25bcd	1.41	0.28
F		0.98	1.04	0.13		
P		0.35	0.33	0.74		
Metarhizium anisopliae	1×10^6	60.0 ± 4.20fgh	64.8 ± 4.57efgh	68.8 ± 3.17defg	0.24	0.80
	1×10^8	52.0 ± 4.31h	64.0 ± 4.43efgh	56.0 ± 4.60gh	0.43	0.66
F		0.35	0.00	1.05		
P		0.57	1.00	0.34		
Lecanicillium lecanii	1×10^6	84.8 ± 3.32abc	89.6 ± 3.38ab	96.0 ± 0.80a	0.82	0.47
	1×10^8	76.8 ± 4.21bcde	80.8 ± 3.89bcd	88.0 ± 2.77ab	0.48	0.63
F		0.45	0.58	1.54		
P		0.52	0.47	0.25		

Within each column, means with different letters are significantly different; in all cases, $df = 2, 14$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

Within each row, means with different letters are significantly different; in all cases, $df = 1, 9$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

Table 7 Sporulation (Mean ± SE, $n = 5$) from dead cadavers of *Nilaparvata lugens* adult exposed to entomopathogenic fungi in two concentrations by three treatments after 14 days of exposure interval

Entomo-pathogenic fungi	Concentration (conidia ml^{-1})	Conidial suspension of EPF sprayed on stem pieces	Conidial suspension of EPF sprayed on adults of *N. lugens*		*F*	*P*
			Without stem pieces	With stem pieces		
Beauveria bassiana	1×10^6	149.2 ± 4.17gh	169.8 ± 3.32cde	173.2 ± 3.91cd	2.31	0.15
	1×10^8	133.2 ± 3.20ij	141.8 ± 2.71hi	164.6 ± 3.29de	5.56	<0.05
F		1.85	8.52	0.57		
P		0.22	<0.05	0.48		
Metarhizium anisopliae	1×10^6	135.2 ± 1.49i	141.8 ± 1.85hi	153.4 ± 1.71fg	5.93	<0.05
	1×10^8	116.2 ± 3.18k	139.4 ± 2.39hi	124.6 ± 1.90jk	4.26	<0.05
F		5.84	0.13	25.4		
P		<0.05	0.73	<0.01		
Lecanicillium lecanii	1×10^6	179.0 ± 2.94bc	195.4 ± 2.57a	205.8 ± 2.01a	5.67	<0.05
	1×10^8	161.4 ± 2.95ef	172.4 ± 3.20cd	184.6 ± 2.56b	3.17	0.08
F		3.57	6.27	8.46		
P		0.10	<0.05	<0.05		

Within each column, means with different letters are significantly different; in all cases, $df = 2, 14$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

Within each row, means with different letters are significantly different; in all cases, $df = 1, 9$ ($P < 0.05$), Tukey's HSD test, comparisons across all treatments

trend was also observed on other two treatments, conidial suspension of *L. lecanii* sprayed on adults without the provision of stem pieces as food (195.4 and 172.4 conidia ml^{-1}) and conidial suspension of *L. lecanii* sprayed on stem pieces (179.0 and 161.4 conidia ml^{-1}). While, minimum mycosis from dead cadavers of *N. lugens* was recorded in both conidial suspension of *M. anisopliae* sprayed on adults with the provision of stem pieces as food (153.4 and 124.6%), while similar trend was also observed on other two treatments, conidial suspension of *M. anisopliae* sprayed on adults without the provision of stem pieces as food (141.8 and 139.4 conidia ml^{-1}) and conidial suspension of *M. anisopliae* sprayed on stem pieces (135.2 and 116.2 conidia ml^{-1}) (Table 7).

Discussion

There are numerous studies on the use of EPF for the management of *Nilaparvata lugens* (Hywel-Jones and Gillespie 1990; Jin et al. 2008; Song and Feng 2011). Adult insects were used in this study on the basis of previous study of Geng and Zhang (2004) who reported that adult *N. lugens* were more susceptible to EPF infection than their nymphs and the young nymphs were most resistant to the fungal infection. Based on the present studies, *M. anisopliae* proved effectiveness against *N. lugens* and responsible to caused 82.67–93.33% mortality, so these results are in accordance with the findings of Mohan et al. (2016) who reported that highest conidial concentration of *Metarhizium* (M1) strain (NBAIR) (a strain of *M. anisopliae*) is responsible to cause 76.67% mortality of *N. lugens* under *in vitro* conditions. Similar findings were also reported by Li et al. (2012a) that maximum mortality of *N. lugens* was recorded due to the *M. flavoviride* (Mf82) and gradually increased with the increase in exposure interval. This virulent isolate of *M. flavoviride* is concluded as a promising candidate for microbial control of *N. lugens*. Shaikh and Mohite (2015) reported that field application of *M. anisopliae* with highest conidial concentration was the most consistently effective for the control of *N. lugens*. Kirkland et al. (2004) reported that *M. anisopliae* and *B. bassiana* have the potential for controlling populations of *Ixodes scapularis* and *Rhipicephalus sanguineus*, and nymphal and adult mortality of these ticks due to these EPF was increased as the exposure interval increased.

Results indicated that maximum mortality of adult *N. lugens* was observed when EPF was sprayed on adult body and provided stem pieces as a food. The application method of EPF formulation is the most important factor in its efficacy against insect pests. Mortality was faster when the fungus was applied directly on the adult body (Kavallieratos et al. 2014). Similar finding was also reported by Rizwan et al. (2019a) that 74% mortality of *Cnaphalocrocis medinalis* larvae was recorded when dipped in *M. anisopliae* concentration for 10 s after 10 days. Likewise, Kavallieratos et al. (2014) reported that when higher concentration of EPF sprayed on adult *Sitophilus oryzae*, it caused 100% mortality after 14 days exposure interval. Kassa et al. (2002) found that adult *Sitophilus zeamais* dipped in *B. bassiana* suspension for 5 s caused 100% mortality after 4 days exposure interval. Cherry et al. (2005) reported that when

adults of *Callosobruchus maculatus* were dipped in conidial suspension of *M. anisopliae* or *B. bassiana* for 5 s, 100% mortality was recorded after 6 and 8 days exposure interval. Rizwan et al. (2019b) found that higher concentration of *B. bassiana* caused 31.67% mortality of *Tribolium castaneum* after 21 days exposure interval.

Results from the application methods of EPF in this study indicated that direct application of the EPF on the adult body of *N. lugens* is more effective as compared to contact of the insect body with the EPF-treated substrate (stem pieces) may be due to the penetration of these pathogens in insect body through their cuticle. This difference is more apparent after 6 days exposure interval, while all treatments had similar efficacy level at shorter exposure intervals. Therefore, on the basis of the present findings, it is assumed that at lengthier exposure interval (14 days), the application of *M. anisopliae* on stem pieces could result in 93% mortality, at least in the cases of *B. bassiana* and *L. lecanii*. These findings were in accordance with Kavallieratos et al. (2014) that maximum mortality of *S. oryzae* adults was recoded when *B. bassiana, M. anisopliae* and *Isaria fumosorosea* were applied on insect body rather than sprayed on food and the efficacy of these EPF were increased as the exposure interval increased. A huge number of publications are dealing with the mode of action and the infection process of EPF (Sanjaya et al. 2013, 2015; Ramirez et al. 2018). The mode of action is very unique as conidial germination starts when these pathogens attach to the outer surface of insect cuticle (Liu et al. 2009; Ment et al. 2010; Leao et al. 2015) and producing germ tube (Lovett and Leger 2015). This tube can penetrate the insect cuticle towards the insect hemocoel where the fungal cells attain yeast like forms called as "hyphal bodies" that attack the host insect throughout a sequential process and insect becomes mummified (Mannino et al. 2019). These cells have the ability to secrete small toxic molecules that aids as immunosuppressive compounds, facilitating fungal infection (Pedrini 2018). Numerous studies are also supporting the infection of these EPF by oral ingestion route as an alternate to the cuticle penetration for entrance to the insect body (Wei et al. 2017; Batta 2018; Rafaluk-Mohr et al. 2018) which ultimately increases the significance of these pathogens.

Insecticidal activity of all tested EPF had increased with the exposure interval which ultimately increases the mortality rate of *N. lugens*. Viability of fungal conidia weakened with time (Moore et al. 2000). Batta (2004) found that the fungal conidial viability of *M. anisopliae* decreased with time when used against *S. oryzae*, but its insecticidal efficacy was not severely affected. Results from present study indicated that percent mycosis and sporulation (conidia ml^{-1}) achieved the maximum in treatment where EPF were applied at low conidial suspension. Among them, maximum results of both parameters were recorded in *L. lecanii* while minimum results were recorded in *M. anisopliae*. These results are in accordance with Riasat et al. (2011) who reported that maximum mycosis and sporulation was observed in the dead cadavers of *Rhyzopertha dominica* at the lowest conidial suspension of *B. bassiana*. These results are also supported by other scientists (Sufyan et al. 2019; Tefera and Pringle 2003). The lowest percent mycosis and sporulation at higher conidial suspensions may be attributed to the self-inhibiting mechanism of fungal spores at higher suspensions (Garraway and Evans 1984; Tefera and Pringle 2003).

Conclusion

The results indicated that all tested EPF, *B. bassiana, M. anisopliae* and *L. lecanii* were effective against *N. lugens* but *M. anisopliae* was more effective than *B. bassiana* and *L. lecanii*. Furthermore, the application of the EPF directly on insect bodies is more effective than application on stem pieces. Such application would also be more consumer friendly than the "pathogen-treated food" approach.

Author Contribution B. Atta, M. Rizwan and A.M. Sabir planned the research and designed the methodology; B. Atta conducted the experiments; B. Atta and M. Rizwan wrote the manuscript; M.D. Gogi and M.A. Farooq statistically analyzed the data; A.M. Sabir, M.D. Gogi, M.A. Farooq and Y.A. Batta reviewed the manuscript and gave suggestions and comments for its improvement. All the authors have read and approved the final manuscript.

Compliance with ethical guidelines

Conflict of interest B. Atta, M. Rizwan, A.M. Sabir, M.D. Gogi, M.A. Farooq and Y.A. Batta declare that they have no competing interests.

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