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Toxicity of Extracellular Proteins from *Beauveria bassiana* and *Metarhizium anisopliae* on *Spodoptera litura* (Lepidoptera: Noctuidae)

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The present study describes the *in vitro* toxicity of extracellular partially purified soluble proteins (PPSPs) obtained from indigenous isolates of *Beauveria bassiana* and *Metarhizium anisopliae* on *Spodoptera litura*. Toxicity was determined by injecting PPSPs at six different concentrations into 5th instar larvae of *S. litura*. The larvae were susceptible to different protein concentrations in a dose-dependent manner. Among 77 isolates of *B. bassiana*, NBAII Bb-12, 47, 48 and 49 showed significantly higher mortality (80-87%) at the dose of 1.25 mg protein ml^{-1} with LC_{50} values of 0.35-0.85 mg ml^{-1}. Among 55 isolates of *M. anisopliae*, NBAII Ma-4 and 42 showed similar mortality rates (80-87%) at 1.75 mg protein ml^{-1} with LC_{50} values of 0.49-0.71mg ml^{-1}. Microscopic observations revealed the presence of dark melanized spots on dorsal, ventral surface and inner side of tracheal wall in treated larvae indicating the toxic effects of PPSPs.

Key words: *Spodoptera litura*, *Beauveria bassiana*, *Metarhizium anisopliae*, Insecticidal proteins, Toxicity.

Spodoptera litura is a polyphagous insect pest which affects several agricultural crops worldwide and causes extensive economic loss by feeding on leaves of host plants (Guo *et al*., 2007). At present, controlling any insect pest is largely dependent on application of synthetic chemical insecticides, acaricides and to some extent botanical insecticides (Niassy *et al*., 2012). However, *S. litura* has developed resistance to various chemical insecticides which has led a way to investigate for an alternative, eco friendly method using microbial biocontrol agents (Pawar and Borikar, 2005). Biocontrol studies on *S. litura* have been mainly focused with nuclear polyhedrosis viruses (NPVs) and *Bacillus thuringiensis* (Jayanthi and Padmavathamma, 2001; Prabagaran *et al*., 2002). More than 700 species of fungi from around 90 genera were also found pathogenic to several insect pests (Khachatourians and Sohail, 2008). Among entomopathogenic fungi (EPF), *Beauveria bassiana* and *Metarhizium anisopliae* are the important class of natural insect pathogens which are largely exploited for the development of mycoinsecticides (Faria and Wraight, 2007). Studies have shown that conidial suspensions of *B. bassiana* and *M. anisopliae* caused larval mortality and reduce pupation rates of *S. litura* in a dose-dependent manner (Malarvannan *et al*., 2010; Sahayaraj and Borgio, 2010). However, limited

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studies have been carried out with *B. bassiana* and *M. anisopliae* effect on insecticidal activity against *S. litura* with special emphasis on secretion of insect toxic fungal proteins. Hence, the present study was taken up to investigate the ability of different indigenous isolates of *B. bassiana* and *M. anisopliae* to produce insect-toxic protein(s) and their effect on *S. litura*.

MATERIALS AND METHODS

Fungal strains

Seventy seven isolates of *B. bassiana* (NBAII Bb-1 to 77) and 55 isolates of *M. anisopliae* (NBAII Ma-1 to 55) isolated from different insect cadavers and soil samples collected from various parts of India were used in study. The identity of each isolate was confirmed through morphological characters and molecular characterization. Pure culture of each isolate was grown on slants of sabaroud's dextrose yeast extract agar (SDYA) medium (Dextrose 40 g, peptone 10 g, yeast extract 10 g, agar 20 g in 1L of distilled water) supplemented with Streptomycin (0.1%) to prevent bacterial contamination. The slants were incubated at 27±2ºC for 15days and then stored at -20ºC until further use.

Insect host

S. litura larvae were reared on fresh castor (*Ricinus communis*) leaves at 25–27 ºC. The leaves were surface sterilized with aqueous solution of sodium hypochlorite (0.5% v/v) followed by washing twice with sterile distilled water before use. Adult males and females were mated and the eggs laid after mating were incubated at 25 ºC. After hatching, the first instar larvae were fed on castor foliage until they reached the fifth instar stage (Supakdamrongkul *et al*., 2010).

Obtaining partially purified proteins for toxicity assay

Each isolate was grown on to SDYA medium for 15 days at 25ºC. Conidial suspensions were prepared by scraping the conidia into sterile water containing 0.1% Tween-80. One ml of conidial suspension from each isolate (1×10^7 conidia ml^{-1}) was inoculated separately into 100 ml Erlenmeyer flask containing 25 ml of Adamek's liquid medium and incubated at 25ºC on a orbital shaker at 110 rpm for 4 days. Cell-free culture filtrate was obtained by removing the mycelia through filtration (Whatman No. 3 filter paper). The cell-free culture filtrate was subjected to ammonium sulphate precipitation (90% saturation) and centrifugation at 10,000g for 30 min. The resulting pellet was dissolved in sterile double distilled water and desalted by dialyzing against 40 volumes of sterile double distilled water for 24 h at 4ºC using dialysis tubing with a 6-8kDa cut-off membrane. The desalted fraction was centrifuged (10,000g for 10 min) at 4ºC and then filtered through syringe filters (0.45µm) for obtaining partially purified soluble proteins (Urquiza *et al*., 2010). The concentration of protein was determined by Folin-Lowry assay with bovine serum albumin (BSA) as the standard (Lowry *et al*., 1951). The protein solution was diluted with known quantity of sterile double-distilled water to obtain the required concentrations for toxicity assays.

Toxicity assay against *S. litura*

Toxicity assay of PPSPs obtained from different isolates was carried out on 5th instar larvae of *S. litura*. A micro-injector device was used to inject 8 µl (per larva) of PPSPs from each concentration (0.5, 0.75, 1.00, 1.25, 1.5 and 1.75mg protein/ml) through the inter-segmental membrane between the second and third abdominal segments. Control insects were injected with same quantity of sterile distilled water (Urquiza *et al*., 2010). The experiment was performed with five replications and each replication had 15 larvae. The treated larvae were placed in sterile plastic containers with castor leaf bits as feed. After seven days, observations on larval mortality in each treatment were recorded. Dead larvae were dissected and observed under a binocular stereo-zoom microscope (Leica EZ 40) for signs of toxicity such as melanization.

Statistical analysis

The percent mortality was corrected using Abbott's formula (Abbott 1925) before statistical analysis and data from each treatment were analyzed in completely randomized block design after arcsine transformation with ANOVA and least significant difference (LSD) test was used to compare means ($p \leq 0.01$). Median lethal concentration (LC_{50}) was calculated by probit analysis. Statistical analyses were performed using AgRes statistical software, version 3.0 and SPSS software 8.0 for Windows.

RESULTS AND DISCUSSION

The larvae of *S. litura* were susceptible to all six concentrations of PPSPs from *B. bassiana* and *M. anisopliae* isolates used in the bioassay in a dose–dependent manner. Among six concentrations of PPSPs of *B. bassiana* tested, highest larval mortality (80-87%) was observed at the concentration of 1.25 mg protein ml^{-1} and above. In case of *M. anisopliae*, the highest mortality (80-87%) was observed at 1.75 mg protein ml^{-1}. Among different isolates of *B. bassiana*, NBAII Bb-12, 47, 48 and 49 isolates showed significantly higher mortality (80-87%) at 1.25 mg protein ml^{-1} with LC_{50} values ranging from 0.35-0.91 mg ml^{-1}. Among *M. anisopliae* isolates, NBAII Ma-4 and 42 isolates also showed higher mortality (80-87%) at 1.75 mg protein ml^{-1} with LC_{50} values ranging from 0.49-0.71 mg ml^{-1}. The details of other *B. bassiana* and *M. anisopliae* isolates are mentioned in table 1. No mortality was observed in control insects injected with sterile water. Microscopic studies of dead insects revealed the formation of dark melanized spots on dorsal, ventral surface and inner side of tracheal wall in PPSPs injected larvae compared to healthy larvae which showed no signs of melanization. Higher intensity of melanization was observed in dead larvae treated with PPSPs of *B. bassiana* (NBAII Bb-12, 47, 48 and 49 isolates) and *M. anisopliae* (NBAII Ma-4 and 42 isolates) which showed significantly higher mortality rates of *S. litura* larvae (Fig 1). The details on source of isolation and identification of these virulent isolates have been provided (Table 2).

Infection of EPF on insects includes cuticle degradation by hydrolytic enzymes like

Table 1. Effect of PPSPs from different isolates of *B. bassiana* and *M. anisopliae* on mortality *S. litura* larvae

S. No	*B. bassiana* isolates	% Mortality @ 1.25mg protein ml^{-1}	LC_{50} value
1.	Bb-12,47,48,49 (4 isolates)	80-87 [a]	0.35-0.91 [a]
2.	Bb-10,19,24,40,42,43,44,45,46, 50,51,55,60,62,63,66,72,74,75,76 (20 isolates)	40-60 [b]	1.04-1.82 [b]
3.	Bb3,9,4,5,11,13,15,17,18,21,22, 23,25,26,29,32,33,34,36,38,39,41, 53,56,61,64, 65,67,68,71,77 (31 isolates)	13-33 [c]	2.04-2.87 [c]
4.	Bb-1,2,6,7,8,14,16,20,27,28,30, 31,35,37,52,54,57,58,59,69,70,73 (22 isolates)	0-7 [d]	3.24-3.90 [d]
5.	Control	0.00 [d]	-
S. No	*M. anisopliae* isolates	% Mortality @ 1.75mg protein ml^{-1}	LC_{50} value
1.	Ma-4, 42 (2 isolates)	80-87 [a]	0.49-71 [a]
2.	Ma-5,8,11,15,16,19,20,21,22,24, 27,31,32,34,35,36,41,44,45,46,48, 47,49,50,51,54,55 (27 isolates)	67-73 [b]	0.89-1.0 [b]
3.	Ma-2,3,6,7,9,10,12,13,14,17,18,23,25, 26,28,29,30,33,37,38,39,40,43,52,53 (25 isolates)	53-60 [c]	1.02-1.50 [c]
4.	Ma-1(1isolate)	47 [d]	1.61 [d]
5.	Control	0.00 [e]	-

Percent mortality values given are mean of five replications and figures with same letter are satisfactorily non-significant according to LSD ($p \leq 0.01$)

chitinase, protease and lipase, which leads to the penetration, proliferation and production of toxin(s) inside the insect host (Shahid *et al*., 2012). The virulent isolates of *B. bassiana* and *M. anisopliae* are known to secrete low molecular weight fungal secondary metabolites like beauvericin, bassianolide, dipicolinic acid, oosporein destruxins, cytochalasin C, D, helvolic acid etc. which are attributed for toxic effect against insect hosts (Kirkland *et al*., 2005; Xu *et al*., 2008, 2009). However, these EPF also have potential to produce high molecular weight compounds such as proteins, which are poorly studied and can also cause insecticidal effect on different insect hosts. In the present study, it was observed that even extracellular protein(s) from different isolates of *B. bassiana* and *M. anisopliae* have shown insecticidal activity against larvae of *S. litura*. Partially purified proteins from different isolates of *B. bassiana* and *M. anisopliae* showed varying toxic effect on *S. litura*. *B. bassiana* (NBAII Bb-12, 47, 48 and 49 isolates) showed 80-87% mortality at 1.25 mg protein ml^{-1} and *M. anisopliae* (NBAII Ma-4 and 42 isolates) also showed 80-87% mortality rates at 1.75 mg protein ml^{-1}. This dose-dependent effect of PPSPs indicates that a minimum threshold concentration of protein is required to cause higher mortality, which varied from one group of organism to another. Among *B. bassiana* isolates, it was observed that further increase (above 1.25 mg ml^{-1}) in protein concentrations did not have any significant effect on larval mortality. Presence of intense melanization spots in dead insects treated with PPSPs from virulent isolates of *B. bassiana* and *M. anisopliae* also supported for toxic effect of proteins. The insecticidal protein(s) are macromolecular in nature as they were retained by dialysis cut-off 6-8 kDa for globular proteins. In a similar study, extra-cellular crude soluble protein extracts of two isolates of *M. anisopliae* and *B. bassiana* showed a dose-dependent mortality and anti-feedant activity in leaf disc assays against *S. littoralis* larvae (Moraga *et al*., 2006). A proteinaceous, thermolabile, negatively charged toxin produced by *B. bassiana* induced a fast and intense melanization on the cuticle of integument and tracheal wall of *Galleria mellonella* (Fuguet and Vey, 2004). Fuguet *et al*., (2004) reported that hydrophilic protein, Bclp (28 kDa) from *B. bassiana* caused the formation of brownish spots on the

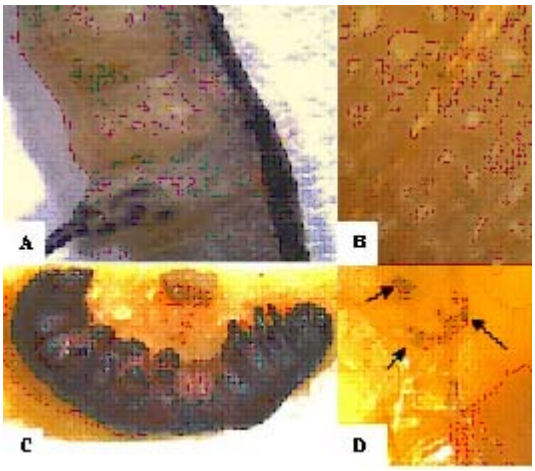

Fig.1. Melanization of the integument and tracheal wall observed after injecting toxic protein(s) secreted by virulent isolates of *B. bassiana* and *M. anisopliae*. A & B: Healthy larvae showing no melanization on integument and tracheal wall. C & D: Dead larvae with melanisation observed after the injection of PPSPs. Arrows point melanized spots on the tracheal wall.

Table 2. Virulent isolates of *B. bassiana* and *M. anisopliae* identified based on toxicity studies

Isolates	Source of isolation	Place	GenBank Acc.
B. bassiana			
Bb-12	Cadaver of red spider mite (*Tetranychus urticae*)	Karnataka	JF837121
Bb-47	Soil sample	Andra Pradesh	JF750393
Bb-48	Soil sample	Andra Pradesh	JF837101
Bb-49	Soil sample	Andra Pradesh	JF837085
M. anisopliae			
Ma-4	Cadaver of cashew stem borer (*Plocaederus ferrugineus)*	Karnataka	JF837157
Ma-42	Soil sample	Gujarat	JQ866688

integument of *G. mellonella* and exhibited clear sequence homologies with fungal chitosanases of *Fusarium solani*. A proteinaceous macro-molecular insecticidal toxic component was secreted by *B. bassiana* (90/2-Dm isolate) *in vitro*, which was found toxic against *Locusta migratoria* in bioassays (Moraga and Vey, 2002).

Our study indicated the secretion of insect-toxic protein(s) by *B. bassiana* and *M. anisopliae* isolates under *in vitro* and their involvement in causing larval mortality of *S. litura*. Among all the isolates tested, *B. bassiana* (Bb-12, 47, 48 and 49 isolates) and *M. anisopliae* (NBAII Ma-4, & 42 isolates) showed higher mortality, lower LC_{50} values and intensive melanization, which indicates the potential of these EPF in causing insecticidal activity against *S. litura*. Further studies on purification and characterization of these toxic protein(s) and gene(s) encoding them will expand the knowledge of their nature and mechanism of action. The potential of these virulence factors can be exploited through genetic engineering for producing hyper-virulent EPF strains and also for expression in plants for combating insect pests in various agricultural crops under field conditions.

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