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Protein Levels in *Metarhizium anisopliae* (Metschn.) Sorokīn Infested Tomato Fruit Borer *Helicoverpa armigera* Hubner (Lepidoptera; Noctuidae)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The tomato fruit borer, *Helicoverpa armigera* Hubner (Lepidoptera; Noctuidae), is responsible for significant yield losses in tomatoes 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*

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anisopliae is an important entomopathogen used worldwide against many lepidopteran and coleopteran pests. The potential of *M. anisopliae* depends on its ability to overcome the host defence mechanisms. The 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 was 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 trends in response to *M. anisopliae* infestation. The virulent ICAR SBI VS 8 strain can resist the host insect's immune response and cause infection to the *H. armigera*.

Keywords: H. armigera; M. anisopliae; protein; median lethal concentration.

1. INTRODUCTION

In agricultural production, pests have long been recognised 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 polyphagous and polymorphous pest infesting over 400 agricultural and horticultural crops [1]. *H. armigera* infests tomato fruits and renders them unsuitable for human consumption, resulting in a 55 per cent loss in crop production and destroying crops worth Rs. 1000 crore [2]. Timely management is a prerequisite to avoid monetary loss to farmers, and they mostly rely on the sequential application of insecticides for management.

The calendar-based insecticide application leads to unwarranted environmental issues in the crop ecosystem. Hence, adopting integrated management strategies is vital to maintain the crop ecosystem balance [3]. Biological control involving entomopathogens is essential to 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. Identifying 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]. Many factors govern this process, and proteins in the pathogen are one of the essential 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 essential for making it commercially feasible. The entomopathogenic fungi degenerate due to 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 immunomodulatory proteins [8,9]. Proteomic analysis of larval plasma proteins reveals the formation of immune complexes and immune signalling systems in haemolymph [10]. Estimating proteins in the *M. anisopliae* infected host insects will help identify potential strains that 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 studied under laboratory conditions to find out the potential strain against the target insect.

2. MATERIALS AND METHODS

2.1 Mass Culturing of *Helicoverpa armigera*

The *H. armigera* laboratory 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 was 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 were kept in the adult

chambers and provided with adult feed consisting of sugar and honey (1:1). These populations were maintained in the laboratory at 28°C and 70-80% relative humidity for five generations to establish homogenous laboratory population [13]. The first three instars were reared in groups, and later instars were individually maintained in a semisynthetic diet. For group rearing, plastic trays (35x27x 6 cm) and for individual rearing, glass vials (30 x 40 mm) were used. The diet in the individual glass vials changed daily, and any diseased or malformed larvae were discarded. The pupae in the vials were 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 sterilised 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 28ºC 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% 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 uniform size were collected from the untreated tomato plants and cut into 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 were exposed to the median lethal concentrations, two higher and two lower of four *M. anisopliae* isolates [17]. The leaf discs were dipped in the fungal spore suspension for 30 seconds and air-dried. For each isolate, 30 second and fourth instar larvae were released, observed for mycosis, and kept at room temperature (30±2°C). The mycosid larvae were used for protein estimation.

2.5 Enzyme Preparation

The whole bodies of the second and fourth instar from each treatment were homogenised 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 analyse 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 the mean, standard error of the mean (SEM) of three replicates. Two-way ANOVA was used to determine the time effect and treatment on total protein levels. A one-way ANOVA was used to examine protein changes between infected and control larvae. 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 out to find out the immune response. The protein

estimation indirectly determines the protease activity 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 concentration increased with a decrease in *M. anisopliae* strain concentration for all the strains in the present investigation (Fig. 1). Among the strains, the ICAR SBI MA4 strain recorded the highest median lethal concentration (127.85 mg/g).

The more virulent *M. anisopliae* strain ICAR SBI VS 8 showed different responses with respect to an 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 the lowest protein concentration, whereas the least potent strain recorded a higher protein concentration in the second instar *H. armigera* larvae. The least effective *M. anisopliae* strain recorded a mixed response with decreased spore concentration. The 10^6 concentration recorded the lowest protein concentration. The hydrophobins 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].

Table 1. Mean lethal concentration (LC_{50}), and 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}

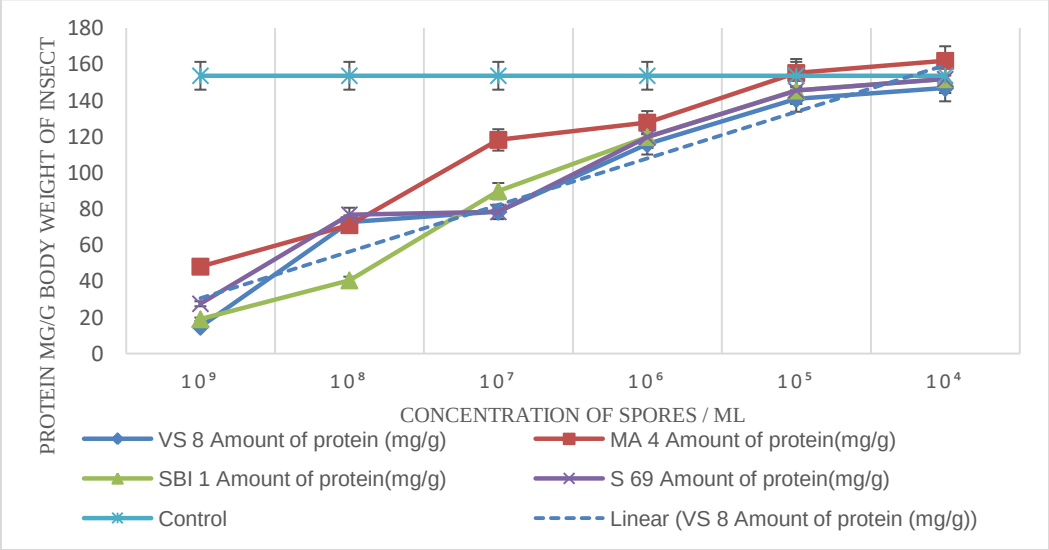

Fig. 1. Protein Levels in *H. armigera* second instar in response to *M. anisopliae* infection

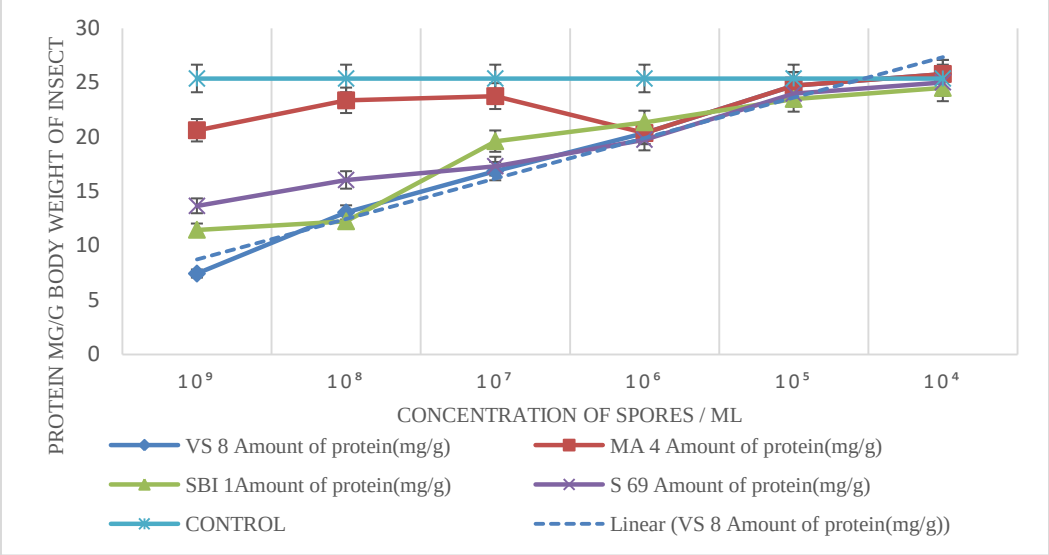

Fig. 2. Protein Levels in *H. armigera* fourth instar in response to *M. anisopliae* infection

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 the 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 the host insect to the infestation of entomopathogen. The virulent strain ICAR SBI VS 8 showed a linear response in *H. armigera* larvae. The lowest protein concentration of 7.43 mg/g was recorded for 10^9 spores/ml concentration, whereas the highest protein concentration was 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 a decrease in fungal spore concentration. The dose-dependent response of *Rhipicephalus miroplus* to entomopathogen was documented by Camargo et al., [24]. Higher proteases in the *M. anisopliae* increase its efficacy against host insect [25]. The initial point of fungal infection depends on protease activity [26]. The virulent strain ICAR SBI VS 8 infected *H. armigera* recorded lower protein levels even at lower conidial concentrations, demonstrating 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 the lowest protein concentration among the isolates. The protein concentration increases with the decrease in the spore concentration of the fungus. The virulent strain can overcome the defense mechanism of host insects.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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