

# Pathogenicity of Three Entomopathogenic Fungi against *Helicoverpa armigera*

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## Abstract

The agricultural pests and pathogens known so far include 2,000 species of insects and 800 fungi. Among the insect pests *Helicoverpa armigera*, a lepidopteran insect causes more than 50% loss in yield of important crops such as cotton, pulses, vegetables and sunflower in India. In recent years, agricultural production of pulses repeatedly have suffered average yield loss of about 67% due to high levels of insecticide resistance in *H. armigera*. In the present study, *Metarhizium anisopliae*, *Beauveria bassiana*, and *Nomuraea rileyi* are used to control the insect pest. The mass production of cell wall was carried out by two stage fermentation for the production of various enzyme and its inhibitors. The *M. anisopliae* and *B. bassiana* isolates from fields showed greater than 70% mortality of *H. armigera* in the bioassay the percentage mortality declined; there was a decrease in the enzyme activities of *B. bassiana*. *N. rileyi* isolates did not exhibit detectable chitinase levels even up to 120 h.

**Keywords:** *Helicoverpa armigera*; Lepidopteran insect; *M. anisopliae*; *B. bassiana*; chitinase

## Introduction

Soil borne pathogens have a broad host range and persist for longer periods in soil by resistant resting structures. Chemical control of soil borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms. Therefore, biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing [1].

With every major outbreak of plant pathogen, the concept of plant pathogen has been revolutionized. Disaster in India caused by fungal attack on potatoes in 1840s necessitated the development of control agents as one of the means for sustainable agriculture. While in 1960s, the insect pest on cotton crop in India when became tolerant to organophosphate insecticides triggered the process of rethinking about the use of chemicals for pest control. Now the cost of global insect control has been raised by almost 40%.

The agricultural pests and pathogens known so far include 2,000 species of insects and 800 fungi. Among the insect pests *Helicoverpa armigera*, a lepidopteran insect causes more than 50% loss in yield of important crops such as cotton, pulses, vegetables and sunflower in India. In recent years, agricultural production of pulses repeatedly have suffered average yield loss of about 67% due to high levels of insecticide resistance in *H. armigera* [2]. Additionally, legumes such as groundnut, cowpea, etc. are prone to attacks by soil-borne plant pathogenic fungi such as *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani* [3] and others. The development of resistance to chemical pesticides and negative impact of pesticides on the environment has prompted several researchers to evaluate alternative methods for the control of important agricultural pests. Thus, there is a need to develop biopesticides that are effective, biodegradable and do not leave any harmful effect on the environment.

Some insect pathogenic fungi have restricted host ranges while other fungal species have a wide host range for example, *Metarhizium anisopliae*, *M. flavoviridae*, *Paecilomyces farinosus*, *Beauveria bassiana* and *B. brongniartii*. This host specificity may be associated with the physiological state of the host system [4].

Viruses, bacteria and fungi can act as biocontrol agents against

insects and fungi. The viral and the bacterial control agents infect insects via their digestive tract while fungi make entry into the host through the cuticle that is the outermost covering in insect and fungal cell wall [5]. Insect-pathogenic fungi that act by contact and with no records of resistance developed so far could be a viable alternative. Louis Pasteur first recognized the potential of fungi for insect control. Over 400 species of fungi have been known to parasitize living insects. Most fungi that attack insects first make contact with the host in the form of conidia. Once the conidium attaches to the host, the fungus penetrates the insect cuticle with the help of hyphae produced from conidia. The fungal enzymes such as chitinases, proteases, lipases and others that weaken the cuticle accelerate the physical process of penetration. The cause of the insect's death is extensive fungal growth and production of different toxins in the haemolymph.

Our objectives of the present investigation are the use of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Nomuraea rileyi* for the control of insect pest, *H. armigera* on pulses under pot conditions.

## Materials and Methods

### Isolation of entomopathogenic fungi

The soil samples were collected from the different regions around Namakkal, Tamilnadu. For the isolation of entomopathogenic fungi two different methods namely, soil dilution method [6] and *Galleria* bait method [7] were used and the basis of conidial morphology [8] isolates were identified as *M. anisopliae*, *B. bassiana* and *N. rileyi*. The *N. rileyi* strains were isolated from mycosed *Spodoptera litura* (Fabricius) larvae found in sugar beet fields near Namakkal.

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## Maintenance

All the *M. anisopliae* and *B. bassiana* isolates were maintained on PDA, while for the maintenance of *N. rileyi* isolates, SMYP agar was used. Numbering of the isolates was done with respect to the field numbers, plot numbers and sample numbers. The stock cultures were maintained at 4°C until used. Sub culturing was done on the host insect (*H. armigera*) after every three months and the passaged culture was re-isolated and maintained on potato dextrose agar and preserved by lyophilisation. The passaged stock of entomopathogenic fungi was used for all the experiments.

## Mass production of conidia of entomopathogenic fungi by solid state fermentation

Three strains namely *M. anisopliae*, *B. bassiana* and *N. rileyi* were selected for the large-scale production of conidia. The mass production of conidia was carried out in flasks filled with 2 kg of rice soaked overnight in 1000 ml distilled water, as a substrate. After autoclaving at 121°C for 40 minutes, flasks were inoculated with 200 ml of biomass grown on YPG medium for *M. anisopliae* and *B. bassiana* and SMYP for *N. rileyi* in shake flasks for 48-72 h. The inoculated flasks were incubated with 25±2° C for 14 days. After 14 days, the sporulated substrate was dried at 30°C for 2-3 days to reduce the moisture content. The conidia were then harvested and stored at 4°C until used.

## Formulation studies of conidia of entomopathogenic fungi

The viability and virulence (% mortality of *H. armigera*, of conidia for *M. anisopliae*, *B. bassiana* and *N. rileyi* were tested in different formulations such as diesel, sunflower oil, diesel: sunflower oil in the ratio 7:3, 0.1% Tween 80 etc. For this study, the conidial suspensions (1 x 10<sup>7</sup> conidia/ml) were prepared in different formulations, kept at room temperature for 1 h and their germination was monitored on YPG agar for *M. anisopliae* and *B. bassiana* at 25 ± 2°C for 24 h and on SMYP agar for *N. rileyi* at 25 ± 2°C for 72 h. The formation was observed under the microscope (40X) and the percent germination was then calculated.

## Germination studies of conidia of entomopathogenic fungi in presence of chickpea leaf extract

The conidial germination was carried out on YPG agar for *M. anisopliae* and *B. bassiana* and on SMYP agar for *N. rileyi* with and without chickpea leaf extract. The chickpea leaf extract was prepared by crushing 5g chickpea leaves in 100 ml water. The germination studies were carried out at 25 ± 2°C and incubated for 24 h for *M. anisopliae* and *B. bassiana* and for 72 h for *N. rileyi*.

## Enzyme Production

### Production of cuticle degrading enzyme complex of entomopathogenic fungi

The cuticle degrading enzyme complex was produced by growing entomopathogenic fungi in chitin medium using chitin as the sole carbon source. The flasks were inoculated with 1 x 10<sup>7</sup> spores/ml and incubated at 28°C for 96 h in case of *M. anisopliae* and *B. bassiana* isolates and for 120 h in case of *N. rileyi* isolates. The culture filtrate was used for the estimation of chitinase, protease and lipase activities [9].

### Chitinase assay

The chitinase activity in the culture supernatant was estimated as described earlier using acid swollen chitin as the substrate [9]. To prepare acid swollen chitin, the chitin (10g, purified powder from crab shells) was suspended in chilled O-phosphoric acid (88%,

w/v) and left at 0°C for 1h with stirring. The acid swollen chitin was repeatedly washed with chilled distilled water, followed with a 1% (w/v) NaHCO<sub>3</sub> wash and further dialyzed against cold distilled water. After homogenization in Waring blender (1 min), 50 mM acetate buffer, pH 5.0, was added to homogenization in warming the suspension so that 1ml of suspension contained 7 mg of chitin. The reaction mixture for chitinase assay contained 1ml 0.7% acid swollen chitin, 1ml 50 mM acetate buffer, pH 5.0 and 1ml enzyme solution that was incubated at 50°C for 1h. The GlcNAc produced was estimated calorimetrically with *p*-dimethyl amino benzaldehyde (DMAB) [10]. One international unit was defined as the activity that produced 1µmol of GlcNAc per min.

### Protease assay

Protease activity was measured using casein as a substrate [9]. The reaction mixture, 2ml, contained an aliquot of a suitably diluted enzyme solution, 10mg casein and 0.2mM of sodium carbonate buffer, pH 9.7. Enzyme reaction was carried out at 35°C for 20 min and terminated by the addition of 3ml trichloroacetic acid (TCA) (2.6 ml 5%TCA + 0.4 ml 3.3N HCl). The absorbance of the TCA soluble fraction was measured at 280nm. One international unit was defined as enzyme activity that produced 1 µmole of tyrosine per min.

### Lipase assay

Lipase activity was determined as described by Pignede *et al.*, [11]. The substrate emulsion was prepared with olive oil, 50 ml and gum arabic, 50 ml (10% w/v). The reaction mixture contained 1ml enzyme, 5ml substrate emulsion and 2ml of 50 mM phosphate buffer, pH 6.8 and was incubated for 1h at 37°C with shaking. The reaction was stopped with 4ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50 mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1µmole of fatty acid per min.

### Insect culture

The initial culture of *H. armigera* was established by collecting larval and pupa stages from the fields. The larvae was done individually in plastic vials on vegetable diet (Okra) disinfected for 10 min with 0.5 % sodium hypochlorite as described by Ignoffo *et al.*, 1975 [12]. The temperature and relative humidity in the insect were maintained at 25±2°C respectively.

### Insect bioassay

The fungal isolates were grown on PDA and SMYP agar, incubated in dark for 14 days at 25°C. Viability of conidia powder was tested in different formulations as described by Ibrahim *et al.* 1999 [13]. For the bioassay, conidial suspensions (1 x 10<sup>7</sup> conidia /ml) of the different isolates were made by scraping conidia from 14 days old culture in 0.1 % Tween 80. Susceptibility was evaluated by directly dipping third instar larvae of *H. armigera* in 30 ml of conidial suspension for 5 sec. Three replicates of 20 larvae were used in each experiment. As a control, 3 batches of 20 larvae were treated with 0.1% Tween 80 in sterile distilled water. After treatment, each larva was kept in a separate plastic vial (42 x 65 mm) containing moist Whatman No.1 paper and allowed to feed on disinfected okra pieces and was incubated at 25±1°C. The percent mortality was recorded upto 14 days. The dead larvae were placed in a sterile petri plate containing a moist cotton swab to allow mycelial growth over the cadaver for 3 days. The mortality was recorded.

## Results

A total of 44 cultures were isolated using different methods of

isolation as described. *M. anisopliae*, *N. rileyi* and *B. bassiana* isolates were identified on the basis of morphology and then analyzed on the basis of percent mortality and biochemical activities. All the isolates were tested for their comparative infectivity against *H. armigera* and were also analyzed for the production of cuticle degrading enzymes. For penetration through the insect cuticle, deuteromycetous fungi such as *Metarhizium* and others produce chitinase, protease and lipase, popularly known as cuticle degrading enzymes, in a co-ordinated manner [14,15] *M. anisopliae*, *B. bassiana* species shows higher enzymes (chitinase, protease and lipase) production when compared with other species (Table 1). Table 2 shows that the conidial germination of *M. anisopliae* in sunflower oil, diesel: sunflower oil mixture (7:3) and Tween 80 (0.1%) was greater than 90% in 12 h. The values indicated are the average of three replicates with < 15% variation germination of the selected fungal isolates when compared with the conidial germination on YPG/SMYP medium (Table 3). The conidia of *M. anisopliae* were used for the bioassay with third instar larvae by the dip method. The 70% mortality was observed in 3 days (Figure 1).

## Discussion

A wide range of microorganisms have the potential to produce cell-wall degrading enzymes when chitin or isolated fungal cell wall material are present in the growth medium [12,13]. The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic fungi [16,17,18]. The *M. anisopliae* and *B. bassiana* isolates from fields

Organism	% Mortality	Chitinase U/ml x 10 <sup>-3</sup>	Activity ± SE	
			Protease U/ml	Lipase U/ml
<i>M. anisopliae</i>	>70	2.89±1.0	2.46 ± 0.91	0.72± 0.29
<i>M. anisopliae</i>	50-70	1.26 ± 0.42	0.90 ± 0.48	0.30± 0.17
<i>M. anisopliae</i>	< 50	0.52 ± 0.06	0.32 ± 0.03	0.08± 0.01
<i>B. bassiana</i>	>70	6.56± 0.33	0.91 ± 0.08	0.34± 0.02
<i>B. bassiana</i>	50-70	4.75 ± 1.27	0.54 ± 0.18	0.26± 0.07
<i>N. rileyi</i>	>70	ND	0.75 ± 0.06	0.6 ± 0.04
<i>N. rileyi</i>	50-70	ND	0.58 ± 0.06	0.36 ± .06
<i>N. rileyi</i>	< 50	ND	0.27± 0.14	0.22 ± 0.07

ND- Not detected, SE- Standard error The values presented are the average of three replications

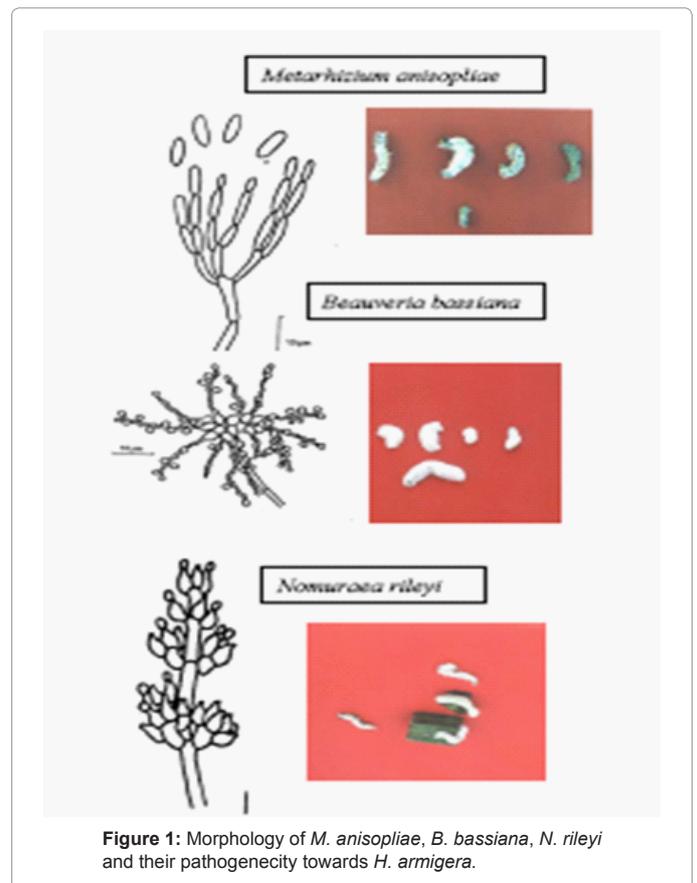
**Table 1:** Comparative analysis of insect bioassay and biochemical characteristics of *M. anisopliae*, *B. bassiana*, and *N. rileyi* isolates

Conidial germination (%) ± SE			
Formulation	<i>M. anisopliae</i>	<i>B. bassiana</i>	<i>N. rileyi</i>
Diesel	87.0 ± 1.1	47.1 ± 2.2	72.2 ± 2.3
Sunflower	92.2 ± 1.9	54.8 ± 1.6	82.2 ± 1.3
Diesel: Sunflower oil (7:3)	95.6 ± 1.7	66.7 ± 1.8	85.1 ± 1.8
Safflower oil	87.1 ± 2.2	42.7 ± 1.3	79.8 ± 1.6
Diesel : Safflower oil (7:3)	78.1 ± 3.1	52.2 ± 2.3	78.0 ± 2.8
Groundnut oil	89.1 ± 1.8	44.2 ± 2.1	50.2± 3.7
Tween 80 .1%)	99.4 ± 0.3	83.9 ± 1.1	84.7 ± 1.6

**Table 2:** Effect of different oil formulations on the germination of conidia of the selected fungal isolates.

Isolate	%Germination±SE	
	YPG agar	SMYP agar
<i>M. anisopliae</i>	95 ± 1.5	95 ± 2.4
<i>B. bassiana</i>	82.11 ± 0.8	78.00 ± 2.4
<i>N. rileyi</i>	74.98 ± 2.2	69.66 ± 1.5

**Table 3:** Effect of chickpea leaf extract on the conidial germination of entomopathogenic fungi.



**Figure 1:** Morphology of *M. anisopliae*, *B. bassiana*, *N. rileyi* and their pathogenicity towards *H. armigera*.

showed greater than 70% mortality of *H. armigera* in the bioassay. Furthermore, all the *M. anisopliae* isolates were isolated by the soil plating method, while *B. bassiana* strains by *Galleria* bait method.

The combination of formulation, application and the selection of the strain is one of the key steps for field trials. Use of different oil based formulations for mycoinsecticides has been extensively studied by Lomer and Lomer [8]. Nicolai and Meyling, 2007 [19] reported that the soil dilution plating method is used the procedure is more tedious than the bait method. Brownbridge et al. 1993 [20] reported that the *Galleria* bait method is relatively simple to use and resulted in isolation of entomopathogenic fungi from 27% of samples. Also, when the percentage mortality declined, there was a decrease in the enzyme activities of *B. bassiana*. *N. rileyi* isolates did not exhibit detectable chitinase levels even up to 120 h. St. Leger et al. 1986 [15] reported that the early appearance of protease and lipase during *in vitro* production of cuticle degrading enzymes in *M. anisopliae*.

The conidial germination of *M. anisopliae* in sunflower oil, diesel: sunflower oil mixture (7:3) and Tween 80 (0.1%) was greater than 90% in 12 h. The percent mortality of *H. armigera* with the three isolates in different oil-based formulations. In the dip method, though all the tested formulations were found to be effective (>50% mortality with all the isolates), in a diesel: sunflower oil mixture and Tween 80 (0.1%) greater than or equal to 90% mortality with all the three isolates was observed. For the field studies, the conidia were mixed with the formulation just before application in the field.

Patel, 1975 [21] suggested that the acidic exudates of the chickpea plant affected the activity of the parasitoids, which was the reason for

their failure. However, as shown in Table 3, presence of the chickpea leaf extract did not affect the conidial.

The conidia of *M.anisopliae* were used for the bioassay with third instar larvae by the dip method. The 70% mortality was observed in 3 days. Rosin *et al.*, 1996 [22] reported that *M.anisopliae* was used for the bioassay with *A.diaperims* larvae ranged between 10 and 97.5% 7 days. Geden *et al.*, 1998 [23] reported that strain of *P.fumosoroseus* and *V.lecanii* were less virulent to *A.diaperinus* larvae, killing a maximum of 20-30% of the treated insect. The result showed that 2<sup>nd</sup> instar larvae were more susceptible to mycoinsecticide than 4<sup>th</sup> instar larvae [24].

## Reference

1. Amin F, Razdan VK, Mohiddin FA, Bhat KA, Banday S, et al. (2010) Potential of *Trichoderma species* as biocontrol agents of soil borne fungal propagules Journal of Phytology 2: 38-41.
2. Ranga Rao GV, Shanover TG (1999) In: ICRISAT Information Bulletin No. 57 International Crop Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India.
3. Deka Boruah HP, Dileep Kumar BS (2003) Rhizoctonia wilt suppression of brinjal (*Solanum melongena* L) and plant growth activity by *Bacillus* BS2. Indian J Exp Biol 41: 627-631.
4. McCoy GW, Samson RA, Boucias DG (1988) Entomogenous fungi in 'CRC Handbook of Natural Pesticides, Part A. Entomogenous Protozoa and fungi.
5. Deshpande MV (1998) In: Novel Biopesticides, CSIR Golden Jubilee Series, CSIR, New Delhi.
6. Goettel MS (1996) Inglis GD. In: Manual of techniques in insect pathology. Academic Press, USA.
7. Zimmermann G (1986) The Galleria bait method for detection of entomopathogenic fungi in soil. J Appl Entomol 102: 213-215.
8. Lomer CH, Lomer CJ (2001) In: LUBILOSA Insect Pathology Manual. CABI Bioscience Publication.
9. Vyas PR, Deshpande MV (1989) J Gen Appl Microbiol 35: 343-350.
10. Reissig JL, Strominger JL, Leloir LF (1955) A modified colorimetric method for the estimation of N-acetyl amino sugars. J Biol Chem 217: 959-966.
11. Pignede G, Wang H, Fudalei F, Gaillardia C, Semon M, et al. (2000) Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*. J Bacteriol 182: 2802-2810.
12. Ignoffo CM, Putter B, Marston NL, Hostetter DL, Dickerson CJ (1975) Invertebr Pathol 25: 135-137.
13. Ibrahim L, Butt TM, Beckett A, Clark SJ (1999) The germination of oil-formulated conidia of the insect pathogen, *Metarhizium anisopliae*. Mycol Res 103: 901-907.
14. Krieger de Moraes C, Schrank A, Vainstein MH (2003) Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Curr Microbiol 46: 205-210.
15. St. Leger RJ, Cooper RM, Charnley AK (1986) Cuticle-degrading Enzymes of Entomopathogenic Fungi: Regulation of Production of Chitinolytic Enzymes. J Gen Microbiol 132: 1509- 1517.
16. Kafetzopoulos D, Martinou A, Bouriotis V (1993) Planta 178: 385-392.
17. Keel C, Defago G (1997) In: Multitrophic interactions in terrestrial systems, 27-46, Gange AC, Brown VK edn. Blackwell Scientific Publishers.127.
18. Khale A, Deshpande MV (1992) Dimorphism in *Benjaminiella poitrasii*: cell wall chemistry of parent and two stable yeast mutants. Ant van Leeuwenhoek 62: 299-307.
19. Nicolai, Meyhing V (2007) Methods for isolation of entomopathogenic fungi from the soil environment. Department of Ecology, Faculty of life sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.
20. Brownbridge M, Ilumbar RA, Parker BL, Skinner L (1993) Fungal entomopathogens recovered from Vermont forest soils. Mycologia 85: 358-361.
21. Patel RC (1975) Final tech. Report, GAU, Anand, Gujarat, India, 55.
22. Rosin F, Shapiro DI, Lewis LC (1996) Effect of fertilizers on the survival of *Beauveria bassiana*. J.Invertebr Pathol 68: 194-195.
23. Geden CJ, Arends JJ, Rutz DA, Steinkraus DC (1998) Laboratory evaluation of *Beauveria bassiana* (Moniliales: Moniliaceae) against the lesser mealworm, *Alphitobius diaperinus*, (Coleoptera: Tenebrionidae), in poultry litter, soil, and a pupal trap. Biological Control 13: 71-77.
24. Amer MM, Sayed TI, Bakheit HK, Moustafa SA, Yasmin A, et al. (2008) Pathogenicity and genetic variability of five entomopathogenic fungi against *spodoptera littoralis*. Res J Agri Biol Sci 4: 354-367.