Identification and molecular analysis of proteins associated with non-spinning disease in the silkworm *Bombyx mori* L induced by BmNPV and BmDNV

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ABSTRACT

Aims: The present study combines morphological and molecular biology investigations to uncover the underlying causes of the non-spinning syndrome in silkworms. To understand the molecular mechanism and immune mechanisam after infection of viral pathogen in silkworm *Bombyx mori* L

Study design: infected silkworm samples were collected from the field and isolated the genomic DNA and total proteins. Analyszed the samples by using genomic tools and proteomic tools.

Place and Duration of Study: Central Sericultural Research and Training Institute, Mysuru, Karnataka.

Methodology: Our findings indicate that infections of DNV and NPV are associated with abnormal growth of the silk gland, leading to the non-spinning phenotype. To confirm pathogen infection in non-spinning silkworms, we collected genomic DNA and conducted PCR analysis using specific primers for DNV and NPV.

Results: Our results showed amplification of both viral infections in the isolated samples. SDS-PAGE analysis revealed differential regulation of several proteins involved in physiological and immunological processes in the hemolymph of non-spinning silkworms. MALDI-TOF/MS was used to identify an up-regulated protein of interest, which was found to be a Lepidopteran low molecular weight lipoprotein. This protein is known to play an important role in insect immune mechanisms.

Conclusion: Our study aimed to identify major disease-causing agents such as DNV and NPV infections and investigate their impact on protein expression patterns in the hemolymph, shedding light on the factors contributing to non-spinning behavior in silkworms.

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Keywords: Non-spinning, proteomics, viral infection, BmDNV, BmNPV

1. INTRODUCTION

Traditionally, sericulture has focused on silk production from spinning silkworms, which go through a complicated silk-spinning process to produce cocoons. Non-spinning silkworms, a distinct and rare subtype of silkworm, have recently gotten much attention. Despite their inability to produce silk, these non-spinning silkworms have distinct biochemical features that open up new avenues for research and potential applications. These conditions might give rise to reduced silk yield, thinner silk strands, and even silkworm death. The non-spinning syndrome is one of the uncommon disorders noticed in the farmer's field that affects the silk productivity of the country [1]. During the metamorphic event, the spinning behaviour of healthy silkworms changed abruptly. The silkworm normally enters the spinning phase after a few moults, where it creates a protective cocoon out of silk threads. On the other hand, the non-spinning syndrome occurs when the silkworm fails to begin or complete the spinning process,

resulting in significant economic losses for sericulturists. The non-spinning condition might manifest itself in a variety of ways. In certain circumstances, the silkworms do not spin at all and instead stay restless and disturbed [2].

In the case of non-spinning incidents, early identification and remediation are very critical. The occurrence of non-spinning syndrome in silkworms is typically complex, and many factors are not favouring the organism to begin its metamorphic event from later stages of larval to early pupae formation [3]. The physiologically induced changes may interact to cause this metamorphic failure condition, called the non-spinning condition. To lower the frequency of non-spinning syndrome in sericulture, a comprehensive approach including disease management practices, nutritional optimization, environmental control, and genetic selection is required, and proper mulberry cultivation and silkworm rearing strategies need to be adopted [4]. From a disease perspective, non-spinning conditions can be caused by two viruses: BmDNV (Bombyx mori densovirus) and BmNPV (Bombyx mori nuclear polyhedrosis virus) [5]. Let's take a closer look at these viruses and their role in silkworms. To combat DNV, BmNPV, and other viral infections impacting sericulture, researchers are working to develop diagnostic tools, immunizations, and antiviral treatments. DNV is a non-enveloped DNA virus in the Parvoviridae family [6]. It is one of the most prevalent viral infections associated with non-spinning silkworm conditions. DNV primarily affects silkworm digestion, creating a disruption in the activity of the silk gland and, as a result, limiting spinning behavior. The virus replicates within the cells of the silk gland, causing cell damage and lowering silk manufacturing. In silkworms, DNV infection causes a number of symptoms, including decreased appetite, sluggish growth, abnormal feeding behaviour, and failure to begin or complete the spinning process. The severity of the symptoms varies, and the level of viral infection in the silkworm population determines the impact on silk output. Ongoing research and developments in sericulture techniques continue to contribute to a better understanding of the causes of non-spinning syndrome in silkworms and effective prevention approaches. DNV is a non-enveloped DNA virus in the Parvoviridae family [6]. It is one of the most prevalent viral infections associated with non-spinning silkworm conditions. In-depth work related to the disease infection and altered expression of proteins needs to be studied with respect to non-spinning behavior. Hence, the current study concentrated on the detection of infections and the identification of protein regulation in both healthy and non-spinning silkworm samples. We found significant results for the identification of infectious pathogens using SDS-PAGE analysis and PCR analysis.

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2. MATERIAL AND METHODS

2.1. Samples collection

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The silkworms were bled by cutting their caudal horn, gently squeezing the hemolymph out of their bodies, and collecting it in a chilled Eppendorf tube. The collected hemolymph was kept in the freezer. The midgut of the larvae was collected and stored at -20°C for subsequent genetic investigation.

2.2. DNA Isolation from the Silkworm Midgut

Crushed silkworm midgut tissue was transferred to an Eppendorf tube. The genomic DNA extraction method was followed by the GeNei protocol. After soaking the DNA pellet in TE buffer, it was treated with RNase A before being stored at -20°C.

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2.3. PCR analysis

Prepared the PCR master mix according to the normal protocol and placed it in the thermal cycler. The PCR (thermal cycling) conditions were: 94 °C, 30 s; 50 °C, 40 s; 72 °C, 90 s. Start a thermal cycling protocol for PCR amplification, remove tubes after completion, and assess results using 1.8% agarose gel electrophoresis.

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2.4. Protein Determination

Isolated protein samples were carried out for quantification by advanced nanodrop spectrophotometer analysis.

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2.5. SDS-PAGE analysis

The experiment was carried out in accordance with our previous standardized protocol. Hemolymph protein was mixed with sample buffer (4% SDS, 20% glycerol, 10% 2-Mercaptoethanol, 0.004% Bromphenol Blue, and 0.125 M Tris HCl, pH 6.8) and heated at 100 °C for 5 minutes. We used 15% of the polyacrylamide gel. A Bio-rad protean tetra cell was used for electrophoresis at 150 V, 30 mA, and 1.30 min. The gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis.

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2.6. Sample preparation for MALDI-TOF/MS

The 5th ~~th~~ day post-infection hemolymph protein 47 kDa band was selected for examination after being isolated from SDS-PAGE gel. Protein bands were extruded from the gel, kept in 7% acetic acid, and subjected to MALDI-TOF/MS analysis.

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2.7. In-gel digestion and MS analysis

Spots of interest were extruded from the gels 6 hours after staining and digested with trypsin, as described by our standard protocol (Gadwala et al., 2023) [7]. 70% acetonitrile and 3% formic acid were used to extract the tryptic peptides. Peptides were desalted with Zip Tip C18 microtips after 10 minutes of sonication and eluted in 4 µl of 50% acetonitrile. Ultraflex TOF/TOF (Bruker Daltonics, Germany) was used to analyze tryptic peptides on a MALDI-TOF/MS.

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2.8. Database search

The MALDI-TOF/TOF MS detection files were searched against the SwissProt database using the MASCOT search engine (version 2.0; Matrix Science, London, UK) and GPS Explorer software (version 3.0; Applied Biosystems, Foster City, CA, USA).

3. RESULTS AND DISCUSSION

3.1. PCR amplification

To identify the viral infections in non-spinning larvae, DNA isolation and PCR were performed using DNV and NPV virus-specific primers. In field samples, DNV2 amplification was seen at 900 bp and BmNPV amplification at 100 bp, showing the existence of both DNV and BmNPV. In field samples, DNV1 amplification was observed at 400 bp, DNV2 amplification at 900 bp, and BmNPV amplification at 100 bp, indicating the presence of both DNV and BmNPV. These results indicate that non-spinning diseases are caused by both the BmNPV and BmDNV viruses.

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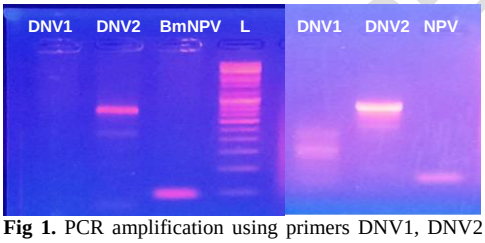

Fig 1. PCR amplification using primers DNV1, DNV2 and BmNPV

3.2 SDS-PAGE analysis

Hemolymph samples revealed the distinction between control and non-spinning silkworms. There were 23 protein bands found in all of the samples. Only 05 (15 kDa, 18 kDa, 29 kDa, 35 kDa, and 50 kDa) protein bands are down-regulated in non-spinning samples out of 23 bands. Specific proteins of 12 kDa, 40 kDa, and 47 kDa are up-regulated in non-spinning samples.

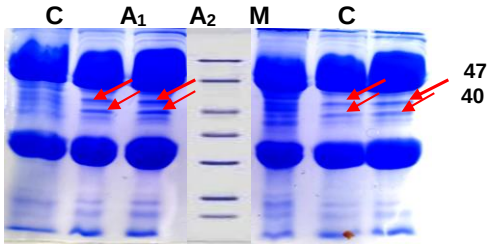

Fig.2. 15% SDS-PAGE analysis, observed changes in the protein banding pattern where 47 KDa and 40 kDa proteins were up-regulated. A1 and A2: Field A samples; B1 & B2: Field B samples.

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3.3 Identification by MALDI-TOF-MS

Based on the SDS-PAGE results, we extruded the 47 kDa protein based on its expression level in the various staining methods and subjected it to MALDI-TOF analysis. MALDI-TOF was used to examine the expressed protein band. The protein had an 89% similarity with the Homologous protein name, Lepidopteron low molecular weight lipoprotein.

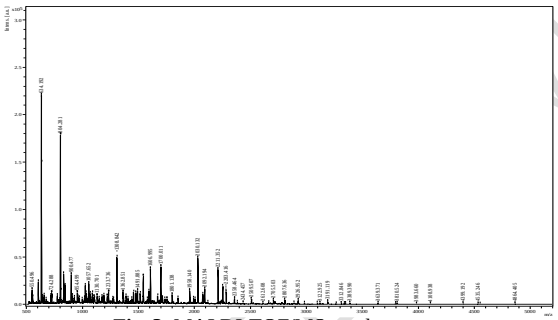

Fig. 3. MALDI-TOF/MS results

4. DISCUSSION

The study of non-spinning silkworm biochemistry is contributing significantly for a variety of reasons. To begin with, it gives information on the physiological and genetic variables that contribute to silk synthesis and silk conversion in silkworms. By examining the molecular differences between these two types of silkworms, we can gain a better understanding of the regulatory processes involved in silk spinning and unravel the complicated interplay between gene expression and protein regulation. The biochemical makeup of non-spinning silkworms was studied using several analytical techniques. The current work aims to analyze the biochemical changes in non-spinning silkworms through a detailed investigation of their protein profiles using SDS-PAGE. By comparing these profiles to those of spinning silkworms, we hope to clarify the molecular differences that strengthen the identification of the problem. Furthermore, we will investigate the potential ramifications of our findings for both basic research and applied sectors.

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In order to identify diseases associated with the problem, we analyzed both healthy and non-spinning silkworms in proteomics and genetic research. We discovered that the non-spinning silkworm is infected with both DNV and NPV viruses. We observed that these infections are implicated in non-

spinning using viral-specific primers. In field samples, DNV2 amplification at 900 bp and BmNPV amplification at 100 bp were observed, showing the presence of both DNV and BmNPV. In field samples, DNV1 amplification was discovered at 400 bp, DNV2 amplification was detected at 900 bp, and BmNPV amplification was detected at 100 bp, indicating the presence of both DNV and BmNPV. Based on the results, we found that both BmNPV and BmDNV cause the non-spinning disease in the silkworm.

We identifiedy and defined the proteins found in this specific insect using SDS-PAGE analysis of protein samples acquired from non-spinning silkworms. This approach detects protein variations and allows comparisons of spinning and non-spinning silkworms, illuminating the underlying molecular differences. In our study, SDS-PAGE analysis showed prominent results in hemolymph after the pathogen was confirmed. Some proteins are up-regulated following infection (12 kDa, 40 kDa, and 47 kDa). MALDI-TOF/MS was used to identify specific protein (40 kDa) in the up-regulated protein band. The targeted protein result shows that lepidopteron low molecular weight lipoprotein has an 89% homology. This protein is synthesized by peripheral fat body tissue for utilization during adult development as storage proteins [8]. The innate immune system and the transportation of lipid and/or sugar are both mediated by lepidopteran low-molecular-weight lipoproteins [9]. After infection with BmNPV, some specific proteins, like serine protease [10], lipase [11], BmNOX [12], arginine kinase [13], and amylase [14], are induced by the midgut tissues. These proteins are active against the virus in the insects and show antiviral activity. Overall, this study adds to the expanding body of knowledge about silkworm biology and lays the groundwork for future research on non-spinning silkworms.

Apart from the pathogens, chemicals like pyriproxyfen, a juvenile hormone pesticide, are widely employed in cultivations around the world, and exposure to them frequently results in silk yield loss and non-cocooning. The effect of pyriproxyfen exposure on cocooning and gene expression levels in the silk gland of *B. mori* has not yet been studied. Exposure to pyriproxyfen can injure silk glands and reduce silk output and cocooning speed. Furthermore, genes involved in the manufacture of silk protein showed substantially lower expression levels. The PI3K/Akt and CncC/Keap1 pathways govern the expression of key genes that can be enhanced by pyriproxyfen exposure, and the two pathways showed comparable change trends [15]. Furthermore, after pyriproxyfen exposure, detoxification enzyme activity (P450, GST, and CarE) and gene expression levels increased, indicating that detoxification enzymes may be important in pyriproxyfen detoxification in the silk gland. These findings provided potential explanations for the silk gland damage and changes in gene transcriptional levels in the silkworm after pyriproxyfen exposure [15].

Juvenile (JH) and Ecdysone hormones well regulate insect metamorphic processes during their life cycles. IGRs, or insect growth regulator hormones, are synthetic analogues heavily used in the pest management of agricultural activities [16]. IGRs usually prolong the larval life cycle and inhibit metamorphosis in insect pests. These residual concentrations will flow into the food chain of the silkworm, leading to biomagnifications in the silkworm's body and an abrupt end to its life cycle. Non-

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spinning syndrome has been observed in other countries as well as on farms where silkworms and breeding cocoons are raised [2]. It is evident from the studies carried out by Jyothi et al. (2019) on pesticide residual effects on silkworms that feeding pesticide-contaminated mulberry leaves to silkworms will cause non-spinning syndrome [17]. In detail, studies are required to dig out the hormonal regulation with respect to disease and pesticide exposure on silkworms and any prominent changes in the biochemical pathways associated with the larval and pupal metamorphosis in silkworm *B~~ombyx~~ mori* [3, 18, and 19].

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Understanding the biochemical makeup of non-spinning silkworms not only adds to our basic understanding of silkworm biology, but it also has potential implications for a variety of practical applications. The identification and characterization of certain proteins in non-spinning silkworms may pave the way for the development of novel silk-like materials with various characteristics. Furthermore, research into protein regulation in these organisms may provide insights into biotechnological applications like recombinant protein production employing non-spinning silkworms as expression hosts.

5. CONCLUSION

Non-spinning is a serious, threatening issue that creates an economic loss to sericulture farmers and industry. The present study found that pathogens such as NPV and DNPV are causing the non-spinning nature of silkworms through the identification and comparative analysis of protein expression studies using SDS-PAGE and MALD-TOF analysis with healthy spinning larvae. The study revealed that Lepidopteron low molecular weight lipoprotein (47 kDa) expression was higher in non-spinning larvae than in healthy or normal silkworms. The presence of the pathogens DNV and Bm NPV in the silkworms identified as an important causative factor will cause non-spinning behaviour in mulberry silkworms. The proper adaptation of technologies relevant technologies and awareness about the IGRs and other chemicals in the mulberry gardens will rescue the farmers from silkworm crop loss.

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