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Comparative vulnerability of *Helicoverpa armigera* (Hubner) larvae to selected entomopathogenic fungi

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Abstract
The larvae of *Helicoverpa armigera* (Hubner), (Lepidoptera: Noctuidae), a polyphagous pest affecting different crops in India, were treated with variable concentrations of conidia of two soil isolates and two commercially available entomopathogenic fungi belonging to two species, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) in a laboratory. The suspension of conidia (10^9 conidia/ml) collected from Sabouraud dextrose agar media with yeast extract (SDAY) plates resulted in the highest mortality (98.3%) with *Beauveria bassiana* (PSC-13) and the lower mortality (75%) with *Metarhizium anisopliae* (Ag-CM). Using the larval immersion method, the values of LC_{50} and LC_{90} reveal that *Beauveria bassiana* was the most virulent species while *Metarhizium anisopliae* was the less virulent species in between the entomopathogenic fungi used in bioassay. The local strain of *Beauveria bassiana* (PSC-13) and *Metarhizium anisopliae* (PSC-11) was more virulent than the tested commercial strain.

Keywords: Entomopathogenic fungi, *Helicoverpa armigera*, Biocontrol, Bioassay, *Beauveria bassiana, Metarhizium anisopliae.*

Introduction
The legume pod borer or cotton bollworm *Helicoverpa armigera* (Hubner), (Lepidoptera: Noctuidae) is a cosmopolitan polyphagous pest of more than 182 plant species including cotton, pigeon pea, chickpea, peas, cowpea, field beans, tomato, sorghum, groundnut, tobacco, maize and a range of vegetables, fruit crops and trees species (Gowda, 2005) [12]. A chemical insecticide is the main pest control agent in developing countries like India, currently increasing cases of resistance reduces the vulnerability of *H. armigera* to many synthetic chemical insecticides reported throughout the world (Ahmad *et al*. 1997; Gunning *et al*. 1998; Martin *et al*. 2000) [3, 13, 18]. Entomopathogenic fungi are one of the alternatives of chemical insecticide to manage the *H. armigera*. The entomopathogenic fungi are well known for environment safety and pest selectivity (Jayaraj *et al*. 1989) [17]. Entomopathogenic fungi having unique advantages to control insect-pest because they are capable to attack all four stages of insect pest (Ferron 1978) [10]. Biopesticides are more efficacious (70-90%) as compare to chemical insecticides (20-50%) (Ansari *et al*., 2007) [6]. In the genus *Beauveria*, *B. bassiana* (Balsamo) Vuillemin is recognized as a common species with a broad ecological host range of more than 700 arthropod species, which covers most orders of the class Insecta (Feng *et al*., 1994) [9] (Hajek, 2000) [14].
The objective of this study was to evaluate the effectiveness of entomopathogenic fungi for the control of *H. armigera* larvae. So, the effectiveness of several species of entomopathogenic fungi *B. bassiana*, *M. anisopliae* commercial as well as local strain were evaluated. Further, the best-performing strain of each tested species was used to characterise.

Materials and Methods
Rearing of *Helicoverpa armigera*.
The larvae of the *H. armigera* were collected in 2018 from chickpea farms of the Patna City area in Bihar, India. To avoid cannibalism larvae are kept separately in sterile plastic containers. For feeding of larvae, following the procedures of Abbasi *et al*. (2007) [1] with minor modification. The artificial diet consists of chickpea flour 171g., 5.7g. active yeast, 7.1g. agar, 2.9g. methyl-para-hydroxy-benzoate, 0.86g. Sorbic acid, 2g. Ascorbic acid, 1.42g.

Vitamin mixture for insects, 40mg. Streptomycin sulphate, and 2ml Formaldehyde in 3.5 litres of distilled water. Larvae were kept in the sterile plastic container on a layer of tissue paper supplemented with a prepared diet.

The emerged adults were transferred to a rearing cage and fed on cotton buds soaked with 10% honey for oviposition. To avoid mortality due to unhygienic conditions, the rearing chamber was cleaned, and fresh food was provided daily. Rearing was carried out in a controlled chamber at 25 ± 2 °C, 70 ± 5% relative humidity with a photoperiod of Light (L) 16: Dark (D) 8. Third instar larvae were used in experiments.

Isolation and Selection of Entomopathogenic fungi

The *Beauveria bassiana* and *Metarhizium anisopliae* were isolated in the laboratory using "*Galleria* bait method" (Zimmerman 1986) [24]. The infected *Galleria mellonella* (Greater wax moth) larvae were selected (trapped EPF) and commercially available *B. bassiana* and *M. anisopliae* were cultures on Sabouraud dextrose agar media including Yeast extract (dextrose 40g, peptone 10g, agar 15g, yeast extract 10g for 1000ml of media. Chloramphenicol 125mg/litre was used to prevent bacterial growth. The plates were incubated at 25 ± 2 °C with 65 ± 5% relative humidity for 15-20 days in the dark [Goettel and Inglis].

Table 1: Source of Entomopathogenic fungi used in Bioassay

Entomopathogenic fungi (Strain)	Source	Location
Beauveria bassiana (PSC-13)	Soil	Patna Science College, Bihar, India
Beauveria bassiana (Ag-CB)	Agrizone-Green Beauveria	Bio-Pesticide Pollachi, Tamil Nadu, India
Metarhizium anisopliae (PSC-11)	Soil	Patna Science College, Bihar, India
Metarhizium anisopliae (Ag-CM)	Agrizone -Green Met	Bio-Pesticide Pollachi, Tamil Nadu, India

Preparation of Conidial suspension and viability test

Conidia of entomopathogenic fungi were harvested by scraping the medium surface by inoculation loop needle and transferred to a test tube containing 1 ml of 0.1% Tween 80. Homogenize the conidia using a Vortex mixer. Conidial concentration was determined by counting using a Neubauer haemocytometer (Alves & Moraes, 1998) [4]. Using serial dilution method 10^4 – 10^9 conidia/ml were prepared using distilled water containing Tween 80 (0.1% v/v). The viability of conidia was tested by plating 200 µl conidial suspension (containing 1×10^6 conidia ml^{-1}) on SDAY medium and incubated at 25 ± 2 °C and 65 ± 5% RH in dark for 24 hrs. The germination was checked by staining with lactophenol cotton blue and viewed under the microscope (Nikon, x450) (Goettel and Inglis, 1997) [11].

Bioassay Methodology

The bioassay technique was used to evaluate the virulence of entomopathogenic fungi. 2 isolates of *B. bassiana* and 2 isolates of *M. anisopliae* were used in the experiment. *Helicoverpa armigera* third instar larvae were dipped into six different spore concentrations (10^4 – 10^9 spore ml^{-1}) for 10 sec. as described by Goettel and Inglis (1997) [11]. For control larvae were dipped into 0.1% Tween 80 solution. Larvae were air-dried by allowing them to freely crawl in laminar airflow for 5-10 minutes and transferred to sterile plastic vials containing a freshly prepared diet. The plastic vials were kept in a BOD incubator at 25 ± 2 °C, 65 ± 5 °C RH. Mortality of larvae and conidial sporulation were examined daily for 15 days. The dead larvae were transferred to a sterilized petri plate having wet cotton to stimulate sporulation. Twenty larvae were used in each treatment and experiments were repeated three times.

Statistical Analysis

Correct mortality percentage was calculated by applying Abbott's formula (Abbott 1925) [2] and before analysis, mortality percentage was transformed and LC_{50} and LC_{90} values were determined by the Probit analysis using Microsoft excel-2016 software.

Results

B. bassiana was more virulent than *M. anisopliae* against third instar larvae of *H. armigera* at different concentrations. However, at 1×10^9 spore ml^{-1}, all the strain of *B. bassiana* and *M. anisopliae* shows a high degree of mortality after 15 days. Significantly 98.3% virulence was recorded in *B. bassiana* (PSC-13) with LC_{50} value (1.8×10^6 spores ml^{-1}) followed by *M. anisopliae* PSC-11 (85.00%) with LC_{50} value (5.07×10^6 spores ml^{-1}) after 15 days of treatment (Table 2).

The experimental data reveal that there was a minimum threshold conidial concentration required for the higher mortality which varied from species to species and even within two isolates of the same species. Minimum LC_{50} and LC_{90} were observed with *B. bassiana* (PSC-13) followed by *M. anisopliae* (PSC-11).

The value of LC_{50} and LC_{90} reveals that *B. bassiana* (PSC-13) was the most virulent fungal strain followed by *M. anisopliae* (PSC-11). Lowest Possible virulent among the four strains of two species used in the bioassay was *M. anisopliae* (Ag-CM) whereas *B. bassiana* (Ag-CB) had virulence of intermediate level.

Table 2: LC_{50} and LC_{90} of the four fungal isolates used in bioassay against the third instar larvae of *H. armigera*

Isolate	LC_{50}	LC_{90}
B. bassiana (PSC-13)	1.8×10^6	8.4×10^7
B. bassiana (Ag-CB)	7.5×10^6	2.72×10^9
M. anisopliae (PSC-11)	5.07×10^6	1.9×10^9
M. anisopliae (Ag-CM)	1.09×10^7	7.5×10^9

Fig 1: Percent mortality of *H. armigera* larvae 15 days post-incubation with a conidial suspension of (a) *B. bassiana* (PSC-13) (b) *B. bassiana* (Ag-CB) (c) *M. anisopliae* (PSC-11) (d) *M. anisopliae* (Ag-CM) as a function of conidial concentration. The percentage mortality values show the mean of three replicate experiments.

Microscopic View of dead larvae

Photographs of larvae showing mycosis indicated that the presence of profusely growing mycelia of respective fungi used in bioassay (Figure 2). The characteristic white mycelia appeared on the surface of *H. armigera* larvae when treated with *B. bassiana*, while green mycelia appeared on the surface of larvae when treated with *M. anisopliae*. The *B. bassiana* shows higher mycosis (up to 82%) as compared to *M. anisopliae* (up to 62%).

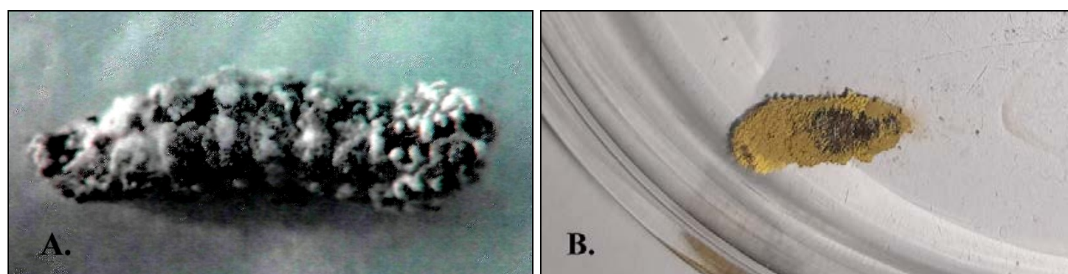

Fig 2: Third instar larvae of *H. armigera* showing mycosis when treated with (a.) *B. bassiana* (PSC-13) (b.) *M. anisopliae* (PSC-11).

Features of Entomopathogenic fungi recovered from infected larvae

The pure culture of all entomopathogenic fungi used in bioassay was recovered from the dead larvae on SDAY media. The recovered isolates showed the respective mycelial and conidial characteristics of entomopathogenic fungi used in the bioassay. Very few contaminations also appeared during entomopathogenic fungi culturing which was insignificant.

Discussion

Several local strains of *B. bassiana* have been reported to be pathogenic to *H. armigera* (Sandh *et al*. 2001). The present study shows that local isolates of *B. bassiana* (PSC-13) and *M. anisopliae* (PSC-11) were more virulent than commercial strain *B. bassiana* (Ag-CB) and *M. anisopliae* (Ag-CB) under

laboratory conditions. This may be due to successive sub-culturing and preservatives used for long life. As per the report of Ana *et al*. (2018) [5], the fall armyworm, *Spodoptera flugiperda* (J.E Smith) (Lepidoptera: Noctudae) is highly susceptible to *B. bassiana* isolates causing 100% larval mortality at 10^8 conidia/ml.

The *B. bassiana* fungal isolate PSC-13 had the highest virulence against the third instar larvae of *H. armigera* because it had a lower LC_{50} and LC_{90} value. These results were compared with those of Swathi *et al*. (2017) [21] say that the lower the LC_{50} value, the highest (100%) larval mortality of *H. armigera* from *B. bassiana* (strain-4). Various research shows that *B. bassiana* and *M. anisopliae* express insecticidal activity against *Rhynchophorus ferrugirous* (El Kichaoui, Abu & El-hindi, 2017) [8] *H. armigera* (Douro *et al*. 2012) [7], *Bemisia tabaci* biotype B (Mascarin *et al*. 2013) [19], *Spodoptera exigua* (Wraight *et al*. 2010) [21] and *Plutella xylostella* (Xia *et al*. 2013) [22].

According to the study by Quesada, Moraga *et al*. (2006) [19] the efficiency of the entomopathogenic fungi began clearly after 48 hrs. of inoculation and the hyphae penetrated the integument, trachea, and epithelial cell. Entomopathogenic fungi sporulation on cadavers is a key factor for proliferation and disease spread within pest population (Hajek *et al*. 1994) [15], (Inglis *et al*. 2001) [16].

In this study, high mortality level (98.3%) was achieved for *H. armigera* third instar larvae treated with 10^9 spores/ml. of *B. bassiana* (PSC-13) at 15 days of treatment under laboratory. For effects on larvae, an adequate concentration of the pathogens is required. It also reveals that there is an increase in mortality with increment in dosage of entomopathogenic fungi. Biopesticides could be promising biological control agents against *H. armigera* larvae as an alternative to chemical insecticides in the leguminous plant including chickpea. Further efforts to develop more potent biopesticides for controlling *H. armigera* in chickpeas are required to screen new, possibly more virulent isolates.

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