



THE DYNAMICS OF FREE AMINO ACIDS LEVELS IN THE SILKWORM BOMBYX MORI L. INFECTED WITH THE FUNGAL PATHOGEN BEAUVERIA BASSIANA (BALS) AND TREATED WITH PLANT POWDERS

Entomology

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ABSTRACT

The silkworm (*Bombyx mori*) is an economically vital biological machine responsible for producing mulberry silk, yet its health and cocoon yield are often severely impacted by various pathogens. Because biomolecular traits such as protein dynamics reflect an organism's physiological state, biochemical markers serve as effective tools to evaluate host health. Among these threats, the fungus *Beauveria bassiana*, the causative agent of white muscardine disease, poses a major risk during rainy and winter seasons by confining itself to and proliferating within the host hemolymph until death. To address this, the present study examined changes in free amino acid and protein levels in the hemolymph of *B. mori* following sublethal inoculation with *B. bassiana* immediately after the fourth moult, followed by treatment with slaked lime combined with varying concentrations (25%, 50%, 75%, and 100%) of nine herbal powders: *Phyllanthus emblica* L., *Azadirachta indica* A.Juss., *Ocimum tenuiflorum* L., *Cymbopogon citratus* (DC.) Stapf, *Psidium guajava* L., *Murraya koenigii* (L.) Spreng., *Sapindus mukorossi* Gaertn., *Manilkara zapota* (Sapota) and *Lawsonia inermis* L. Compared to pathogen-only controls, infected silkworms treated with these eco-friendly herbal formulations exhibited a gradual increase in hemolymph protein levels. These findings demonstrate that natural, plant-based interventions offer effective, sustainable protection against fungal infection while enhancing silkworm health and overall cocoon quality.

KEYWORDS

Bombyx Mori, *Beauveria Bassiana*, *Azadirachta Indica*, Haemolymph

INTRODUCTION

The silkworm, *Bombyx mori*, is an economically important beneficial insect widely reared for the production of natural silk fibre. Silk is primarily composed of two major proteins, fibroin and sericin, which are synthesized and secreted by specialized regions of the silk gland. Fibroin forms the structural core of the silk fibre, whereas sericin surrounds and binds the fibroin filaments. The synthesis of these silk proteins is a highly regulated physiological process that depends on the availability and utilization of nutrients, particularly amino acids, obtained from the mulberry diet. Recent studies have demonstrated that nutritional status and amino-acid metabolism strongly influence silk-protein synthesis and silk yield in *B. mori*. Nutritional deficiency can reduce the expression of silk-associated proteins and consequently decrease silk production, indicating a close relationship between nutrient availability and silk biosynthesis (Cao *et al.*, 2024).

The haemolymph functions as an important transport medium in *B. mori*, carrying proteins, free amino acids, carbohydrates, lipids, hormones, enzymes and other metabolites between different tissues. Haemolymph metabolites undergo substantial developmental changes during larval growth, moulting, spinning and metamorphosis. Recent metabolomic investigations have shown that changes in dietary conditions can significantly modify the haemolymph amino-acid profile of fifth-instar *B. mori*. Wang *et al.* (2023) identified significant changes in 17 amino acids in the haemolymph following dietary restriction, demonstrating that haemolymph amino-acid metabolism is closely associated with nutritional and physiological status.

Free amino acids in the haemolymph are particularly important because they provide substrates for protein biosynthesis, energy metabolism and tissue development. During larval development, amino-acid concentrations fluctuate according to nutritional intake and the metabolic requirements of developing tissues. These changes are especially important during the fifth instar, when the silk gland undergoes rapid growth and silk-protein synthesis. Recent research has further demonstrated that the amino-acid composition within the silk gland is associated with the distribution and production of fibroin and sericin. Valisalmi *et al.* (2024) reported spatial differences in amino-acid composition within the middle silk gland that corresponded to changes in fibroin and sericin content, emphasizing the importance of amino-acid availability in silk-protein formation.

The synthesis of fibroin and sericin is therefore dependent on the efficient uptake and utilization of amino acids supplied through the

haemolymph. In particular, amino acids such as glycine and serine are important components of fibroin. Alterations in amino-acid metabolism can influence fibroin synthesis and ultimately affect silk production. Recent molecular evidence indicates that disturbances in amino-acid and glutathione metabolism can reduce the availability of glycine and serine and consequently suppress the synthesis of fibroin heavy chain, fibroin light chain and P25 proteins (Qin *et al.*, 2022).

Furthermore, the relationship between nutrition, haemolymph metabolism and silk synthesis is regulated by intracellular nutrient-sensing pathways. Cao *et al.* (2024) demonstrated that nutritional regulation of silk-protein synthesis in *B. mori* involves the PI3K-Akt-SGF1-Dimm pathway, and that nutritional deficiency reduces silk-protein expression and silk yield. These findings indicate that changes in haemolymph amino-acid availability may have direct consequences for silk-gland metabolism and silk-protein biosynthesis. Thus, the determination of free amino-acid levels in the haemolymph provides an important biochemical indicator for assessing nutritional status, physiological condition, metabolic alterations and silk-producing capacity in *B. mori*.

METHODOLOGY

For the study, bivoltine double hybrid (CSR2 X CSR27) X (CSR6 X CSR26) was selected, and chawki worms were procured from Chawki Rearing Centre (CRC), Vedurukuppam, Chittoor (Dist.). Silkworm stock was maintained according to Dandin *et al.* (2003) for the experiment with meticulous synchronisation of several factors required for the healthy growth of silkworms. The silkworms were taken to experiment with the stock at the beginning of the 5th instar.

Beauveria bassiana culture was maintained on potato dextrose agar media. After achieving the required growth, conidia from a single colony were harvested from the *Beauveria bassiana* culture and transmitted to PDA slants, and the pure culture was maintained through repeated transfers every 15 days. The culture of *Beauveria bassiana* was maintained under absolutely sterile conditions.

Fresh and matured leaves of *Phyllanthus emblica* L., *Azadirachta indica* A.Juss., *Ocimum tenuiflorum* L., *Cymbopogon citratus* (DC.) Stapf, *Psidium guajava* L., *Murraya koenigii* (L.) Spreng., *Sapindus mukorossi* Gaertn., *Manilkara zapota* (Sapota) and *Lawsonia inermis* L. were collected in fresh condition and taken to the laboratory of the Department of Biosciences and Sericulture, SPMVV, Tirupati. The leaves were thoroughly cleaned with distilled water, and then the

leaves were shade-dried. The dried leaves were ground into a fine powder and kept in airtight containers for the experimentation.

The fine herbal powders of the nine medicinal plants have been mixed with slaked lime in four ratios: 25% (25 grams of herbal powder + 75 grams of slaked lime), 50% (50 grams of herbal powder + 50 grams of slaked lime), 75% (75 grams of herbal powder + 25 grams of slaked lime), and 100% (100 grams of herbal powder) for the experiment.

Silkworms were inoculated with *Beauveria bassiana* inoculum in sublethal concentration (2.15×10^6 conidia/ml) per treatment. After 2 hours of inoculation, the silkworms were treated with herbal powders and slaked lime mixtures in four concentrations, i.e., 25%, 50%, 75%, and 100% @ 5 grams/sq. ft. Every day before the first feeding, silkworms were dusted with an herbal mixture. from the 1st to the 6th day of the 5th instar silkworm. Three replications were maintained @ 100 silkworms/replication. Two controls were maintained, i.e., silkworms inoculated with a fungal pathogen and another normal control without any treatment.

Haemolymph was collected daily from the first to the sixth day from silkworms inoculated with the fungal pathogen and treated with 9 herbal powders mixed with slaked lime in 4 different ratios, along with both control groups. The silkworms were selected randomly for the collection of haemolymph, then the worms were cleaned and kept in ice for 2-10 minutes. Haemolymph was collected by cutting the 3-5 abdominal legs in pre-chilled centrifuge tubes. Then the haemolymph has been taken for the assessment of total proteins in the experimental and control batches. Free amino acids were estimated by the method of Moore and Stein method (1968). The data have been recorded and statistically analyzed by using three-way ANOVA, and the amino acids levels were expressed as mg/ml of haemolymph.

RESULTS

Amino acids play a key role in maintaining the correct osmotic balance in the haemolymph of arthropods (Sutcliffe, 1963). The nature and arrangement of amino acids influence the physical, chemical, and biochemical features of proteins. These amino acids and their derivatives are involved in various intracellular functions, including nerve transmission, regulation of cell growth, and the biosynthesis of compounds such as hormones, purines, pyrimidines, porphyrins, and certain vitamins (Rodwell, 1993). Amino acid quality is the best diagnostic tool for assessing the cell's physiological state. Changes in total amino acid content also affect protein balance in the silkworm because amino acids are intermediates in protein synthesis. The effect of disease on total and individual amino acid content varies depending on the host and pathogen interactions.

Free amino acid levels (Table -1) were analyzed in the haemolymph of the silkworm *Bombyx mori* inoculated with the fungal pathogen *Beauveria bassiana* and treated with selected herbal powders in combination with slaked lime powders in four concentrations, viz., 25%, 50%, 75%, and 100%, during the 5th instar, with reference to both controls.

A significant decrease in free amino acid levels was observed in the silkworms inoculated with fungal pathogens and treated with almost all herbal powders compared with infected worms (33.81 ± 0.60 mg/ml). However, the elevation of amino acids was recorded relative to the healthy control haemolymph, which was 21.41 ± 0.41 mg/ml.

The gradual depletion of amino acid levels was observed in silkworms inoculated with a fungal pathogen and treated with *Phyllanthus emblica* powder combined with slaked lime at ratios of 25%, 50%, 75%, and 100%. The respective amino acid levels recorded were 32.24 ± 0.65 , 29.74 ± 0.25 , 24.86 ± 0.11 , and 21.35 ± 0.65 , compared to *Beauveria bassiana*-inoculated silkworms (33.81 ± 0.60). However, when compared with the healthy control group (21.41 ± 0.4), the amino acid levels in the experimental groups were elevated.

A similar trend was noticed in *Beauveria bassiana* - inoculated silkworms treated with *Azadirachta indica* powder in combination with slaked lime; the values recorded in the four ratios are 32.25 ± 0.64 , 29.77 ± 0.20 , 24.89 ± 0.02 , and 21.36 ± 0.40 at 25%, 50%, 75%, and 100% ratios, respectively. However, when compared with the healthy control group (21.41 ± 0.4), the amino acid levels in the experimental groups were elevated; however, a decline in amino acid content was recorded in *Beauveria bassiana*-inoculated silkworms (33.81 ± 0.60).

Ocimum tenuiflorum also demonstrated gradual recovery of amino acid levels, viz., 32.26 ± 0.56 , 29.83 ± 0.29 , 24.89 ± 0.19 , and 21.38 ± 0.46 at 25%, 50%, 75%, and 100% ratios, respectively, on par with the healthy control (21.41 ± 0.4). However, gradual drops in amino acid levels were noticed compared to experimental silkworms (33.81 ± 0.60).

A similar pattern of amino acid levels was noticed in the experimental silkworms, i.e., silkworms inoculated with *Beauveria bassiana* and treated with *Cymbopogon citratus* in different concentrations, viz., 32.29 ± 0.59 , 29.86 ± 0.39 , 24.92 ± 0.17 , and 21.38 ± 0.51 at 25%, 50%, 75%, and 100% concentrations, respectively, with reference to the healthy control (21.41 ± 0.4). Nonetheless, a gradual decline in amino acid levels was noticed compared to experimental silkworms (33.81 ± 0.60).

The silkworms dusted with *Psidium guajava* powder in combination with slaked lime in four ratios significantly recovered amino acid levels in a concentration-dependent manner with reference to the healthy control. The amino acid levels recorded in the treated silkworms with 25%, 50%, 75%, and 100% concentrations were 32.29 ± 0.55 , 29.88 ± 0.42 , 24.96 ± 0.17 , and 31.05 ± 0.46 , respectively.

Treatment with *Murraya koenigii* powder in disease-inoculated silkworms significantly restored the amino acid levels on par with those of healthy silkworms. The amino acid levels recorded in four treatments are viz., 32.36 ± 0.44 , 29.94 ± 0.47 , 24.96 ± 0.07 , and 29.88 ± 0.96 at 25%, 50%, 75%, and 100% concentrations, respectively.

The amino acid content in silkworms treated with *Sapindus mukorossi* was recorded as 32.39 ± 0.39 , 29.96 ± 0.47 , 24.98 ± 0.22 , and 21.39 ± 0.52 at different concentrations of 25%, 50%, 75%, and 100%, respectively. *Manilkara zapota* treatment resulted in amino acid levels of 32.76 ± 0.34 , 30.00 ± 0.37 , 24.98 ± 0.22 , and 21.40 ± 0.66 , while *Lawsonia inermis* treatment produced values of 32.32 ± 0.28 , 30.07 ± 0.34 , 27.65 ± 1.24 , and 31.06 ± 0.84 at concentrations of 25%, 50%, 75%, and 100%, respectively.

Overall, the results demonstrate that pathogen infection markedly increased free amino acid levels in silkworm larvae, while treatment with medicinal plant extracts progressively reduced these elevated levels. Among the evaluated botanicals, *Phyllanthus emblica* showed the greatest recovery throughout the treatment period, followed closely by *Azadirachta indica*, *Ocimum tenuiflorum*, *Cymbopogon citratus*, *Sapindus mukorossi*, *Manilkara zapota*, *Psidium guajava*, *Murraya koenigii*, and *Lawsonia inermis*, all of which restored free amino acid content to values nearly identical to the healthy control.

DISCUSSION

The superior performance of *Phyllanthus emblica* may be attributed to its high concentration of vitamin C, emblicanins, gallic acid, ellagic acid, and other polyphenolic compounds that possess strong antioxidant and antimicrobial activities. These compounds are known to neutralize ROS, protect cellular membranes from oxidative damage, and maintain normal enzyme function during microbial challenge (Ahmad *et al.*, 2025). Similarly, *Azadirachta indica* contains azadirachtin, nimbin, nimbolide, quercetin, and other limonoids that exhibit broad-spectrum antimicrobial activity and immunomodulatory properties. These phytochemicals may reduce microbial proliferation and consequently decrease infection-induced physiological stress in silkworm larvae (Isman, 2021 and Li *et al.*, 2023).

The effectiveness of *Ocimum tenuiflorum* and *Cymbopogon citratus* may be explained by their abundance of phenolic compounds and essential oils. Eugenol, rosmarinic acid, ursolic acid, citral, and geraniol have been reported to possess antioxidant, antibacterial, and anti-inflammatory activities (Keshab Bhattarai *et al.*, 2024 and Abdelmonem Siddiq *et al.*, 2026). These bioactive constituents can preserve membrane integrity, reduce oxidative injury, and improve metabolic homeostasis during infection. Likewise, the efficacy observed for *Sapindus mukorossi* may be related to its saponin-rich composition, which disrupts microbial cell membranes and suppresses pathogen growth. Saponins and other plant secondary metabolites are widely recognized for their antimicrobial and immune-enhancing activities (Hasan *et al.*, 2022). *Manilkara zapota* also contains flavonoids, tannins, and phenolic compounds that may contribute to cellular protection and restoration of physiological balance. *Psidium*

guajava, *Murraya koenigii*, and *Lawsonia inermis* also possess well-documented antimicrobial phytochemicals; their extracts restore the measured parameter to the control level.

Previous studies have demonstrated that supplementation with medicinal plant extracts improves the health and productivity of *Bombyx mori* by enhancing antioxidant enzyme activities, stimulating innate immune responses, reducing oxidative stress, and improving larval growth and silk production (Xia *et al.*, 2014; Zhang *et al.*, 2023). Plant-derived phenolics and flavonoids have been shown to enhance the activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and peroxidase, thereby protecting insect tissues from oxidative damage during microbial infection (Wang *et al.*, 2023; Li *et al.*, 2023). The concentration-dependent response observed in the present study is consistent with these reports and supports the potential application of botanical therapeutics in sustainable sericulture.

Table 1: The Free Amino Acids (mg/ml) Levels in the Haemolymph of Silkworm *Bombyx Mori* L. Treated with the Fungal Pathogen *Beauveria Bassiana* (Bals.), Treated with Nine Selected Herbal Powders Mixed with Slaked Lime in Four Concentrations (25%, 50%, 75%, and 100%) During the Fifth Instar Compared with Controls.

Plants	Control	Infected	Treatments 1 (25%)	Treatments 2 (50%)	Treatment 3 (75%)	Treatment 4 (100%)
Phyllanthus Emblica - Usiri	21.41 ± 0.41	33.81 ± 0.60	32.24 ± 0.65	29.74 ± 0.25	24.86 ± 0.11	21.35 ± 0.66
Azadirachta Indica - Neem	21.41 ± 0.41	33.81 ± 0.60	32.25 ± 0.64	29.77 ± 0.20	24.89 ± 0.02	21.36 ± 0.40
Ocimum Tenuiflorum - Tulasi	21.41 ± 0.41	33.81 ± 0.60	32.26 ± 0.56	29.83 ± 0.29	24.89 ± 0.19	21.38 ± 0.46
Cymbopogon Citratus - Lemmon Grass	21.41 ± 0.41	33.81 ± 0.60	32.29 ± 0.59	29.86 ± 0.39	24.92 ± 0.17	21.38 ± 0.51
Psidium Guajava - Guava	21.41 ± 0.41	33.81 ± 0.60	32.29 ± 0.55	29.88 ± 0.42	24.96 ± 0.17	21.05 ± 0.46
Murraya Koenigii - Curry Leaf	21.41 ± 0.41	33.81 ± 0.60	32.36 ± 0.44	29.94 ± 0.47	24.96 ± 0.07	29.88 ± 0.96
Sapandus Mukorossi - Indian Soap Berry	21.41 ± 0.41	33.81 ± 0.60	32.39 ± 0.39	29.96 ± 0.47	24.98 ± 0.22	21.39 ± 0.52
Manilkara Zapota - Sapota	21.41 ± 0.41	33.81 ± 0.60	32.76 ± 0.34	30.00 ± 0.37	24.98 ± 0.22	21.40 ± 0.66
Lawsonia Inermis - Henna	21.41 ± 0.41	33.81 ± 0.60	32.32 ± 0.28	30.07 ± 0.34	27.65 ± 1.24	31.06 ± 0.84

Note: Values are Significant at P<0.01 level

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CONCLUSION

Overall, the present findings indicate that medicinal plant extracts can effectively ameliorate infection-induced physiological disturbances in *Bombyx mori*. All the tested botanicals, *Phyllanthus emblica*, *Azadirachta indica*, *Ocimum tenuiflorum*, *Cymbopogon citratus*, *Sapindus mukorossi*, *Manilkara zapota*, *Psidium guajava*, *Murraya koenigii*, and *Lawsonia inermis*, demonstrated the highest efficacy, producing values that closely approached those of the healthy control at 100% concentration. These results suggest that these plants may serve as promising eco-friendly alternatives for improving silkworm health and reducing disease-associated metabolic stress. Further studies involving phytochemical characterization, gene expression analysis of immune and antioxidant pathways, and field-scale validation are recommended to elucidate their mechanisms of action and practical application in sericulture.