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Virulence of Metarhizium anisopliae and Beauveria bassiana Against Common White Grubs in Nepal【Research report】

黑殭菌 (Metarhizium anisopliae) 與本土白殭菌 (Beauveria bassiana) 對白蠐螬之毒性於尼泊爾之研究【研究報告】

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Abstract

Entomopathogenic fungi Metarhizium anisopliae and Beauveria bassiana were isolated from soils and white grubs cadavers from farmlands in Nepal. In the mortality studies, 8 isolates infected more than 80% of the grubs, another 65 isolates infected more than 50% of the grubs and the rest of the isolates had a low level of pathogenicity with 107 conidia/mL concentration. Five isolates were highly pathogenic against third instar larvae of Maladera affinis. The isolate, M. anisopliae M1 was the most virulent strain of all the evaluated isolates. LT50 of the tested isolates varied between 2-9 weeks. Isolates M1 and M6 had the highest mortality rates and required the shortest time to kill the white grub larvae. Assessment with fungus inocula with conidiospores and blastospores against three different instars of white grubs showed that a significantly larger numbers of second instar larvae were infected with the fungi than first and third instars. A comparison of indigenous and commercial isolates of M. anisopliae and B. bassiana with third instar larvae of Anomala dimidiata, Adoretus lasiopygus and Phyllognathus dionysius demonstrated that native isolates were more virulent to the above three white grubs species tested than the exotic fungi. Based on this study, the indigenous virulent isolates have the potential to be biocontrol agents against white grubs in Nepal.

摘要

在尼泊爾，從田間土壤和蠐螬屍體中分離出的蟲生真菌：Metarhizium anisopliae (黑殭菌) 與Beauveria bassiana (本土白殭菌)。於致死試驗中，有8個分離株感染了超過80%的蠐螬，另有65個分離株感染了超過50%的蠐螬，而其餘濃度為107 conidia/mL的分離株致病力低。有5個分離株對Maladera affinis的3齡幼蟲有高度致病力。分離株M. anisopliae M1是所有分離株中檢測出毒性最強的品系。菌株的LT50測試結果介於2到9週之間。分離株M1與M6有最高的致死率，殺死白蠐螬的時間也最短。以真菌分生孢子與芽生孢子為接種原對抗3種不同齡期白蠐螬進行評估，顯示出2齡幼蟲遠較1齡與3齡幼蟲更容易被感染。比較本土與商品化的分離株M. anisopliae與B. bassiana對Anomala dimidiata、Adoretus lasiopygus與Phyllognathus Dionysius等3種白蠐螬3齡幼蟲的毒性，證明本土分離株高於外來真菌。根據此研究，尼泊爾原生的毒性分離株有成為對抗當地白蠐螬的生物防治劑之潛力。

Key words: Beauveria bassiana, Metarhizium anisopliae, biological control, white grubs

關鍵詞: 本土白殭菌、黑殭菌、生物防治、白蠐螬。

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Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* Against Common White Grubs in Nepal

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ABSTRACT

Entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* were isolated from soils and white grubs cadavers from farmlands in Nepal. In the mortality studies, 8 isolates infected more than 80% of the grubs, another 65 isolates infected more than 50% of the grubs and the rest of the isolates had a low level of pathogenicity with 10^7 conidia/mL concentration. Five isolates were highly pathogenic against third instar larvae of *Maladera affinis*. The isolate, *M. anisopliae* M1 was the most virulent strain of all the evaluated isolates. LT_{50} of the tested isolates varied between 2-9 weeks. Isolates M1 and M6 had the highest mortality rates and required the shortest time to kill the white grub larvae. Assessment with fungus inocula with conidiospores and blastospores against three different instars of white grubs showed that a significantly larger numbers of second instar larvae were infected with the fungi than first and third instars. A comparison of indigenous and commercial isolates of *M. anisopliae* and *B. bassiana* with third instar larvae of *Anomala dimidiata, Adoretus lasiopygus* and *Phyllognathus dionysius* demonstrated that native isolates were more virulent to the above three white grubs species tested than the exotic fungi. Based on this study, the indigenous virulent isolates have the potential to be biocontrol agents against white grubs in Nepal.

Key words: *Beauveria bassiana, Metarhizium anisopliae,* biological control, white grubs

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Introduction

White grubs are serious agriculture pests across the farming areas of Nepal (GC and Keller, 2002). Entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria brongniartii* were isolated from white grubs (Zimmermann, 1992; Keller, 2000). However, their infectivity differs depending on the fungus species, host species and developmental stage of the insects (Samson, 1981). Epizootics of *M. anisopliae* and *B. bassiana* were reported from several scarab species and other soil inhibiting Coleoptera (Fleming, 1968; Hurpin and Robert, 1972; Young, 1974; Glare, 1992). The pathogenicity however varies with the isolates (Aizawa, 1987), pathogen population, dispersal and survival in the host's environment as well as inoculum density and spatial distribution (Tanada and Fuxa, 1987).

In Nepal, more than eighty different beetle species are reported as pest, however, the control of those pests is either ignored or chemical pesticides are applied due to a lack of suitable control options (Baker and Gyawali, 1994). Furthermore, there have been no reports on the isolation, culture and evaluation of indigenous pathogenic fungi isolates against the white grub species. Therefore, this study aimed to isolate, culture, and evaluate indigenous isolates, *M. anisopliae* and *B. Bassiana*, for use against the white grub *Maladera affinis,* and to compare them with a commercial dust formulation of *M. anisopliae* and *B. bassiana* under laborotory conditions in Nepal.

Materials and Methods

Origin and rearing of white grubs

Three species of white grubs (Coleoptera: Scarabaeidae) (*Anomala dimidiata*, *Adoretus lasiopygus* and *Phyllognathus dionysius*) were used in this study. The white grub larvae were collected from the farm areas of Gunganagar and Saradanagar, villages located in central Nepal, at a low altitude. The grub larvae were reared separately in individual poly pots (4.5 cm diameter and 6 cm height) at 22±1°C and 70% RH under completely dark conditions. They were fed weekly with slices of carrots, potatoes, tree yam or ground nuts. The rearing vials were covered with muslin and tied with rubber bands to ensure good aeration. The moisture level was maintained by gentle moistening when the soil became dry. After six weeks of rearing, healthy white grubs were used for our studies.

Origin of fungi

To obtain the indigenous fungi isolates, 46 soil samples were collected from a soil core from the farmland in the Parbat, Syangja, Tanahun and Chitwan districts of Nepal. From these 46 soil samples, 23 were collected from arable land (cultivated) and 23 were collected from grassland (none cultivated).

Thirty soil samples, ten each from Rising Patan, Pang and National Maize Research Programme were analyzed by plating the soil suspension on a selective medium (Keller *et al.*, 2000). The *Galleria* bait method (GBM) from Zimmermann (1986) was adapted. A 60 mL soil/sample was placed into each of 4 cylindrical plastic vials (4.5 cm diameter × 6 cm high) and 1 large *Galleria* larvae was added to each tube. The samples were kept under dark condition at 22 ± 1°C and 70% RH. During the first five days, the tubes were turned daily to keep the larvae moving in the soil. After 16-18 days the larvae were examined, fungus infections were recorded and the fungus from infected larvae was isolated.

The soil plating method was adapted from Fornallaz (1992). A 10 g soil/sample of fresh soil was shaken for 3 h at 140 rpm on a rotary shaker in 250 mL Erlenmeyer flasks with 50 mL tap water with 1.8 g l000 mL^{-1} tetra - Sodiumdiphosphate-Decahydrate ($Na_4P_2O_7*10H_2O$) to disaggregate the soil. After 15 seconds of sedimentation,

0.1 mL of the suspension from the upper 5 mm was distributed with a Drigalsky spatula on a Petri dish with selective medium. Three replicates/soil samples were prepared. After 8-10 days at 20°C under dark condition, the colonies of *M. anisopliae* were counted and the density per g fresh soil calculated and expressed as colony forming units (CFU). The soil suspension was not diluted, even if there were high fungal densities (exceeding 600 colonies/Petri dishes).

The fungus isolates were isolated and maintained on selective medium (SM) as reported by Strasser *et al.* (1996) as 10 g Peptone from meat pancreatically digested, 20 g Glucose, 18 g agar-agar, dissolved in 1L distilled water and autoclaved at 120°C for 20 minutes. At a temperature of 60°C; 0.6 g Streptomycin, 0.05 g Tetracycline and 0.05 g Cyclohexamide previously dissolved in distilled, sterile water and 0.1 mL Dodine were added.

Bioassay

The virulence of the insect pathogenic fungi was tested at three different levels. In the first set of experiments, the efficacy of 70 isolates was determined by using a single dose (10^7 conidia/mL) against third instar larvae of *Maladera affinis*. After selecting the most virulent isolates from the initial screening, dose-mortality studies with four different concentrations (10^9 conidia/mL, 10^5 conidia/mL, 10^2 conidia/mL and control) were conducted in the second set of experiments. In addition, the infectivity to the larval instars (L1, L2 and L3) with different forms of inocula (blastospores and conidiospores) was also studied. In the third set of experiments, indigenous isolates and an Indian strain from commercial formulations of *M. anisopliae* (Com Ma) and *B. bassiana* (Com Bb) were compared.

A completely randomized design was used for the present study, and poly pots and sterile soils were the experimental units. The larvae were reared in the laboratory for 6 weeks before they were used in the experiment. For each strain, 30 larvae were dipped individually into the conidial suspension for five seconds (Goettel and Inglis, 1997) by holding them loosely at the leg with forceps. Excess liquid was allowed to drop off and the larvae were returned individually to the rearing vials containing sterile soil, and were incubated at a temperature of 22-22-24°C. The lid was perforated for air circulation. One larva was left untreated as the control; the other larvae were dipped in water. The larvae were fed with slices of potato and checked for mortality every third day for ten weeks. Dead larvae were observed under the stereomicroscope for fungal infections. Larvae with *M. anisopliae* fungal growth (white mycelia and green spores) were recorded as mycosed larvae (Mazodze and Zvoutete, 1999). The LT_{50} was determined by probit analysis. Mortality, infection rate and LT_{50} were compared using the GENSTAT (GenStat, 1998) computer program.

Results

Fungus isolates

Since mid-2002, a total of 70 different isolates of *M. anisopliae* and 8 isolates of *B. bassiana* were recovered from 4 different sources areas in Nepal (see Table 1). The highest number of *M. anisopliae* were obtained from GBM (41) followed by white grubs (24) and SM (5). Similarly, the highest numbers of *B. bassiana* were obtained from GBM (6) followed by white grubs (2), however, no *B. bassiana* isolates were obtained from SM (see Table 1).

The natural infection of white grubs by *M. anisopliae* was low (0-2%); however, in Nepal it is widespread compared to *B. bassiana* (0.4%). From the 23 soil samples obtained from the arable land, 11 (48%) contained entomopathogenic fungi. From these fungi 10 isolates of *M. anisopliae* and 2 isolates of *B. bassiana* were isolated. From the 23 soil samples obtained from

Table 1. Origin of the insect pathogenic fungi isolated 2002-2005 from different localities in Nepal.

Fungus species	Geographic origin	Number of isolates	Isolated from		
			White grubs	Soil/GBM*	Soil/SM**
M. anisopliae	Total	70	24	41	5
	Chitwan	25	10	15	0
	Parbat	35	7	23	5
	Tanahun	7	4	3	0
	Nawalparasi	3	3	0	0
B. bassiana	Total	8	2	6	0
	Chitwan	7	2	5	0
	Parbat	1	0	1	0
	Tanahun	0	0	0	0

*GBM: *Galleria* bait method

**SM: selective medium

Table 2. Mortality and infection rate of the 70 isolates of *M. anisopliae* after treatment with 10^7 conidia/mL.

LT_{50} (week)	Number of isolates	Range of mortality (%)	Range of infection (%)
2-3	2	100	87-93
4	10	90-97	70-87
5	18	63-100	57-83
6	16	77-100	50-87
7	16	73-93	53-73
>8	8	50-90	33-70
Water treatment		20	0

the grasslands 13 (57%) contained entomopathogenic fungi. From these fungi 10 isolates of *M. anisopliae* and 5 isolates of *B. bassiana* were isolated. These results proved that the *Galleria* bait method was suitable for obtaining the entomopathogenic fungi from soils in a short period of time.

Screening of fungus isolates

All 70 isolates of *M. anisopliae* were observed as pathogenic for the test insect with 33-93% infection rates and 50-100% mortality rates (see Table 2). The LT_{50} of M1 and M6 was two and three weeks respectively, however the LT_{50} of the remaining isolates was four or more weeks. The infection rate of only two isolates was below 50%, however they caused a high level of mortalities. The M1 isolate was the most virulent one, causing 100% mortality, had a 93.3% infection rate and a significantly lower LT_{50} ($p < 0.001$) than the other isolates tested (see Table 2).

Based on their virulence, five isolates (M1, M6, M18, M48 and M50) were selected for the dose-mortality study (see Table 3). Isolate M1 had the highest mortality and mycosis at a concentration of 10^7 conidia mL^{-1} (67.50% mortality, 40.83% mycosis) among the isolates evaluated. Mycosis caused by the different isolates ranged from 26.7% to 40.8% (see Table 3). A possible reason for this low infection rates might be due to the unfavorable environmental conditions as suggested by the high control mortality. Therefore, M1 isolate was selected for further pathogenicity assessments with different doses of conidiospores against third instars larvae of *Maladera affinis*.

Table 3. Pathogenicity of different isolates of *M. anisopliae* against third instar larvae of *M. affinis* at 22-24°C at a concentration of 1 x 10^7 conidia/mL.

Fungus strain	Fungus origin	Mortality* (%)	Mycosis* (%)
M1	White grub	67.5a	40.8a
M6	White grub	64.2ab	38.3ab
M18	Soil/GBM	59.2b	26.7b
M48	Soil/GBM	58.3b	29.2ab
M50	White grub	59.2b	30.8ab
Untreated (water)		58.1b	0.00c
LSD (p = 0.01)		6.953	10.92
SEM		2.357	2.713
CV%		10.81	23.14

*Numbers in the same column followed by the same letters are not significantly different at $p < 0.01$ by DMRT

Table 4. Pathogenicity of different doses of isolates M1 of *M. anisopliae* against third instar larvae of *M. affinis* at 22-24°C after 70 days.

Fungus doses	Mortality* (%)	Mycosis* (%)
10^9 conidia/mL	79.3a	52.0a
10^5 conidia/mL	72.7ab	45.3a
10^2 conidia/mL	70.0b	35.3b
Control	24.7c	0.00c
LSD (p = 0.010)	8.483	9.764
SEM	2.108	2.427
CV%	10.81	23.14

* Numbers in the same column followed by the same letter are not significantly different at $p < 0.01$ by DRT

The results also revealed a positive correlation between dose and mortality as well as between dose and mycosis (10^9 conidia/mL, 52%; $p < 0.01$) (Table 4).

Furthermore, the results obtained with two types of inocula (conidia and blastospores) on different instars of white grubs showed no difference in the grubs' mortality, although they differed in infection rate (Table 5). Similarly, conidia caused a higher infection rate to the first instar larvae compared to the second and third instar larvae. The blastospores caused infections in all instars, however, a significantly higher infection rate ($p < 0.05$) was only observed in the second instar larvae. Blastospores caused significantly higher mortalities (82.2%) to all instars, with an infection rate of 70.0% ($p < 0.001$), while conidia caused 66.7% mortality with a 46.7% infection rate (Table 5).

Comparison of indigenous isolates with commercial products

The pathogenicity of different indigenous isolates of *M. anisopliae* and that from two commercial products against third instar larvae of *Anomala dimidiata, Adoretus lasiopygus* and *Phyllognathus dionysius* (10^7 conidia/mL) was tested in the laboratory (Table 6).

The study revealed that most of the indigenous isolates of *M. anisopliae* caused significantly higher mortalities and infection rates than the exotic *Metarhizium* and *Beauveria* commercial products towards three species of scarab larvae. Among the isolates, M1 was found to be the most

Table 5. Effect of fungus inocula (1x10^7 conidia/mL) on different larval instars (L1/L2/L3) of *M. affinis* after 70 days.

Inoculum	Mortality (%) of different instars			Infection (%) of different instars		
	1st	2nd	3rd	1st	2nd	3rd
Conidia	73.3	70.0	56.7	60.0	40.0	40.0
Blastospores	86.7	86.7	73.3	70.0	83.0	56.0
LSD (5%)		16.63			16.63	
SEM		3.85			3.85	
CV%		9.00			11.40	

Table 6. Comparative study of the efficacy of indigenous and commercial isolates of insect pathogenic fungi against three different species of white grubs

Isolate	Tested species*								
	Anomala dimidiata			*Adoretus lasiopygus*			*Phyllognathus dionysius*		
	Mortality	Infection	LT_{50} (days)	Mortality	Infection	LT_{50} (days)	Mortality	Infection	LT_{50} (days)
M1$^\gamma$	90.0 d	70.0f	26.0a	76.6bc	66.6d	24.3	90.0	70.0c	22.3a
M6	80.0cd	46.7cde	36.6ab	80.0bc	46.6bcd	34.3	83.3	56.6b	24.0a
M18	76.7bcd	40.0bc	40.3b	83.3c	46.6bcd	45.3	76.6	50.0b	26.3ab
M48	83.3cd	56.7e	33.3ab	73.3bc	53.3c	33.3	80.0	56.6b	29.0abc
M50	66.7bc	43.3bcd	42.3b	63.3b	36.6b	35.3	86.6	53.3b	28.6abc
M70	66.7bc	53.3de	43.0b	70.0bc	50.0bc	38.3	90.0	56.6b	28.3ab
Com. Ma$^\delta$	60.0b	33.3b	38.3ab	66.6bc	36.6b	34.0	80.0	50.0b	31.0bc
Com. Bb$^\lambda$	60.0b	43.3bcd	39.6b	63.3b	40.0bc	34.0	86.6	46.6b	34.6bc
Control	26.7a	0a	45.3b	36.6a	0.0a	35.0	73.3	0.0a	39.0d
Grand mean	67.8	43.0	38.5	68.1	41.9	34.9	83.0	48.9	29.15
SEM	5.98	3.85	5.77	5.21	3.85	4.74 (ns)	7.54 (ns)	4.44	2.9
LSD	17.78	11.44	12.12	15.48	11.4	14.08	15.83	9.34	6.2
CV %	15.3	15.5	18.4	13.2	13.2	23.5	11.1	11.1	12.4

* Numbers in the same column followed by the same letters are not significantly different at $p < 0.001$ by DMRT

$^\gamma$ M: *Metarhizium anisopliae*

$^\delta$ Com Ma: commercial *M. anisopliae*

$^\lambda$ Com Bb: commercial *Beuveria bassiana*

virulent one among the isolates (see Table 6). It induced the highest mycosis as well as the shortest LT_{50} in all the grub species, followed by M48 and M70. The efficacy of the exotic isolates was found at par with the rest of the native isolates. Furthermore, the result demonstrated that larvae of *P. dionysius* were more susceptible to the tested fungal isolates compared with other white grub species (Table 6).

Discussion

The entomopathogenic fungi *M. anisopliae* and *B. bassiana* are widely distributed in Nepal, and was proven by the large number of isolates obtained from white grubs from different parts of Nepal (see Table 1).

Blastospores caused significantly higher number of mortalities than the conidia of *M. anisopliae* and *B. bassiana* tested. The production of blastospores was also faster,

easier in small amounts. In addition, the hydrophilic nature makes them better suited for bioassays. Kessler (2004) demonstrated that bioassays with blastospores and conidia resulted in a comparable ranking of the isolates. Also, Lane *et al.* (1991) and Bidochka *et al.* (1995) reported that blastospores germination goes faster than the respective conidia. Blastospores are produced in-vivo inside the insect body, and they multiply faster in order to escape from the host defense system. However, conidia are built on the surface of the killed insect and are adapted to survive in a highly competitive soil environment, which therefore may result in different induction mechanisms of spores germination or host penetration (Aregger-Zacadil, 1992).

Some of the native isolates were more virulent than the imported ones. We thus continued the consequent studies with indigenous isolates only. A possible reason for the lower virulence of the exotic isolates might be the loss of virulence during manufacturing, transportation and the storage process of the imported isolates. Other reasons could be their original host, the formulation procedure and environmental factors. Arthurs and Thomas (2001) reported that the efficacy of fungal entomopathogens is highly dependent on suitable climatic conditions, in particular the availability of a high level of environmental moisture.

In this study, more numbers of second instar larvae were infected with the fungi than the first and third instar. Ferron (1967) and Vandenberg *et al.* (1998) reported that the possible reason for this might be the molting of the larvae. Early mounting soon after inoculation may lead to shedding of the spores together with the cuticle, as has been observed for *Plutella xylostella* and *Beauveria bassiana*.

The reason for the higher susceptibility of *P. dionysius* larvae might be attributed to their larger body size compared to the other two species tested. The larger body size of *P. dionysius* grubs provides more space to pick up conidia than for grubs with a smaller body size such as *A. lassiopygus* and *A. dimidiata.* The cuticle of *P. dionysius* is very firm. Therefore, the chances to be infected may be higher in larvae of *P. dionysius* compared to the other species. The variation of pathogenicity according to different isolates might be due to fungus-specific characteristics. A number of dead larvae in the untreated grub population without any mycosed grubs suggest that mortality may be induced by other factors such as infections with other pathogens or sub-optimal rearing conditions. In addition, the variation in pathogenicity within isolates in this study might be due to the adaptation process of different grub populations (Keller and Zimmermann, 1989). In conclusion, the indigenous virulent isolates have the potential to be biocontrol agents against white grubs in Nepal.

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黑殭菌（*Metarhizium anisopliae*）與本土白殭菌（*Beauveria bassiana*）對白蠐螬之毒性於尼泊爾之研究

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摘　要

在尼泊爾，從田間土壤和蠐螬屍體中分離出的蟲生眞菌：*Metarhizium anisopliae*（黑殭菌）與 *Beauveria bassiana*（本土白殭菌）。於致死試驗中，有 8 個分離株感染了超過 80%的蠐螬，另有 65 個分離株感染了超過 50%的蠐螬，而其餘濃度爲 10^7 conidia/mL 的分離株致病力低。有 5 個分離株對 *Maladera affinis* 的 3 齡幼蟲有高度致病力。分離株 *M. anisopliae* M1 是所有分離株中檢測出毒性最強的品系。菌株的 LT_{50} 測試結果介於 2 到 9 週之間。分離株 M1 與 M6 有最高的致死率，殺死白蠐螬的時間也最短。以眞菌分生孢子與芽生孢子爲接種原對抗 3 種不同齡期白蠐螬進行評估，顯示出 2 齡幼蟲遠較 1 齡與 3 齡幼蟲更容易被感染。比較本土與商品化的分離株 *M. anisopliae* 與 *B. bassiana* 對 *Anomala dimidiata*、*Adoretus lasiopygus* 與 *Phyllognathus Dionysius* 等 3 種白蠐螬 3 齡幼蟲的毒性，證明本土分離株高於外來眞菌。根據此研究，尼泊爾原生的毒性分離株有成爲對抗當地白蠐螬的生物防治劑之潛力。

關鍵詞：本土白殭菌、黑殭菌、生物防治、白蠐螬。

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