Biological Forum – An International Journal **16(8): 173-177(2024)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Effect of Pesticide's Residue on the Biochemical Parameters of the Silkworm (*Bombyx mori* L.) Haemolymph

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(Received: 17 May 2024; Revised: 12 June 2024; Accepted: 05 July 2024; Published: 14 August 2024)
(Published by Research Trend)

ABSTRACT: The organic constituents of haemolymph (proteins, carbohydrates) play an important role in biochemical processes underlying growth and development of insects. The biochemical parameters and activity of enzymes was estimated in haemolymph of silkworm, *B. mori* from first day to sixth day of fifth instar which were fed with mulberry leaves treated with pesticides. The total protein content in haemolymph was found significantly decreased in pesticide treatments from first day to sixth day of fifth instar. The total carbohydrate content in the haemolymph was found significantly increased from first day to sixth day in all the treatments. The results indicated that, novluron 10EC and chlorfenapyr 10SC were highly toxic even after waiting period.

Keywords: Silkworm, haemolymph, protein, carbohydrate, pesticides, novluron.

INTRODUCTION

The process of silk production is sequential and interlinked process, as food taken by silkworm is converted into protein during the course of development and protein into silk fibre. Therefore, the silk fibre is completely dependent on the protein content of the silkworm. The fifth instar haemolymph protein contributes towards silk protein biosynthesis in the silk gland and the final products of silk proteins are fibroin and sericin which form the main components of silk fibre (Shivkumar and Subramanya, 2015). The growth and development of silk gland depend on the health of silkworm and its nutritional status (Kumar and Gangwar, 2010). Carbohydrates are the major fuel in insects, which provide the energy for their survival. The dietary carbohydrates meet the energy demand during their growth and metamorphosis (Shivakumar and Shamitha, 2013). The origin, nature and fate of carbohydrate during growth, metamorphosis, flight, reproduction and embryonic development are varied in different insect species. In most of the insects, carbohydrates are present as glycogen and trehalose which can be readily converted into glucose. Carbohydrates are necessary for normal functioning of the male and female reproductive system as well as the development of embryo (Yamashita and Hasegawa, 1974).

MATERIALS AND METHOD

A. Experimental details

Crop : Mulberry
Variety : Victory-1 (V-1)
Silkworm breed : PM×CSR2
No. of treatment : 9
No. of replication : 3
Design : RCBD

Table 1: Treatment details.

Treatments	Details	Dosage
T_1	Carbofuron 3G	12g/plant
T_2	Dimethoate 30EC	2ml/L
T_3	Novluron 10EC	0.5ml/L
T_4	Azadirechtin 0.03EC	2ml/L
T_5	Fenazaquin 10EC	1.5ml/L
T_6	Dinotefuron 20 SG	0.25g/L
T_7	Chlorfenapyr 10EC	1.5ml/L
T_8	Water spray	1L
T_9	Absolute control	-

B. Collection and storage of haemolymph

The haemolymph was collected from the first day to sixth day of fifth instar in each treatment. From each replication ten larvae were randomly selected. For extracting the haemolymph in fifth instar, third abdominal legs were amputated with sterilized blade and the haemolymph thus bled was immediately drawn into pre-cooled eppendorf tubes containing phenyl thiourea at 1mg/tube. Phenyl thiourea was used to avoid the activity of prophenol oxidase that causes melanization of the haemolymph samples (Etebari *et al*., 2006). To ensure complete extraction of haemolymph, the larvae were gently pressed from anterior and posterior ends simultaneously until no more haemolymph oozed out of the wound. The samples were centrifuged at 3000 rpm for 15 minutes to separate out the phenylthiourea crystals and haemocytes. The supernatant was used for the estimation after proper dilution (Mahesha *et al*., 2000).

The samples were labelled and then preserved in deep freezer at - 20°C till further use (Plate 2).

C. Estimation of proteins

The total proteins in the haemolymph was estimated by using Lowry's method (Lowry *et al*., 1951) using crystalline Bovine Serum Albumin (BSA) as standard (Plate 3).

(i) Principle. The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau's reagent by the amino acids tyrosine and tryptophan present in the protein together with the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate is measured in the Lowry's method.

(ii) Reagents

1. 2% Sodium carbonate in 0.1N sodium hydroxide (Reagent A).
2. 0.5% Copper Sulphate ($CuSO_4 \cdot 5H_2O$) in 1% potassium sodium tartarate (Reagent B).
3. Alkaline copper solution: Mix 50 mL of Reagent A and 1 mL of Reagent B prior to use (Reagent C).
4. Folin-Ciocalteau's reagent (Reagent D).
5. Protein solution (Stock Standard): Dissolve 50mg BSA in 50mL of 0.1N NaOH in a volumetric flask. Take 10mL of this stock standard and dilute to 50mL in another flask for working standard solution. One mL of this solution contains 200 µg proteins.

(iii) Procedure for estimation of protein

1. 0, 200, 400, 600, 800, 1000 µL of the working standard was taken into a series of test tubes and the final volume was made up to 1 mL in each test tube. A test tube with 1.0 mL of water served as the blank.
2. 100 µL of haemolymph sample was taken in a 5 mL volumetric flask and the volume was made up.
3. 50 µL of this sample was taken in a test tube and the volume was made up to 1 mL.
4. 5 mL of reagent C was added to each tube including the blank. It was mixed well and allowed to stand for 10 minutes.
5. Then 0.5 mL of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes, till a blue colour is developed.
6. The absorbance of the blue colour developed was read against the blank at 660nm on spectrophotometer.
7. The values were expressed in terms of mg of protein per ml of haemolymph, by employing proper dilution factors.

(iv) Calculation. A standard graph was drawn by taking the concentration of BSA on X-axis and spectrophotometer reading on Y-axis. From the graph the concentration of protein in the sample was calculated.

D. Estimation of carbohydrates

The quantitative estimation of total carbohydrate in the haemolymph of silkworm was done by Anthrone method (Dubois *et al*., 1956) using glucose as standard.

(i) Principle. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone having absorption maximum at 600 nm.

(ii) Reagents

1. **Glucose stock standard:** 100 mg of glucose was dissolved in 100 mL of water in a standard flask.
2. **Working standard:** 10 mL of the stock was diluted to 100 mL. Such that 1 mL of this solution contained 100 µg of glucose.
3. **Anthrone reagent:** 0.2% anthrone dissolved in ice cold concentrated sulphuric acid. Prepared fresh before use.

(iii) Procedure

1. The standards were prepared by taking 0, 200, 400, 600, 800, 1000 µL of the working standards in a series of test tubes and volume was made up to 1 mL in all the test tubes. 1mL of water was used as the blank.
2. 100 µL of haemolymph sample was taken in a 5 mL volumetric flask and the volume was made up to 5 mL with double distilled water.
3. 100 µL of this sample was taken in a test tube and the volume was made up to 1 mL.
4. To all the test tubes (both standards and samples) 4 mL of freshly prepared Anthrone reagent was added.
5. The test tube was kept in boiling water bath for 15 minutes for the reaction to complete.
6. The samples were cooled immediately to room temperature.
7. The absorbance of the green to dark green colour developed was read against the blank at 600nm on spectrophotometer.
8. The values were expressed in terms of mg of carbohydrate per mL of haemolymph, by employing proper dilution factors.

(iv) Calculation. A standard graph was drawn by taking the concentration of glucose on X-axis and spectrophotometer reading on Y-axis. From the graph the concentration of glucose in the sample was calculated based on the absorbance.

RESULTS AND DISCUSSION

The results and discussion on the research topic entitled "Effect of pesticide's residue on the biochemical parameters of the silkworm (*Bombyx mori* L.) haemolymph" conducted during 2021-2022 at College of Sericulture, Chintamani and biochemical studies were conducted in Advanced Centre for Plant Biotechnology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore, presented below and discussed in the light of earlier reports published.

A. Total protein content (mg/mL)

Total protein content was significantly different among the treatments, from 2nd day to 6th day except on 1st day where it is non-significant. The pooled data of the two rearings revealed that, in treatments carbofuron 3G (T_1), dimethoate 30EC (T_2), novluron 10EC (T_3), azadirechtin 0.03EC (T_4), fenazaquin 10EC (T_5), dinotefuron 20 SG (T_6) and chlorfenapyr 10EC (T_7), the total protein content significantly decreased from first day to sixth day of fifth instar. But in treatments water spray (T_8) and absolute control (T_9) the total protein content significantly increased from first day to sixth day of fifth instar (Fig. 1).

The lowest haemolymph total protein content of 4.02 mg/mL was recorded in novluron 10EC (T_3), followed

by chlorfenapyr 10EC (T_7) recorded 4.10 mg/mL and fenazaquin 10EC (T_5) recorded 4.80 mg/mL which was statistically lower than rest of the treatments on sixth day of fifth instar under study. During the study, it was also reported that highest peak of haemolymph total protein content of 24.66 mg/mL was noticed in absolute control (T_9), followed by water spray (T_8) recorded 23.32 mg/mL and dimethoate 30EC (T_2) recorded 8.79 mg/mL (Table 1) which was statistically higher than rest of the treatments on sixth day of fifth instar.

Table 2: Total protein content (mg/mL) in the haemolymph of fifth instar silkworm (*B. mori*) as influenced by feeding pesticide treated mulberry leaves.

Treatments	Protein content in 5th instar					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
T1 – Carbofuron 3G	14.66	13.95	12.15	10.81	10.16	8.70
T2 – Dimethoate 30EC	14.70	14.00	13.09	11.44	9.93	8.79
T3 – Novluron 10EC	13.96	10.13	7.36	5.19	4.51	4.02
T4 – Azadirechtin 0.03EC	14.48	12.60	10.32	9.12	8.49	7.37
T5 – Fenazaquin 10EC	14.26	11.28	8.83	6.62	5.21	4.80
T6 – Dinotofuron 20SG	14.32	11.99	9.39	8.05	6.19	5.11
T7 – Chlorfenapyr 10EC	14.14	10.41	7.87	5.29	4.75	4.10
T8 – Water spray	14.94	17.11	17.58	20.84	21.64	23.32
T9 – Absolute control	14.75	16.59	17.93	20.66	22.84	24.66
F - test	NS	*	*	*	*	*
SEm ±	-	0.66	0.63	0.59	0.59	0.62
CD @ 5%	-	1.97	1.88	1.75	1.75	1.83
C.V (%)	-	8.78	9.42	9.36	9.78	10.59

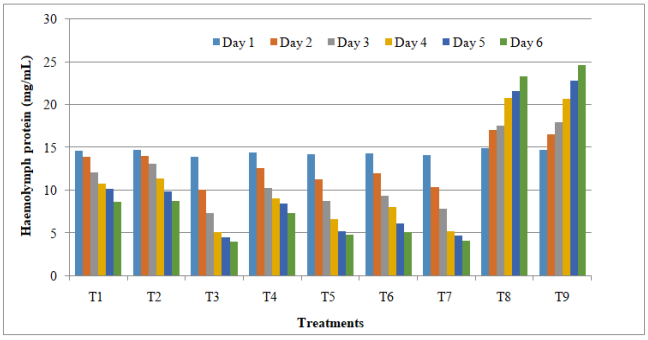

Fig. 1. Total protein content (mg/mL) in the haemolymph of fifth instar silkworm as influenced by feeding pesticide treated mulberry leaves.

In the normal larva of *B. mori*, Malik and Malik (2009) reported that the haemolymph protein concentration increased in a relatively constant pace from the first instar to fourth instar. In the fifth instar larvae, it increased nearly two-fold on the day four and attained a maximum on day nine. In the present study, the increasing trend of protein content was observed in water spray (T_8) and absolute control (T_9). But in case of pesticide exposed treatments, a deviation from this trend was observed and the total haemolymph protein content showed a marked decrease from first day to sixth day of fifth instar silkworms when fed with pesticides exposed leaves. This protein depletion in hemolymph could be due to the breakdown of protein into amino acids, so with the entrance of these amino acids to Krebs cycle as a keto acid, they will help to supply energy for the insect. So, protein depletion in hemolymph may be a physiological mechanism and may be involved in compensatory mechanisms under pesticidal stress, to provide intermediates to the Krebs cycle, by maintaining free amino acid content in hemolymph, these results are in conformity with the findings of Surendranath *et al.* (1997). Similarly, Leonardi *et al.* (1996); Bindu *et al.* (2015) studied the toxicological effect of chlorantraniliprole on the total haemolymph protein and found that it increased with larval age in the untreated control larvae of *B. mori*; meanwhile, in the treated larvae, the protein level is reduced. Monconduit and Mauchamp (1998) reported that application of ultra low doses of JH and the JHA, fenoxycarb and pyriproxyfen induced an inhibition of larval haemolymph protein synthesis in *B. mori* L. An insecticide, Phoxim exposure resulted in a significant reduction in total protein and increase in free amino acid and protease activity in the haemolymph of fifth instar *B. mori* (Li *et al.*, 2012). It was reported that pyriproxyfen did not affect the protein band pattern in treated insects; although it affected the amount of protein concentration (Aribi *et al.*, 2006). So the result obtained in the pesticides treated silkworm shows decreased haemolymph protein is correlated with findings of other scientists.

B. Total carbohydrate content (mg/mL)

Total carbohydrate content was significantly different among the treatments from 1st to 6th day of fifth instar. The results revealed that carbohydrate content in the haemolymph of fifth instar larvae fed with pesticide treated mulberry leaves, increased significantly in all the treatments. However, there was a steadily increase

of carbohydrate content in treatments carbofuron 3G (T_1), dimethoate 30EC (T_2), novluron 10EC (T_3), azadirechtin 0.03EC (T_4), fenazaquin 10EC (T_5), dinotefuron 20 SG (T_6) and chlorfenapyr 10EC (T_7) and in treatments water spray (T_8) and absolute control (T_9) there was a maximum increase of carbohydrate content (Fig. 2).

The lowest total carbohydrate content of 11.15 mg/mL was recorded in novluron 10EC (T_3), followed by chlorfenapyr 10EC (T_7) recorded 11.55 mg/mL and fenazaquin 10EC (T_5) recorded 12.25 mg/mL which was statistically lower than rest of the treatments on first day of fifth instar. However, total carbohydrate content was highest (23.73 mg/mL) in absolute control (T_9), followed by water spray (T_8) (22.38 mg/mL) and fenazaquin 10EC (T_5) (17.59 mg/mL) (Table 2) which was statistically higher than rest of the treatments on sixth day of fifth instar. The results are in support with the findings of Simex and Kodrik (1986), who reported that the haemolymph carbohydrates increased with the advancement of age of larvae and reached at its peak on the last day of fifth instar larvae. Mishra *et al*. (2010) reported that high concentration of carbohydrates in haemolymph is maintained during larval development as energy reserve to be utilized later during metamorphosis, pupal and adult stage. Surendranath (2000) reported that organophosphorus insecticides decrease the amount of carbohydrate in silkworm haemolymph. Further, it has been reported that different stresses can decrease the amount of total carbohydrates in silkworm haemolymph (Etebari and Matindoost 2004). Several insecticides have been shown to affect carbohydrate reserves in insects. Therefore, any change of carbohydrate metabolism can be expected under phoxim toxicity (Li *et al*., 2012).

Table 3: Total carbohydrate content (mg/mL) in the haemolymph of fifth instar silkworm (*B.mori* L.) as influenced by feeding pesticide treated mulberry leaves.

Treatments	Carbohydrate content in 5th instar					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
T1 – Carbofuron 3G	14.15	14.52	14.93	15.35	16.36	17.07
T2 – Dimethoate 30EC	14.48	15.04	15.15	16.34	16.97	17.20
T3 – Novluron 10EC	11.15	12.08	12.99	13.73	14.73	15.25
T4 – Azadirechtin 0.03EC	13.71	14.26	14.82	15.13	16.14	17.02
T5 – Fenazaquin 10EC	12.25	12.72	15.15	15.84	16.52	17.59
T6 – Dinotofuron 20SG	13.28	14.10	14.53	15.06	15.92	16.31
T7 – Chlorfenapyr 10EC	11.55	13.58	14.31	16.15	16.97	17.37
T8 – Water spray	14.68	15.38	16.74	18.35	19.53	22.38
T9 – Absolute control	14.75	17.37	18.62	19.89	22.40	23.73
F - test	*	*	*	*	*	*
SEm ±	0.54	0.58	0.80	0.79	0.89	1.08
CD @ 5%	1.59	1.73	2.37	2.35	2.65	3.21
C.V (%)	6.97	7.05	9.07	8.47	8.92	10.26

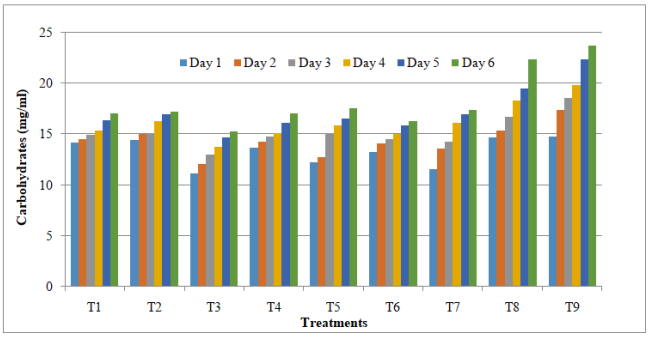

Fig. 2. Total carbohydrate content (mg/mL) in the haemolymph of fifth instar silkworm as influenced by feeding pesticide treated mulberry leaves.

CONCLUSIONS

The study revealed that pesticide exposure significantly decreases total protein content while increasing total carbohydrate content in the haemolymph of silkworms. Treatments with carbofuron, dimethoate, novluron, azadirachtin, fenazaquin, dinotefuron, and chlorfenapyr notably reduced protein levels, whereas carbohydrate levels were elevated across all treatments.

Acknowledgement. We gratefully acknowledge the College of Sericulture, Chintamani, and the Advanced Centre for Plant Biotechnology, University of Agricultural Sciences, Bangalore, for their support and resources. Special thanks to all research staff and contributors for their valuable assistance in this study.

Conflict of Interest. None.

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How to cite this article: Sandhya N., Sithara H., Ranjitha Bai H., Prakash Hariwal and Santosh M. Kolur (2024). Effect of Pesticide's Residue on the Biochemical Parameters of the Silkworm (*Bombyx mori* L.) Haemolymph. *Biological Forum – An International Journal, 16*(8): 173-177.