ZOOLOGY RESEARCH ARTICLE

Mitigation of Non-spinning syndrome in silkworm: Synergistic effects of spinach extract and heat shock treatment

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Abstract: Non-spinning syndrome in *Bombyx mori* larvae leads to significant silk crop failures, primarily due to pesticide exposure and environmental stress, causing hormonal and oxidative imbalances. Conventional remedies are often ineffective, necessitating innovative, eco-friendly interventions. This study evaluates the synergistic effect of spinach (*Spinacia oleracea*) extract and heat shock treatment on silkworm maturation and silk gland functionality. Fifth instar larvae from affected areas were divided into control and three treatment groups: heat shock + spinach extract (T1), spinach extract alone (T2), and heat shock alone (T3). Heat shock was applied at 40°C for 1 hour on the fourth day of the fifth instar, while spinach extract-treated mulberry leaves (1:25 dilution) were fed throughout the instar. SDS-PAGE analysis, using a Biobharati pre-stained protein molecular weight ladder (14–120 kDa), followed by gel documentation and densitometric analysis via Bio-Rad Image Lab software, was conducted to evaluate protein expression. T1 showed the highest maturation rate (96%), followed by T2 (93%) and T3 (23%), while the control exhibited no maturation. Protein profiling revealed HSP bands (74, 30 and 27 kDa) in heat shock-treated groups, aligning with Vasudha *et al.* (2006). The results support the role of HSP90 and HSP70 in enhancing steroid receptor functionality (Pratt & Toft, 1997). This synergistic approach combines biochemical (phytoecdysteroids) and physiological (HSP-mediated stress tolerance) pathways, offering a sustainable solution for improving silkworm health and productivity in pesticide-prone areas. Future studies should further investigate the molecular interactions between phytoecdysteroids, HSPs, and ecdysteroid receptors to refine this integrated strategy.

Keywords: Non-spinning syndrome; Silkworm; Spinach Extract; Sericulture

1. Introduction

Non-spinning syndrome in silkworms (*Bombyx mori*) represents a significant challenge for the sericulture industry, manifesting as the inability of larvae to spin cocoons. This disorder results in considerable economic losses, particularly in tropical and subtropical regions where silkworms are highly susceptible to environmental stressors and diseases. The precise causes of non-spinning syndrome remain an area of active research, but mounting evidence implicates hormonal imbalances induced by adverse weather conditions and the indiscriminate use of pesticides in non-sericulture agriculture as contributing factors.

2. Hormonal imbalance and environmental stress

The delicate physiological processes of silkworms are heavily influenced by their hormonal milieu, particularly ecdysteroids and juvenile hormones, which regulate molting and metamorphosis. Disruption of these hormonal pathways can impair the silk gland's function, leading to non-spinning behavior. Adverse environmental conditions, such as high temperature and humidity, exacerbate these disruptions, often triggering oxidative stress and altering metabolic pathways. Heat shock proteins (HSPs), including HSP70 and HSP60, play crucial roles in mitigating stress by acting as molecular chaperones. Studies have shown that heat shock treatments can upregulate these proteins, enhancing thermotolerance and protecting cellular functions during stress periods [1-2].

Indiscriminate pesticide use in neighboring agricultural systems further compounds this issue. Organophosphate insecticides such as dichlorvos and phoxim have been shown to induce oxidative stress in silkworms by increasing reactive oxygen species (ROS) and altering the expression of stress-related proteins, including HSPs and superoxide dismutase (SOD) enzymes [1-3]. While these pesticides are primarily intended for crops like paddy, potato, and jute, their drift into sericulture zones has a profound negative impact on silkworm physiology. The combined stress from abiotic factors and pesticide exposure disrupts the silk gland's ability to synthesize and secrete fibroin, leading to the characteristic non-spinning phenotype.

3. Spinach extract as a mitigative approach

In our previous work, we explored the potential of spinach (*Spinacia oleracea*) extract to counteract the detrimental effects of non-spinning syndrome. Spinach extract, rich in antioxidants and phytoecdysteroids, was found to restore cocoon-spinning behavior in affected larvae. Probably by scavenging ROS, stabilizing cellular membranes and (or) restoring the hormonal balance, the extract effectively mitigated the syndrome and improved the silk gland's functionality. Notably, the supplementation of spinach extract not only enhanced the survival rate of silkworms but also significantly increased cocoon yield and quality. These findings underscore the importance of natural phytochemicals in safeguarding silkworm health and productivity.

4. Focus of the current study

Building on the success of spinach extract, the present study aims to investigate the synergistic effect of heat shock treatment and spinach extract in mitigating non-spinning syndrome. While heat shock treatments have been individually studied for their role in enhancing thermotolerance and inducing HSP expression [2-3], their combined application with bioactive compounds like spinach extract has never been explored. This novel approach is anticipated to provide a dual protective mechanism: enhancing the silkworm's inherent stress-response system through heat shock and directly combating hormonal imbalance via phytoecdysteroid supplementation.

To elucidate the mode of action of these combined mitigation strategies, we will employ advanced molecular biology and biochemical techniques. SDS-PAGE will be utilized to analyze differential protein expression, focusing on heat shock proteins, while enzyme assays will quantify key antioxidant enzymes. These methodologies will provide insights into the cellular and molecular mechanisms underlying the observed phenotypic improvements.

By addressing the interplay between environmental stress, hormonal imbalance, and oxidative damage, this study aims to establish a comprehensive framework for mitigating non-spinning syndrome. The integration of spinach extract and heat shock treatment holds promise not only for enhancing silkworm health but also for improving the resilience and sustainability of sericulture in stress-prone regions.

5. Materials and methods

5.1. Rearing of Silkworms

The study was conducted using *Bombyx mori* L. silkworms of F1 Hybrid, obtained from cross between Nistari Plain and SK6 X SK7 hybrid. Vth instar larvae were procured from affected areas and reared on mulberry leaves procured from affected areas as well. Rearing conditions were maintained following guidelines suggested by The Central Silk Board [4].

5.2. Experimental Design

The study was designed with one control group and three treatment groups to evaluate the effects of spinach extract, heat shock treatment, and their combination on the mitigation of non-spinning syndrome.

- **Control Group (C):** Fifth instar larvae procured from the affected area were reared on mulberry leaves procured from affected areas without any treatment.
- **Treatment Set 1 (T1):** Fifth instar larvae procured from the affected area were subjected to heat shock treatment, administered by placing the larvae in an incubator set at 40°C for 1 hour, as standardized from prior thermal stress studies [5] and were reared on mulberry leaves procured from affected areas treated with spinach extract (1:25 dilution, as standardized in our previous study) [6].
- **Treatment Set 2 (T2):** Fifth instar larvae procured from the affected area were reared on mulberry leaves procured from affected areas treated with spinach extract (1:25 dilution, as standardized in our previous study) [6].

- **Treatment Set 3 (T3):** Larvae from the control group were subjected to heat shock treatment on the fourth day of the fifth instar stage. Heat shock was administered by placing the larvae in an incubator set at 40°C for 1 hour, as standardized from prior thermal stress studies [5].
- Each group consisted of 50 larvae, and three replicates were conducted per group. Maturation rates were recorded daily until the eighth day of the fifth instar to assess the effects of the treatments.

5.3. SDS-PAGE and Densitometric Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted following the protocol described by Vasudha *et al*. [2]. Protein samples were prepared from silkworm larvae homogenates, and proteins were resolved using a 12% polyacrylamide gel. A Biobharati Pre-stained Protein Molecular Weight Ladder (14 kDa–120 kDa) was used as a marker for molecular weight estimation. Following electrophoresis, gels were documented using a Gel Documentation Unit, and densitometric analysis of protein bands was performed using Bio-Rad Image Lab software.

6. Result

6.1. Progression to spinning stage during the 5th instar

- **Control Group (C):** The control group exhibited no maturation up to the eighth day.
- **Treatment Set 1 (T1):** The combined application of **spinach extract and heat shock treatment resulted in the highest maturation rates.** Progression began on the sixth day (87%) and reached **96% by the eighth day**.
- **Treatment Set 2 (T2):** Silkworms treated with **spinach extract alone** exhibited improved progression compared to the control group. Maturation began on the sixth day, with **49% progressing to the spinning stage**. This increased to **93% by the eighth day**.
- **Treatment Set 3 (T3): Heat shock treatment alone** resulted in delayed maturation, with **only 15% of larvae progressing by the sixth day** and **23% by the eighth day**.

Table 1 summarizes the progression of silkworms to the spinning stage under different treatment conditions. The maturation percentage in **Treatment Set 1 (T1)** was significantly higher than in all other groups. By the eighth day of the fifth instar, **96% of larvae in T1** reached the spinning stage, closely followed by **Treatment Set 2 (T2) at 93%**. In contrast, **Treatment Set 3 (T3) and the Control (C) showed significantly lower maturation rates, reaching only 23% and no maturation, respectively**, by the end of the observation period.

These results demonstrate a clear hierarchy in efficacy, with **T1 being the most effective, followed by T2, T3, and the control group**.

Table 1: Effect of different treatments on the Progression of Silkworms to the Spinning Stage During the 5th Instar (up to Day 8)

Sl. No.	Duration in 5th Instar (Days)	Control set (C)	Treatment sets		
			T1	T2	T3
			Maturation (%)		
1.	2nd		Application of SE started	Application of SE started	
2.	3rd				
3.	4th		Heat shock Treatment		Heat shock Treatment
4.	5th			10	
5.	6th		15	49	7
6.	7th		87	71	13
7.	8th	No maturation	96	93	23

** Values in cells represent maturation % of silkworm larvae in respective days of 5th instar stage*

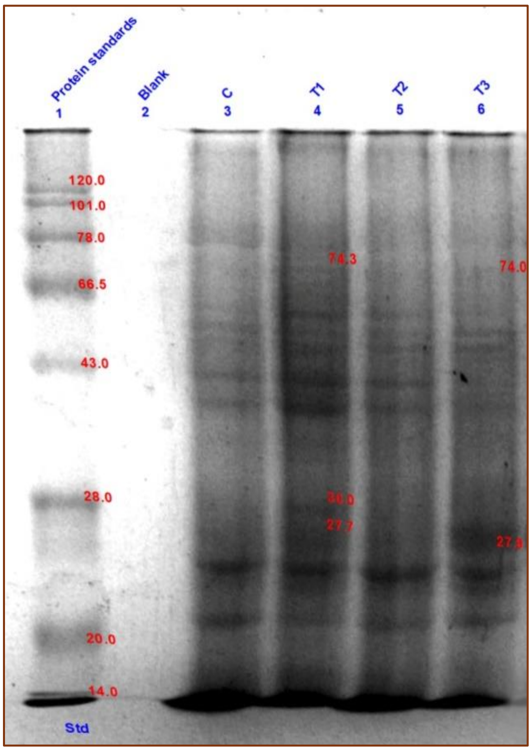

Figure 1: SDS-PAGE profile showing differential protein expression in silkworm larvae subjected to various treatments for mitigating non-spinning syndrome

[Lane 1: Molecular weight marker (Biobharati Pre-stained Protein Ladder, 14 kDa–120 kDa). Lane 3: Control group (C) – larvae from affected areas reared on untreated mulberry leaves. Lane 4: Treatment Set 1 (T1) – larvae subjected to heat shock and reared on spinach extract-treated mulberry leaves. Lane 5: Treatment Set 2 (T2) – larvae reared on spinach extract-treated mulberry leaves without heat shock. Lane 6: Treatment Set 3 (T3) – control larvae subjected to heat shock on the fourth day of the fifth instar stage.]

Table 2: Densitometric Analysis of Differential Protein Expression in Silkworm Larvae Subjected to Various Treatments for Mitigation of Non-Spinning Syndrome

Lane	Band No.	Mol. Wt. (KDa)	Relative Front	Adj. Volume (Int)	Volume (Int)	Rel. Quant.	Band %	Lane %
1	1	120	0.100686	1257306	7633098	9.406326	29.68136	0.736633
1	2	101	0.144165	1239807	7084587	9.27541	29.26826	0.726381
1	3	78	0.19807	421743	3805776	3.1552	9.956133	0.247092
1	4	66.5	0.277492	25080	3466569	0.187632	0.592066	0.014694
1	5	43	0.410263	107673	5969610	0.805538	2.541848	0.063084
1	6	28	0.636156	9576	3472725	0.071641	0.226062	0.00561
1	7	20	0.879569	345135	5080239	2.58207	8.14764	0.202208
1	8	14	0.970252	829692	1865325	6.207203	19.58663	0.486102
4	1	74.34481	0.221968	133666	3113061	1	72.28432	1.058681
4	2	30.01621	0.599542	27136	2113640	0.203013	14.67469	0.214926
4	3	27.73555	0.643021	24115	1793202	0.180412	13.04099	0.190999
6	1	74.00392	0.224256	108136	2603832	0.809002	51.30181	0.137331
6	2	27.89186	0.638955	102648	2057552	0.767944	48.69819	0.130361

Molecular weights (kDa) of major protein bands are provided alongside their respective relative front (Rf), adjusted volume (intensity), volume (intensity), relative quantification, and percentage contribution of each band to the total protein content in each lane. Lane 1 represents the protein profile of the molecular weight marker (Biobharati Pre-stained Protein Ladder, 14 kDa–120 kDa); Lane 4 corresponds to Treatment Set 1 (T1) (Heat shock + Spinach Extract); Lane 6 represents Treatment Set 3 (T3) (Heat shock only). Densitometric analysis was performed using Bio-Rad Image Lab software.

7. Discussion

The results of the present study emphasize the significant potential of combining spinach extract and heat shock treatment (T1) as a synergistic strategy to mitigate non-spinning syndrome in silkworms. The highest maturation rates and improved silk gland functionality observed in the T1 group highlight the effectiveness of targeting both biochemical and physiological stress adaptation mechanisms to enhance silkworm resilience and silk production.

7.1. Role of HSPs in Stress Mitigation

Heat shock proteins (HSPs) are molecular chaperones that play critical roles in stress tolerance and cellular homeostasis. Exposure to thermal stress activates the expression of various HSPs in *Bombyx mori*, which protect cells from damage by assisting in protein folding, preventing aggregation, and facilitating the repair or degradation of denatured proteins. Vasudha *et al*. [2] demonstrated that silkworm larvae subjected to heat shock at 40°C exhibited the induction of multiple HSPs, specifically bands corresponding to molecular weights of approximately 74, 30 and

27 kDa during the fifth instar stage. These HSPs were suggested as molecular markers for thermotolerance evaluation in silkworm strains [2]. The protein expression patterns observed in T1 and T3 groups in our study closely align with this previous research, suggesting that heat shock treatment effectively induced a similar cytoprotective response.

Furthermore, our SDS-PAGE and densitometric analysis identified comparable bands, emphasizing the functional significance of these HSPs in safeguarding cellular functions under stress, ultimately promoting larval progression and cocoon spinning.

7.2. HSPs and Steroid Hormone Receptor Functionality

The link between HSPs and steroid hormone receptor functionality offers additional insights into the molecular underpinnings of the observed improvements in silkworm maturation. HSP90 and HSP70 are essential for the proper folding, activation, and nuclear translocation of nuclear receptors, including insect ecdysteroid receptors [7]. These chaperones maintain receptors in a high-affinity ligand-binding conformation, ensuring that hormonal signaling remains functional under stress conditions. Upon heat shock, upregulation of HSP90 and HSP70 likely facilitated receptor stabilization and improved ecdysteroid receptor functionality, which is vital for the regulation of molting and silk gland development.

Pratt and Toft [7] described the dynamic interaction between steroid hormone receptors and HSP heterocomplexes as a fundamental process regulating receptor activation and nuclear translocation. Our findings align with this model, suggesting that heat shock treatments, through HSP induction, enhance ecdysteroid receptor sensitivity and stability, thereby supporting hormonal pathways crucial for normal silk gland development and cocoon spinning.

7.3. Spinach Extract as a Hormonal Modulator

Spinach (*Spinacia oleracea*) extract is rich in phytoecdysteroids and antioxidants [8-10], which play pivotal roles in mitigating oxidative stress and restoring hormonal balance in silkworms [11-13]. Phytoecdysteroids can mimic insect ecdysteroids, enhancing hormonal signaling pathways associated with molting and silk gland development. In the present study, larvae in T2 (spinach extract alone) and T1 (spinach extract + heat shock) groups exhibited significantly improved maturation rates compared to the control group, highlighting the potential of spinach extract as a biochemical modulator [6].

The enhanced efficacy of the combined treatment (T1) compared to spinach extract alone (T2) suggests that spinach extract and heat shock treatments work synergistically. While spinach extract provides antioxidant and hormonal stabilization, heat shock primes the cellular stress response via HSP induction. Together, these interventions likely create an optimal internal environment that supports silk gland functionality and promotes cocoon spinning, mitigating the detrimental effects of pesticide exposure and environmental stress.

7.4. Comparison with Previous Studies

Our study corroborates the observations made by Vasudha *et al*. [2], who reported that heat shock treatment significantly increased cocoon and shell weights in NB4D2 silkworms. They highlighted the potential of heat shock conditioning as a practical approach to enhance commercial traits in silkworms. Our data reinforce these findings, as T1 treatment not only accelerated larval maturation but also resulted in more uniform cocoon production.

Furthermore, the molecular model proposed by Pratt and Toft [7], emphasizing the chaperone-mediated activation of steroid hormone receptors, provides a mechanistic explanation for the observed synergy between heat shock and spinach extract treatments. The heat shock-induced upregulation of HSPs likely enhanced ecdysteroid receptor stability and ligand-binding capacity, while phytoecdysteroids from spinach extract further augmented receptor activation, culminating in improved silk gland development and cocoon formation.

These findings underscore the importance of integrating biochemical and physiological interventions to develop robust, eco-friendly solutions for managing non-spinning syndrome in sericulture.

8. Conclusion

This study confirms that the combined application of spinach extract and heat shock treatment (T1) is an effective, synergistic strategy for mitigating non-spinning syndrome in silkworms. The molecular basis of this synergy lies in the concurrent enhancement of stress resilience via HSP induction and hormonal homeostasis via phytoecdysteroid supplementation.

The results align with previous findings by Vasudha *et al*. [2], who demonstrated the positive impact of heat shock on HSP expression and cocoon traits. The chaperoning role of HSP90 and HSP70 in stabilizing ecdysteroid receptors, as described by Pratt and Toft [7], further substantiates our observations.

T1 emerged as the most effective intervention, achieving the highest maturation rates and demonstrating the potential for integrating physiological (heat shock) and biochemical (phytoecdysteroids) pathways to support silkworm development. This approach provides a sustainable, cost-effective strategy for improving silkworm health and productivity, especially in regions affected by pesticide exposure and climatic stress.

Future research should focus on elucidating the precise molecular interactions between phytoecdysteroids, HSPs, and ecdysteroid receptors. Additionally, long-term field trials assessing the practicality and economic viability of this combined treatment across diverse sericulture zones would further validate its applicability in commercial silk production.

9. Future Scope

- **Biochemical Pathways:** Further research is needed to elucidate the exact **biochemical mechanisms** through which spinach extract and heat shock mitigate stress in silkworms.
- **Long-Term Field Trials:** Testing the efficacy of these interventions across **diverse environmental conditions** and silkworm strains would provide insights into their broader applicability in sericulture.
- **Integration with Other Remedies:** Investigating combinations of **spinach extract with other plant-based or synthetic stress mitigation agents** may yield synergistic effects, further enhancing silkworm health and productivity.
- **Economic Analysis:** A **cost-benefit analysis** of the proposed interventions would be beneficial for their practical adoption by farmers.
- **Climate Resilience Strategies:** Exploring the role of these interventions in **mitigating climate-induced stress** on silkworms aligns with broader strategies for sustainable agriculture.

This study provides a foundation for **adopting sustainable, low-cost interventions in sericulture**, ensuring resilience against emerging stressors while promoting silk industry profitability.

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