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Effect of Entamopathogenic fungus, *Nomuraea rileyi* on the Larval Protein Patterns and Proteases Activity of *Helicoverpa armigera* (Hubner)

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Abstract: **The *Helicoverpa armigera* is one of the most serious polyphagus pest of many economically important crops. *Nomuraea rileyi* is an effective entomopathogenic fungus for the control of *H. armigera* has several advantages over other synthetic insecticide. *N. rileyi* LC_{50} concentration of 1.97×10^6 spores/ml was applied to the 4th larval instar of *H. armigera* to investigate their impacts on the total protein and protease activity of body homogenate. Moreover, qualitative analysis of proteins in healthy developmental stages of *H. armigera* larvae and in *N. rileyi* treated larvae was detected using SDS-PAGE. The present investigation reported that there was difference in SDS-protein bands of control untreated and treated groups. The SDS-PAGE analysis of total body homogenate demonstrated that the few of proteins get under regulated upon treatment with *N. rileyi*. The quantitative analysis of total protein content and proteolytic activity was done. The *H. armigera* larvae upon treatment with *N. rileyi* produced significant decrease ($p<0.05$) in the total protein content of larval body while the proteases activity increased ($p<0.05$) significantly in *N. rileyi* treated larvae than the control larvae. The data of present findings help to understand a part how *N. rileyi* be effective in controlling *H. armigera.***

Key words: **Entamopathogenic fungi, *Helicoverpa armigera, Nomuraea rileyi,* Proteases, Protein profile, SDS-PAGE**

I. Introduction

The legume pod borer or cotton bollworm (*Helicoverpa armigera* Hübner) is a major constraint to crop production globally. It is the principle insect pest in the world due to its flexibility, high polyphagy, short life cycle and high regenerative rate (Lawo *et al.,* 2008). It is the most decisive and effective pest of agriculture causes economic threshold of yield losses both quantity and quality, thus leading to various socio economic problems. The control of *Helicoverpa armigera* relies mainly on utilization of chemical insecticides including carbamates, organochlorines, pyrethriods, organophosphates etc. However, *H. armigera* has developed resistance to these unplanned uses of insecticides which is key hurdles in the management (Kranthi, 2002). Therefore, it becomes imperative to search for alternative methods of control which are ecologically sound, reliable, economical and sustainable. Biological control offers a suitable alternative which includes the use of parasites, predators and microbial pathogens. Across the use of the different pesticide of entomogenous fungus, *Nomuraea rileyi* (Farlow) is an option for the management has several advantages over other traditional insecticides.

The protein metabolism is the important phenomenon during growth and development of insects. The different aspects of protein metabolism comprising quantitative changes in haemolymph protein synthesis and metabolic activity of specific enzymes have fascinated the interest of many insect biochemists. The observations from these biochemical studies indicate that protein metabolism is of immense importance in characterizing different stages of insect development (Chen, 1966). 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).

An entamopathogenic fungus causes infection to the host by contact mechanism of action. Once enter, the fungus grew and on response comprised formation of pathogenic structures like extracellular enzymes, production of toxins, secondary metabolites etc., which eventually caused mortality of insects

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(Sinha *et al.*, 2016). Changes are often expressed as metabolic changes with gradual changes in infected tissue and pathogenicity depends on the physiological state of the host. Thus, the separation and characterization of the insect proteins after infection of *N. rileyi* will be efficient in modulation of protein which can be utilized for the control of pests. Therefore, the current study examined the electrophoretic protein patterns in the 4th instar larvae of the *H. armigera* treated with LC_{50} of *N. rileyi*. In addition, estimation of total protein and protease activity were carried out. Qualitative and quantitative evaluation of proteins in larval insect pests was of immense importance for the understanding of different physiological processes. Therefore, it is essential to study the effect of *Nomuraea rileyi* that appeared on *H. armigera* in terms of their toxicity and their impact on proteins and proteases that play a crucial role in relation to pathophysiology of the pest.

II. MATERIAL AND METHODS

A. Insect rearing

H. armigera larvae were collected from chickpea crop field in Satara district. Laboratory temperature was maintained at 25±2 ℃, 75±5% RH and 14-10 (L:D) h photoperiod. Stock culture of the pest was maintained at Yashvantrao Chavan Institute of Science, Department of Zoology, Satara. The larvae were reared individually using chickpea based artificial diet (Sharma *et al.*, 2014).

B. Nomuraea rileyi (biopesticide)

The pure culture of *N. rileyi* obtained from University of Agricultural Sciences, Dharwad. The growth of *N. rileyi* was obtained by periodically subculturing on Sabouraud's Maltose Agar with Yeast extract (SMAY) medium (Morrow *et al.*, 1989). A series of concentrations of 2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7 and 2×10^8 spore/ml were prepared. Bioassay was carried out according to earlier report with some modifications and LC_{50} values were calculated by analysing data using Probit analysis (Ingale *et al.*, 2015).

C. Preparation of sample of with and without N. rileyi treatment to H. armigera

The *N. rileyi*, biopesticide was sprayed topically in the direct spray method. For the test, one ml of each different concentration of fungal spore suspension was directly sprayed. Dosage of 2×10^8 to 2×10^4 spores/ml of *N. rileyi* was applied to 4th instar *H. armigera* larvae. The concentration at LC_{50} 1.97×10^6 *N. rileyi* spores/ml treated to 4th instar larvae. After this treatment, larvae were transferred to separate Petri dishes containing diet. For control set, one 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 were observed and recorded from 24 hours to 7 days after treatment. Finally, Larvae from control and larvae from test were used for the analysis of proteins and proteases.

Fig. 1 Photographs showing A. Rearing of *H. armigera* B. Maintenance of *N. rileyi* fungal culture C. *H. armigera* larvae without treatment D. *H. armigera* larvae after treatment with *N. rileyi.*

D. SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

Homogenate of the whole body of larva was prepared according to the standard protocol, purification of proteins with 90% saturation by ammonium sulphate method (Duong-Ly and Gabille *et al.*, 2014). The supernatant of the larval body homogenate could be used directly and stored at 4 ℃ until needed for later analysis by SDS-PAGE. Separation of protein content of *H. armigera* healthy larvae and *N. rileyi* treated larvae were done by SDS-PAGE method (Laemmli, 1970). The computer analysis was done for the determination of molecular weight of protein bands using Gel analyzer 19.1 software (Lazar I., 2010).

E. Quantitative Estimation of Proteases and Proteins

The larvae were isolated, weighed, and cleaned with distilled water. The samples were homogenized in chilled 0.1 M Phosphate buffer solution at pH 7 and diluted with the same buffer solution so as to get various concentrations. Supernatant collected by centrifugation at 6000 rpm for 10 minutes. The samples were prepared for both control and *N. rileyi* treated *H. armigera* larvae.

Total protein concentration of the freshly prepared larval homogenate of untreated and treated was assayed by the method Lowry *et al.*, (1951) using bovine serum albumin as a standard. The proteolytic activity was determined in untreated and treated whole *H. armigera* larvae as described by Waghmare *et al.*, (2015) using casein as substrate.

F. Statistical analysis

Biochemical data was expressed as a mean ± SD. Data was analyzed for one way analysis of variance (ANOVA) to test the level of significance using SPSS software package version 19. $p < 0.05$ was considered statistically significant.

III RESULTS

A. Effect of N. rileyi on 4th instar larvae of H. armigera

N. rileyi at different concentration were able to cause death of *H. armigera* after treatment. The results were represented in table I. In bioassay studies the doses of 2×10^8 to 2×10^3 were applied to 4th instar larvae and recorded 69.5 to 28.5% mortality. The LC_{50} and slope values were determined represented in Table I. Mortality percentage of larvae was changed with different concentrations. The differences between larval mortality readings were statistically significant ($P<0.001$).

Table I. Effect of concentration of *N. rileyi* spores on larval mortality of *H. armigera*

Concentration *N. rileyi* spores/ml	Mean larval mortality (%)
2×10^8	69.5
2×10^7	60
2×10^6	56
2×10^5	48.5
2×10^4	36
2×10^3	28.5
LC_{50} spores/ml	1.97×10^6
Slope (±SE)	0.27 (0.03)
P-value	< 0.001

B. Effect of N. rileyi on the protein profile of fourth instar H. armigera larvae

The SDS-PAGE analysis of protein content with and without treatment of *N. rileyi* was done. The figure 2, showed the separated protein bands. The treatment of *N. rileyi* significantly affects the expression pattern of 4th instar larvae of *H. armigera*. The SDS-PAGE showed total 12 protein bands in untreated control and their Rf values were mentioned in Table II with their corresponding molecular weight. The protein bands with molecular weight 91 kDa, 53 kDa, 49 kDa, 44 kDa, 28 kDa and 22 kDa were disappeared upon treatment of *N. rileyi* which observed in untreated *H. armigera*. The expression pattern of proteins was significantly affected by the *N. rileyi* treatment to 4th instar larvae of *H. armigera*. The five protein bands with molecular weight 57 kDa, 38 kDa, 36 kDa, 33 kDa and 24 kDa were detected. However the intensity of these bands was found lesser when observed for density as compared to control untreated larvae of *H. armigera*. The protein patterns both *N. rileyi* treated larvae revealed that there were appearance 2 abnormal bands with molecular weight 40 kDa and 12kDa which were not observed in untreated fourth instar larvae. The protein band with molecular weight 70 kDa was observed upon treatment of *N. rileyi* with slightly increasing density which also observed in untreated *H. armigera* larvae. It was showed the up regulation of protein expression.

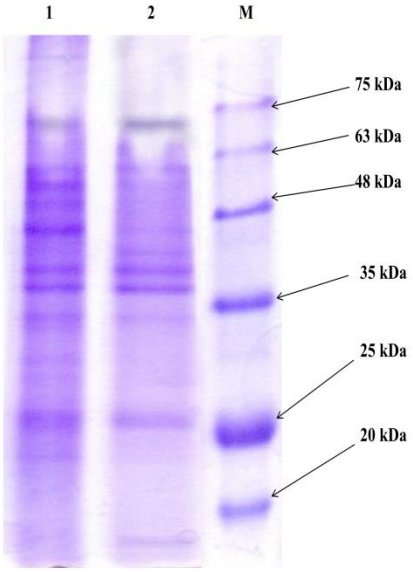

Fig. 2 SDS-PAGE protein patterns of control and *N. rileyi* treated 4th instar *H. armigera* larvae. M: Marker, 1: Control *H. armigera* larvae, 2: *N. rileyi* treated 4th instar *H. armigera* larvae

Table II. Protein profile of control and *N. rileyi* treated *H. armigera* larval body

SR.no.	Rf value	Molecular weight(kDa)	Protein bands	
			Untreated sample	Treated samples with *N. rileyi*
1	0.056	91	+	-
2	0.146	70	+	++
3	0.203	57	++	+
4	0.226	53	++	-
5	0.265	49	++	-
6	0.279	44	++	-
7	0.302	40	-	+
8	0.323	38	++	+
9	0.359	36	+++	+
10	0.393	33	++	+
11	0.453	28	++	-
12	0.525	24	+	+
13	0.602	22	+	-
14	0.983	12	-	+

+ = present, '-' indicates the present or absent of protein bands, + = low, ++ = medium and +++ = high staining intensity.

C. Effect of N. rileyi on protein concentration of H. armigera larvae.

The *H. armigera* larvae upon treatment with *N. rileyi* showed significant decrease ($p \leq 0.05$) in total protein concentration. The

total protein content of the larvae *H. armigera* was recorded after 96 hours post treatment. The *H. armigera* larvae upon treatment with $LC_{50,}$ 1.97×10^6 spores/ml of the *N. rileyi* showed depletion in protein content as (44.69%) of the control group.

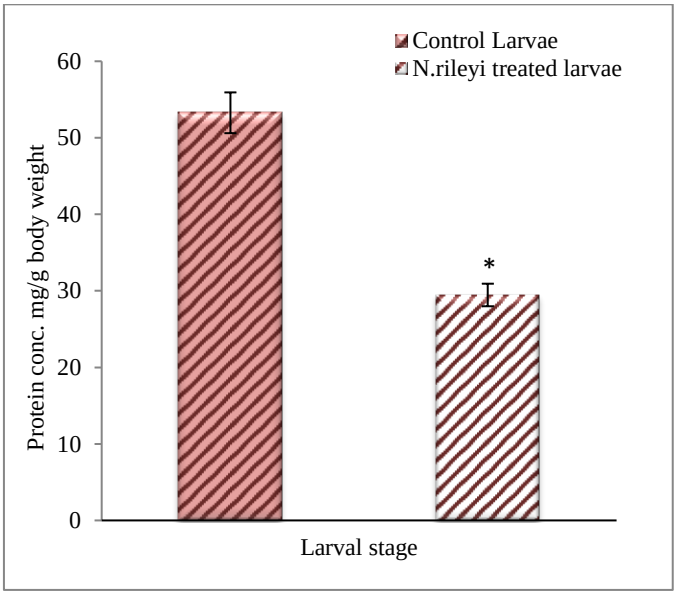

Fig. 3 Protein concentration of control and *N. rileyi* treated *H. armigera* larvae. Significant differences are indicated by asterisk (One way ANOVA; $p\leq 0.05$).

D. Effect of N. rileyi on proteases activity of H. armigera larvae.

The fig.1 illustrates the changes in proteolytic activity of control *H. armigera* larvae was recorded. The 4th instar of *H. armigera* larvae upon treatment with LC_{50} 1.97×10^6 spores/ml of the *N. rileyi* showed rise in proteolytic activity as (24.35%) of the control group. The present study showed a significant increase ($p \leq 0.05$) in proteolytic activity of 4th instar larvae of *H. armigera* treated by *N. rileyi* as compared with the control larvae.

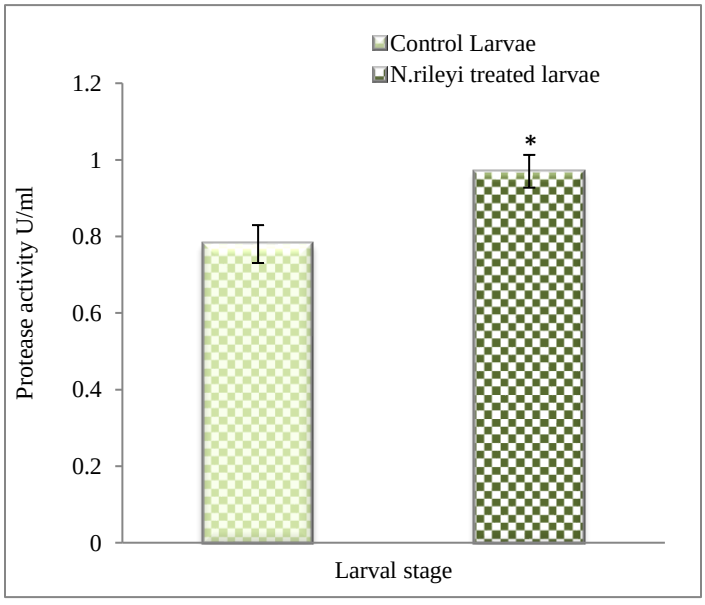

Fig. 4 Proteases activity of control and *N. rileyi* treated *H. armigera* larvae. Statistical differences are shown by asterisk (One way ANOVA; $p\leq 0.05$).
1 U/ml corresponds to 1 µg of tyrosine produced min^{-1} ml^{-1}

IV DISCUSSION

Nomuraea rileyi is a fungal entamopathogen used as an important biological controlling agent worldwide. *N. rileyi* at various concentrations were able to cause death of all stages of *H. armigera*. According to present findings, *N. rileyi* spores caused 50% mortality of *H. armigera* larvae at concentration 1.97×10^6 spores/ml. The present results agreed with Hazarika *et al.* (2016) who studied the effect of *N. rileyi* (local isolate) on 4th instar larvae of *H. armigera* in bioassay study revealed that local isolate of *N. rileyi* was highly virulent. Gundaanavar *et al.* (2005) evaluated the percentage mortality of larval instars of *H. armigera* treated with *N. rileyi*. The concentration dependant mortality percentage was observed.

Proteins are key macromolecules during manifestation of living system. Proteins are important component involved in the framework and metabolism of cells. The data in the present article strongly supports our hypothesis that *N. rileyi*, was more effective biopesticide which causes alteration in the protein content of its host, the *Helicoverpa armigera*. In the present study, biochemical changes upon treatment of *Nomuraea rileyi* showed major changes in the total protein concentration of whole larvae during the course of infection. The alteration in metabolism of any organism is due to physiological anomalies and infection by pathogens. In the present study, the total 9 number of protein bands was observed in healthy *H. armigera* larvae. The 5 protein bands with molecular weight 57 kDa, 38 kDa, 36 kDa, 33 kDa and 24 kDa were appeared upon treatment of *N. rileyi* with lesser intensity. The similar results obtained by Gillespie *et al.*,(2000) who studied the biochemical changes in the desert locust *S. gregaria* on exposure of *M. anisopliae*, the qualitative changes appeared in the protein composition of larval body samples during infection in addition to diminish in the overall protein concentration. The abnormal protein expression was also observed. The protein bands with molecular weight 40 kDa and 12 kDa were found and 70 kDa protein bands was slightly upregulated after treatment of *N. rileyi*. This result in line with, the *Nomuraea rileyi* was supressed the cellular immune response of its host *H. armigera*. The authors reported that haemolymph extracted from *H. armigera* larvae upon infection of *N. rileyi* encompassed toxic metabolites (Zhong *et al.*, 2017). The immunosuppressive compounds were toxic proteins in *N. rileyi* culture fluid against *G. mellonella* (Tseng *et al.*, 2008).

Our findings are in agreement with Sahir-Halouane (2012) showed the effect of *M. anisopliae* and *B. bassiana* on total protein concentration of the 5th nymphal instar of desert locust *S. gregaria*. The protein content in infected insects slightly increased on 2nd day post inoculation and then decreased

significantly on 3rd post infection. The combined effect of these two fungi showed highest reduction in the amount of total protein content. In host-fungus interaction process, host protein biosynthesis was suppressed by entamopathogenic fungi during mycelial tissue invasion. Also it was concluded that the decrease in the concentration of the protein content in the treated larvae may indicate the inhibition of DNA synthesis. Elbanna *et al.*, (2012) reported that *S. gregaria* adults infected with *M. anisopliae* caused physiological imbalances in the host were led to change in enzyme activity and a quantitative reduction in haemolymph protein and other biochemical contents.

Fungi those are entamopthogenic such as *M. anisopliae* and *B. bassiana* who have ability to penetrate the cuticle by releasing extracellular cuticle degrading enzymes including proteases, chitinases and lipases. According to present findings, the result suggests a direct relationship between a high proteolytic activity and an efficient virulence of the fungal strain. Proteases play an important role to break down of proteins into amino acids during food digestion process in the insects. In relation to of these certain aspects the proteolytic activity was observed in developmental stages of insect host, *H. armigera* larvae. The larvae upon treatment with *N. rileyi* showed that the proteolytic activity was increased after death of the host, *H. armigera* (Fig. 4). Entomopathogenic fungi synthesize endo and exo acting proteolytic enzymes in culture and 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).

Our findings corroborate the findings of these mentioned previously cited studies. Didair *et al.*, 2018 studied the biochemical activity of proteases, chitinases and α-&β –esterases in the 4th instar larvae of red palm weevil treated by *Beauveria bassiana*. The author found that the fungus, *B. bassiana*-9894 increases proteases, chitinases and α- & β-esterases enzymes level compared with haemolymph of control larvae. The total protein content was decreased upon treatment of fungus, *B. bassiana*. Decreasing the total protein content may lead to the death of treated insects and this may be one of the reasons of insect mortality. The reduction or the lack of protein leads to retardation of many physiological processes in insects, adult insect requires protein to promote ovulation and egg development supports this conclusion (House, 1963). Reduction of protein content associated with an increase in the level of protease activity was noticed during the progress of fungal infection in the *Helicoverpa armigera.*

CONCLUSION

The results of the present study indicated that *N. rileyi* could infect *H. armigera* larval stage led to death of the host. The *H. armigera* larvae upon treatment with *N. rileyi* (Farlow) biopesticide produced a severe disturbance in the protein metabolism. The depletion in protein content was observed due to the toxic action upon treatment of *N. rileyi* to its host, *H. armigera*. Accordingly, *N. rileyi* can use as natural alternative over synthetic chemical insecticides to control *H. armigera*. The increase in proteolytic activity of the larval body on infection of *N. rileyi* could cause physiological imbalances in the host. The biochemical changes in the protein profiling could be considered as molecular basis of pathophysiology of the pest.

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