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Effect Of *Metarhizium Rileyi* On Proteases Activity Of *Helicoverpa Armigera* (Hubner) (Lepidoptera: Noctuidae)

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Abstract

Helicoverpa armigera is the most severe polyphagous pest of many economically important crops. An extensive and unplanned excess chemical pesticides application has led to adverse effects on human and insecticide resistance. Entomological pests are controlled by entomopathogenic fungi (EPF) as an alternative tool. *Metarhizium rileyi* is an effective entomopathogenic fungus having a specific host range for many insect pests. In the present study, proteolytic activities of *H. armigera* larvae (1st to 6th larval instars) at different days were evaluated for control and test. The current work aimed to focus on studying the virulent *M. rileyi* against *H. armigera* larvae at various instars. After treatment of *M. rileyi* biopesticide, increasing protease activity was observed in all six different stages of larvae than the control.

Keywords: *Helicoverpa armigera*, *Metarhizium rileyi*, Proteases, Biopesticide, Insecticide resistance.

1. Introduction

Helicoverpa armigera Hubner (Lepidoptera: Noctuidae) is a foremost obnoxious insect pest. It has cosmopolitan distribution occurring in different geographical regions. This pest is polyphagous, leading to heavy economic disruption and yield losses of many economically important crops like chickpea, pigeon pea, tomato, etc. To control *H.armigera* mainly used different chemical insecticides. During the last decades, many insect species have developed resistance to insecticides. This recognized resistance is proved to be one of the key hurdles in effectively managing insect pests (Kranthi et al., 2002). An attractive alternative method for chemical insecticide is biocontrol, an effective eco-friendly option for bacteria, viruses, and fungi in controlling insect pests (Dhakal and Singh, 2019). Across the use of the different pesticide of entomogenous fungus, *Metarhizium rileyi* is an option for the management have several advantages over other traditional insecticides. Therefore, it is essential to study the effect of *Metarhizium rileyi* that appeared on *H. armigera* in terms of their toxicity and their impact on proteases that play a destructive role in relation to concentration.

Proteases are key enzymes in the alimentary canal of insects that are responsible for protein catabolism and release amino acids. Proteolysis plays a crucial role in insect physiology and food digestion (Chitgar et al., 2013). Depending on the amino acids or metal ion engaged, proteases are of four types; serine, cysteine, aspartic acid, and metalloproteases. All these classes of proteases have been determined in insects (Terra and Ferreira, 1994). In contrast, some proteases have been shown to play a key role in the fungal penetration process. The proteases like endopeptidases, aminopeptidases, and carboxypeptidase degrade insect cuticles before chitinases. Entomopathogenic fungi synthesize endo and exo acting proteolytic enzymes in culture (Sharma et al., 2020).

Entomopathogenic fungi get nutrients on degradation by aminopeptidases and exopeptidases of soluble proteins into amino acids (Mondal et al., 2016). The enzymatic activity of subtilisin-like serine protease (Pr1) and trypsin-like protease (Pr2) of *N. rileyi* plays a vital part in the pathogenicity of *Anticarsia gemmatis* caterpillar. The enzymatic activity of *N. rileyi* was higher in the cuticle of larva than with other casein and pupal exuvia as a substrate (Nunes et al., 2010). The knowledge about the activity of these proteases after the pathogenicity of *Nomuraea rileyi* to the *H. armigera* is fundamental for future pest control programs. The protease activity of *H. armigera* larvae treated with *M. rileyi* increased in the present study, suggesting a role of these enzymes in the pathophysiology of the caterpillar. Hence, the present study is focused on the efficiency of *M. rileyi* towards *H. armigera*.

2. Material and Methods

2.1Insect rearing

Pure cultures of *H. armigera* larvae were obtained from the chickpea crop field. Stock culture of the pest was maintained at Yashvantrao Chavan Institute of Science, Department of Zoology, Satara. The newly hatched larva of *H. armigera* was tiny, semitranslucent, and creamy in color. The larvae were reared in two phases viz; in the first, neonate larvae were fed on chickpeas having younger, tender leaves for 3-5 days. In the second phase, the grown-ups were reared individually using an artificial diet (Hamed and Nadeem, 2008). The total larval developmental period is 20-23 days. The first to the sixth instar lasted about 3, 3, 4, 5, 5, and 4 days respectively. In a laboratory-controlled environment at 25±2℃, 75±5℃ RH and 14h photoperiod, the larvae were fed on a semisynthetic diet based on chickpea.

2.2 *Nomuraea rileyi*

Pure cultures of *N. rileyi* were obtained from University of Agricultural Sciences, Dharwad, India. The growth of *N. rileyi* was obtained by periodically subculturing on Sabouraud's Maltose Agar with Yeast extract (SMAY) mediu (Morrow et al., 1989). Bioassay was carried out according to an earlier report with some modifications, and LC50 values were calculated by analyzing data using Probit analysis (Ingle et al., 2021).

2.3 Preparation of samples

2.3.1 The biopesticide was sprayed topically using the direct spray method. For the test, 1.0 ml of each different concentration of fungal spore suspension was directly sprayed. After this treatment, larvae were transferred to separate Petri dishes containing diet. For the control set, 1.0 ml of distilled water was sprayed on the larvae. Both Petri dishes with larvae were maintained at temperatures 25 ± 2℃ and 75±5 % RH. Larval mortality of different instars was observed and recorded from 24 hours to 7 days after treatment.

2.3.2 To study the proteases activity of larval developmental stages from all six instars of 2nd and 4th days were taken. The larvae were isolated, weighed, and cleaned with distilled water. The samples were homogenized in chilled 0.1M Phosphate buffer solution at pH 7 and diluted with the same buffer solution so as to get various concentrations for protease activity. After 10 minutes of centrifugation at 6000 rpm, the supernatant of homogenates were employed as the control. The homogenates of the alternate-day larvae were collected at the interval between 24 to 120 hours after treatment were collected. Homogenate made from selected test larvae between 30 to 50 % mortality with different time intervals.

2.4Determination of proteolytic activity

Casein was used as a substrate to evaluate protease activity (Waghmare et al., 2015). 1.0 ml of 1% casein, 1.0 ml enzyme solution, and 0.5 ml carbonate-bicarbonate buffer made up the reaction mixture (pH 10). For 30 minutes, the reaction mixture was incubated at 37℃. 0.5 ml of 1% Trichloroacetic acid (TCA) was used to halt the reaction. The supernatant from the centrifuged reaction mixture (5000 rpm for 10 minutes) was collected further. 2.5 ml of 0.2 M sodium carbonate buffer and 0.5 ml of 1 M Folin-phenol reagent were added to 0.5 ml of supernatant. At 660 nm, the absorbance was measured using tyrosine as a standard. The enzyme activity was expressed in µg tyrosine/ml/min.

2.5Statistical analysis

Analysis of variance (one-way ANOVA) was performed using obtained data and compared at a 0.05 level of significance. All statistical analyses were done by using Microsoft Excel-2010 software.

Table 1: Effect of *Nomuraea rileyi* spore's concentration on different larval stages of *Helicoverpa armigera*

Larval instars	LC50 (Spores/ml)	Concentration (2×10^2 to 2×10^8 Spores/ml)
First instar larvae	1.46×10^2	1.0×10^1 -5.70×10^3
Second instar larvae	2.60×10^4	2.30×10^2-4.30×10^5
Third instar larvae	4.29×10^5	1.80×10^3- 5.25×10^6
Fourth instar larvae	1.64×10^6	4.85×10^2- 4.09×10^7
Fifth instar larvae	2.77×10^7	9.27×10^4- 9.36×10^8
Sixth instar larvae	1.99×10^8	3.27×10^5- 8.76×10^9

Table 2 Comparative analysis of protease activity of six larval instars on different developmental days in untreated and after treatment with *N. rileyi* pathogenicity on different days of *H. armigera* larvae.

Treatment	Protease activity on alternate days (µg /ml)									
	First instar	Second instar	Third instar		Fourth instar		Fifth instar		Sixth instar	
Day	2nd day	3rd day	2nd day	4th day	2nd day	4th day	2nd day	4th day	2nd day	4th day
Control	0.42± 0.02	0.62± 0.00	0.82± 0.02	0.98 ± 0.05	1.22± 0.03	1.44± 0.02	1.53± 0.02	1.38± 0.45	1.04± 0.02	0.97 ± 0.02
Test	0.50± 0.02*	0.83± 0.03*	1.21± 0.01*	1.78± 0.01*	2.13± 0.02*	2.21± 0.03*	2.38± 0.02*	1.76± 0.03*	1.39± 0.02*	1.22± 0.03*

The values are mean of two experiments ± SD significantly different from control at *p ≤ 0.05, **p≤0.01, ***p≤0.001 by one-way analysis of variance (ANOVA) with Tukey-Kramer comparison test.

3. Results and discussion-

Figure 1 illustrates variations in the proteolytic activity of *H. armigera* during growth. In untreated larvae, enzyme activity enhanced as they progressed through the instars. From the 2nd day of the 1st instar to the 4th day of the 4th instar larvae, there was a substantial rise in protease activity (0.42 µg/ml to 1.44 µg/ml) by an average difference of 0.2 µg/ml. On the 2nd day of the 5th instar larvae, the highest protease activity (1.53 µg/ml) was reported, followed by a minor reduction (1.38 µg/ml) on the 4th day of the 5th instar larvae. On the 2nd day (1.04 µg/ml) and 4th day (0.97 µg/ml) of the 6th instar larvae, there was a substantial reduction in protease activity.

The toxicity of dosages (spores/ml) ordered sequentially as per LC_{50} values for each six-instar larval developmental stage of *H. armigera*, according to the findings of the *N. rileyi* treatment bioassay. Deuteromycetus fungi, such as *M. rileyi* and others, synthesize chitinases, proteases, and lipases in a concerted way to penetrate insect cuticles (St. Leger, 1995). When *M. rileyi* caused infection, the percentages of protease enzyme activity increased gradually in all days of the larval instars. Protease activity progressively raised from the 1st instar (0.50 µg/ml) to the 2nd instar (0.83 µg/ml). After inoculation and incubation, the enzyme activity in the 3rd and 4th instar larvae increased gradually. The highest level of protease activity (2.38 µg/ml) was found in larvae of the 5th instar on the 2nd day. Later, in the 6th instar, levels decreased substantially (1.39 µg/ml to 1.22 µg/ml), but that was more than enough to manage the larvae.

Entomopathogenic fungi (EPF) are biologically valuable control agent that have benefits in Integrated Pest Management (IPM) programmes because of their target host selectivity, ease of transmission, safety for non-target species, and potential to survive in the utter lack of a host for extended periods (Sandhu et al., 2012). As soon as fungus comes into contact with an insect host, enzymatic destruction of the host cuticle barrier as well as mechanical pressure are generated. Fungi release cuticle-degrading enzymes such as chitinases and proteases to tear down this barrier, which is one of the most significant activities that occur during the invasion and infection of the host (Balachander et al., 2012).

The maximum average protease activity of *M. rileyi* NIPHM isolate (2.28 U/ml) was reported on the 8th day (Grewal et al., 2021), after which it decreased significantly. The virulence of three different *M. rileyi* isolates was compared, as well as the amount of cuticle-degrading enzyme synthesis. On the 6th day following inoculation in *Oryctes agamemnon* (Burmeister) larvae, the protease activity of *B. bassiana* (2.61 U/ml), *M. anisopliae* (2.97 U/ml), and *L. lecanii* (2.49 U/ml) was maximum (Elgizawy and Ashry, 2019). The efficacy of *Bacillus thuringiensis* strains with endotoxin crystalline proteins against the insect pest red flour beetle, *Tribolium castaneum*, was investigated (Herbst). The protein concentration and digestive enzymes were found

to be lower in the experimental larvae than in the control larvae. In the 2nd instar *Heliothis zea* larvae (Abdul et al., 1983), the proteolytic activity of *Nomuraea rileyi* utilizing casein and insect host cuticle as substrates found that proteolytic activity began on day 4 after inoculation at optimum pH 8.
The activity of alkaline proteases in the pest *Spodoptera litura* larvae's gut was examined (Ahmad et al., 1976); they discovered that protease increased with larval development and reduced after pupation commenced. Fasting 5th instar larvae resulted in a slight upsurge in activity at 4 hours, gradually dropping with continued deprivation. The activity of acidic, neutral, and alkaline proteases during *Chilo partellus* larval growth was investigated. A significant increase in protease activity at the beginning of each instar was observed, followed by a gradual decline at the end of each instar. The largest increase in protease activity was found on the 4th and 5th larval instars in untreated developing days, and it gradually declined until the last days of the 6th instar. Our findings corroborate the findings of the previously cited study (Nalawade and Bakare, 2015) and the proteolytic activity increases on infection of entamopathogenic fungi than control/healthy larval stages. According to this investigation, our results consent with those of (Ragheb et al., 2018), (Charnley, 2003) and (Pelizza et al., 2012).

4. Conclusion

According to the findings of this study, the proteases activity increases when *M. rileyi* infects *H. armigera* and affects all larval stages at various incubation times and concentrations. During the infection process to the host, the fungi produced extracellular proteases that degraded the host cadaver.

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