Protein Levels in *Metarhizium anisopliae* (Metschn.) Sorokīn infested Tomato fruit borer *Helicoverpa armigera* Hubner (Lepidoptera; Noctudiae)

ABSTRACT

The tomato fruit borer, *Helicoverpa armigera* Hubner (Lepidoptera; Noctuidae), is responsible for significant yield losses in tomato and employing chemical pesticides leads to unwarranted environmental issues. The use of entomopathogens in tomato Integrated Pest Management reduces the dependency on chemical insecticides. The green muscardine fungus *Metarhizium anisopliae* is one of the important entomopathogens and worldwide used against many lepidopteran and coleopteran insect pests. The potential of *M. anisopliae* depends on its ability to overcome the host defense mechanisms. The formation immune system in the *H. armigera* in response to *M. anisopliae* infection can be revealed using protein analysis. The order of efficacy of four *M. anisopliae* strains against second and fourth instar *H. armigera* under laboratory conditions were ICAR SBI VS 8 > ICAR SBI I >ICAR SBI S69 > ICAR SBI MA4. The protein concentration increased with a decrease in *M. anisopliae* strain concentration in the second and fourth *H. armigera* larvae. The highly virulent ICAR SBI VS 8 infested *H. armigera* showed a decline in protein concentration up to 168 h (14.99 mg/g). The second and fourth instar *H. armigera* showed similar protein concentration trend in response to *M. anisopliae* infestation. The virulent ICAR SBI VS 8 strain able to resist the host insect immune response and cause infection to the *H. armigera*.

Keywords: H. armigera, M. anisopliae, Protein, Median Lethal Concentration

1. INTRODUCTION

In the realm of agricultural production, pests have long been recognized as formidable adversaries, capable of causing substantial damage to crops and threatening food security. One such formidable pest is *Helicoverpa armigera* Hubner (Lepidoptera; Noctuidae), commonly known as the tomato fruit borer or gram pod borer. It is a highly polyphagous and polymorphous pest infesting more than 400 agricultural and horticultural crops [1]. *H. armigera* infests tomato fruits and renders them unsuitable for human consumption, resulting in a 55 percent loss in crop production and destroys crops worth Rs. 1000 crore [2]. Timely management is a prerequisite to avoie monetary loss to the farmers and they mostly rely on sequential application of insecticides for management.

The calendar-based insecticide application leads to unwarranted environmental issues in the crop eco-system and hence adoption of integrated management strategies is important to maintain the crop ecosystem balance [3]. Biological control involving entomopathogens are an important component of Integrated Pest Management (IPM) [4]. Among the entomopathogens, the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin is one of the most promising microbial control agents against insect pests [5].

A successful biocontrol program depends on the virulence and sustenance of components. The identification of potential *M. anisopliae* strains with unique features are important strategies for their sustained use in pest management activity. The major pathogenicity activities of *M. anisopliae* are adhesion, germination, penetration and dissemination [6]. This process is governed by many factors and proteins in the pathogen are one of the important factors. The commercial success of any mycoinsecticide depends significantly on the virulent nature of the infective propagules, such as conidia, that are sprayed in the field. Moreover, the stability of a fungal strain during repeated conidial sub-culturing on artificial media is very important for making it commercially feasible. The entomopathogenic fungi degenerate as a result of loss of virulence and change in morphology when successively sub-cultured on artificial media [7].

Proteins govern the enzyme and toxin production in the entomopathogens and also immunomodulatory proteins.[8] [9]. Proteomic analysis of larval plasma proteins reveals the formation of immune complexes and immune signalling systems in haemolymph [10]. Estimation of proteins in the *M. anisopliae* will help to identify potential strains which can be employed in Integrated Pest Management [11] [12]. In the present investigation protein levels in tomato fruit borer *H. armigera* in response to four *M. anisopliae* isolates infection was under laboratory conditions to find out the potential strain against the target insect.

2. MATERIAL AND METHODS

2.1. Mass Culturing of *Helicoverpa armigera*

The laboratory gram pod borer *H. armigera* population was established from the fourth and fifth instars collected from pigeonpea and tomato fields in the Dharmapuri Dt. Tamil Nadu. The field-collected population kept in the Insect Rearing Facility, Department of Agricultural Entomology, Coimbatore and observed for any parasitoid emergence and disease incidence. The diseased cadavers and malformed pupa were removed from the culture. The pupae from the field-collected population kept in the adult chambers and provided with adults feed consists of sugar and honey (1:1). These population were maintained in the laboratory at 28°C and 70-80% relative humidity for five generations to establish homogenous laboratory population [13]. First three instars reared in groups and later instars individually maintained in semisynthetic diet. For group rearing plastic trays (35x27x 6 cm) and for individual glass vials (30 x 40 mm) used. The diet in the individual glass vials changed daily and any diseased or malformed larvae were discarded. The pupa in the vials, transferred to oviposition chambers (25x25x32cm) and covered with black muslin cloth. The adult diet containing sugar, honey, and multivitamin (30:5:5) were soaked in sterilized cotton and kept inside the adult chamber. The eggs were collected daily and placed in plastic trays (18x12x6 cm) containing artificial diets.

2.2. Fungal isolates and culture conditions

The *M. anisopliae* fungal isolates *viz*., ICAR SBI VS8, ICAR SBI MA4, ICAR SBI 1 and ICAR SBI S69 collected from ICAR - Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India were used in the present study. These isolates were plated on Potato Dextrose Agar (PDA) medium and incubated in a BOD incubator at 28.2 °C [14]. From the source fungal culture sub-culture was done to get pure culture without any contamination for further studies. The fungal characters were confirmed by studying the conidial and mycelial characteristics using phase -contrast microscope (Make: Euromex iScope – EU 2160058) [15],

2.3. Preparation of spore suspension:

The 10 – 14 days fungal isolates maintained at 28 ºC were used for the study. The fungal spores were scrapped from the plates after full sporulation and suspended in 10 ml sterile distilled water containing 0.05 percent Tween® 80 (Make : Molychem - 19740). The spore count was determined using an upgraded Neubauer hemocytometer (Make: Naudh solutions) [16].

2.4. Exposing the *H. armigera* larvae to *M. anisopliae*

Leaves from the tomato plants maintained under controlled conditions were used to release the larvae. The leaves of unwere iform size collected from the untreated tomato plants and cut in to discs of (2.4 x 1.7 cm) size and kept in the bioassay plates(11.1x8.4x2.2cm) (Precision scientific Co.) The newly moulted II and IV instar *H. armigera* larvae exposed to the medial lethal concentrations, two higher and two lower of four *M. anisopliae* isolates [17]. The leaf discs dipped in the fungal spore suspension for 30 seconds and air dried. For each isolate 30 second and fourth instar larvae released and observed for mycosis and kept in room temperature (30±2 °C). The mycosid larvae used for protein estimation.

2.5. Enzyme preparation

The whole bodies of the second instar and fourth instar for each treatment were homogenized in 0.5 mol L^{-1} Na-phosphate buffer (PB) at pH 7 with 0.1% TritonX-100. Extracted samples were centrifuged at 10,000 ×g for 10 min at 4°C. Supernatants were transferred to new Eppendorf tubes and centrifuged at 15,000 ×g for 20 min at 4°C. (Make : Medline MC-16000R) [18].Then, the supernatants were used to determine the protein concentrations. For protein analysis, Ten II and IV instars from each concentration were tested for each *Metarhizium* strains concentrations [19].

2.6. Protein assay

The protein concentrations of the samples were determined by Bradford's method [20]. It was measured at 595 nm. Bovine serum albumin was used to build a calibration curve. UV-VIS Spectrophotometer (Make: Labtronics; Model LT-2760) was used to record and analyze the readings.

2.7. Data Analysis

Using SPSS Statistics Data Editor Ver.22, IBM software, descriptive analysis was carried out for measured protein at hourly intervals, and the results were reported as mean standard error of mean (SEM) of three replicates. Two-way ANOVA was used to determine the time effect and treatment on total protein levels. To examine protein changes between infected and control larvae, a one-way ANOVA was used. Fisher's least significant difference (Fisher's LSD) was used for mean comparison.

3. RESULTS AND DISCUSSION

The protein estimation in tomato fruit borer *H. armigera* infected by *M. anisopliae* was carried to find out the immune response. The estimation of protein indirectly determines the activity of protease in the host insect in response to the entomopathogenic fungi infection. The larvae were exposed to median lethal concentrations of *M. anisopliae* strains as presented in Table 1. The order of efficacy of different *M. anisopliae* strains in the present investigation was ICAR SBI VS 8 > ICAR SBI I >ICAR SBI S69 > ICAR SBI MA4. The median lethal time presented also showed a similar trend. The protein level in the second instar *H. armigera* in response to *M. anisopliae* infection was presented in Fig. 1. The protein concentration increased with decrease in *M. anisopliae* strain concentration for all the strains in the present investigation (Fig. 1). The highest protein concentration was recorded in ICAR SBI MA 4 strain (127.85 mg/g). Among the strains, the ICAR SBI MA4 strain recorded highest median lethal concentration.

Table 1. Mean lethal concentration (LC_{50}), mean lethal time (LT_{50}) of *M. anisopliae* isolates against *H. armigera* second and fourth instar larvae.

Strain	Second Instar		Fourth Instar	
	LC_{50} ± SE[a] (Conidia ml^{-1})	LT_{50} ± SE[b] (days)	LC50 ± SE[a] (Conidia ml^{-1})	TL50 ± SE[b] (days)
ICAR SBI - VS 8	$2.56 \times 10^6 \pm 2.7 \times 10^6$ a	6.1 ± 0.3 a	$2.23 \times 10^7 \pm 2.7 \times 10^7$ b	6.1 ± 0.3 b
ICAR SBI - MA 4	$2.75 \times 10^7 \pm 3.1 \times 10^6$ b	9.2 ± 0.4 b	$2.56 \times 10^9 \pm 3.1 \times 10^9$ a	9.2 ± 0.4 a
ICAR SBI - SBI I	$9.84 \times 10^6 \pm 5.9 \times 10^5$ a	7.9 ± 0.2 a	$9.71 \times 10^8 \pm 5.9 \times 10^8$ a	7.9 ± 0.2 a
ICAR SBI - S69	$1.59 \times 10^7 \pm 0.8 \times 10^5$ b	8.7 ± 0.4 b	$1.82 \times 10^9 \pm 0.8 \times 10^9$ a	8.7 ± 0.4 a

Means within columns with the same letters are not significantly different (P < 0.05) according to the LSD test.
[a] LC_{50}, calculated after 11 days of the beginning of the experiments.
[b] LT_{50}, calculated at 10^7 conidia ml^{-1}.

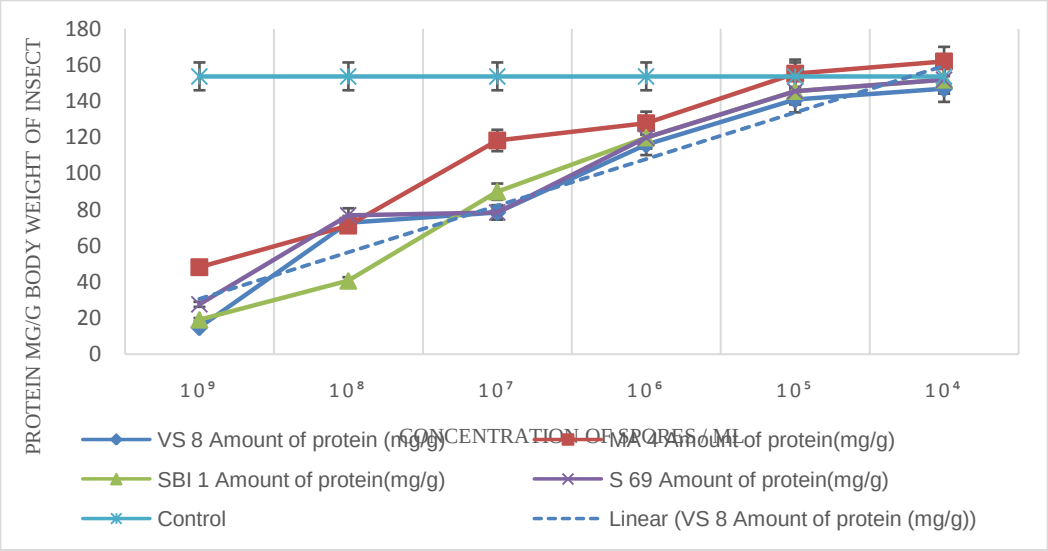

Fig.1. Protein Levels in *H. armigera* second instar in response to *M. anisopliae* infection

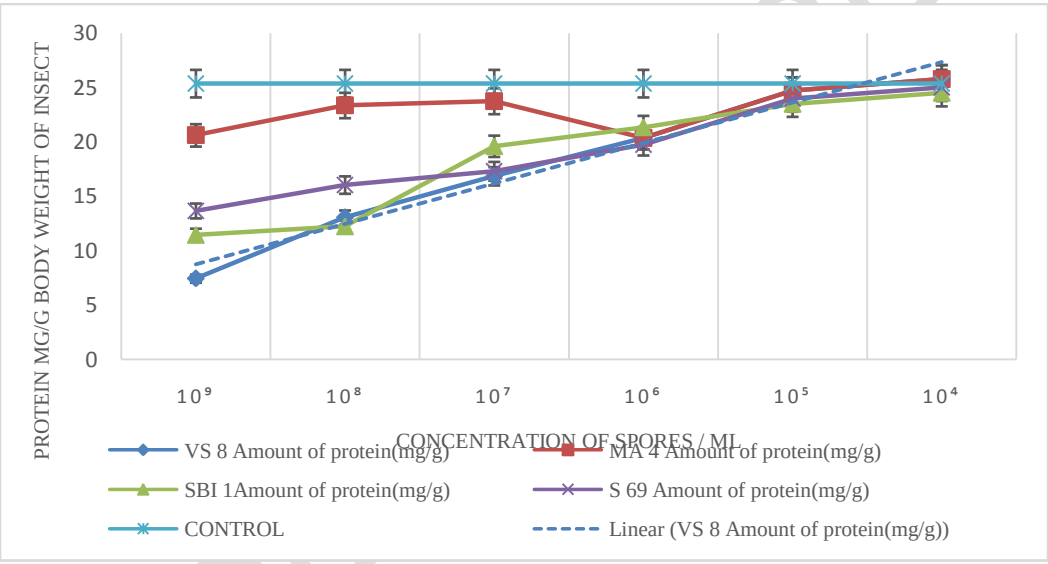

Fig.2. Protein Levels in *H. armigera* fourth instar in response to *M. anisopliae* infection

The more virulent *M. anisopliae* strain ICAR SBI VS 8 showed different responses with respect to increase in spore concentration. The ICAR SBI VS 8 infested *H. armigera* showed a decline in protein concentration up to 168 h with (14.99 mg/g). A similar trend was recorded for the third potential strain ICAR SBI 69. The ICAR SBI 69 recorded 19.10 mg/g at 10^9 spores/ml and 151.88 mg/g at 10^4 spores/ml concentration. The more potent strain recorded lowest protein concentration, whereas the least potent strain recorded a higher protein strain in the second instar *H. armigera* larvae. The least effective *M. anisopliae* strain recorded mixed response with a decrease in spore concentration. The 10^6 concentration recorded lowest protein concentration. The hydrophobins present in the conidial outer layer facilitate its adhesion to the hydrophobic insect cuticle [21]. The proteins such as subtilisins, trypsins, chymotrypsins, and carboxypeptidases digest the protein-rich procuticle of arthropods and allow the fungus to evade the host immune system [22].

Though the fourth instar *H. armigera* showed a similar trend, in response to *M. anisopliae* infestation the levels differed with a decrease in concentration. The ICAR SBI MA 4 strain recorded the lowest protein concentration at 10^6 spores/ml (20.35 mg/g) and the highest concentration at 10^7 spores/ml

(23.74 mg/g) concentration in fourth instar *H. armigera*. The protein levels in ICAR SBI MA 4 treated *H. armigera* fourth instar larvae were higher than the control group, which shows the increased response of host insect to the infestation of entomopathogen. The virulent strain ICAR SBI VS 8 showed linear response in *H. armigera* larvae. The lowest protein concentration of 7.43 mg/g recorded for 10^9 spores/ml concentration, whereas highest protein concentration recorded for 10^4 spores/ml concentration (25.78 mg/g). ICAR SBI 1 strain recorded variable response in *H. armigera* fourth instar larvae (Fig. 2). Rosengaus et al., [23] revealed that, targeting the protein binding sites will increase the susceptibility of termites to *M. anisopliae*. In the present investigation, the host insect protein levels increase with decrease in fungal spore concentration. The dose-dependent response of *R. miroplus* to entomopathogen was documented by Camargo et al., [24]. The presence of higher proteases in the *M. anisopliae* increases its efficacy against host insect [25]. The initial point of fungal infection depends upon the protease activity [26]. The virulent strain ICAR SBI VS 8 infected *H. armigera* recorded lower protein levels even at lower conidial concentrations, which demonstrates its potential to produce more protease to defend the host immune system.

4. CONCLUSION

The order of efficacy of different *M. anisopliae* strains in the present investigation was ICAR SBI VS 8 > ICAR SBI I >ICAR SBI S69 > ICAR SBI MA4. The potent *M. anisopliae* strain ICAR SBI VS 8 treated tomato fruit borer *H. armigera* recorded lowest protein concentration among the isolates. The protein concentration increases with the decrease in the spore concentration of the fungus. The virulent strain is able to overcome the defense mechanism of host insects.

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