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EFFECT OF MICRONUTRIENTS SUPPLEMENTED MULBERRY LEAVES ON THE LARVAL BIOCHEMICAL CHARACTERISTICS OF MULBERRY SILKWORM, *Bombyx mori* LINNAEUS 1758 (LEPIDOPTERA: BOMBYCIDAE)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Nutritive value of mulberry leaf is a key factor besides environment and technology adoption for better growth and development of the silkworm larvae. The present study deals with the effect of micronutrient supplemented mulberry leaves on the biochemical characteristics of PM×CSR_2 hybrid variety of mulberry silkworm, *Bombyx mori* fifth instar larvae. The field experiment was laid out in a randomized block design with twelve treatments including a control (T0 to T11), and the treatments were replicated thrice. Each treatment was supplemented with the desired quantity of the respective micronutrient in single or in combination. Twenty early *Bombyx mori* fifth instar larvae in plastic trays were used for each replicate of the respective treatment, and were fed with the 5-6 fully grown mature mulberry leaves (T0 to T11) twice a day (morning and evening). The amount of carbohydrate and protein present in haemolymph, silk gland, fat body and muscle, and the amount of lipid, trehalose and glycogen in haemolymph and fat body of the fifth instar larvae of control and treated group were estimated. Maximum carbohydrate content in the haemolymph, silk gland, fat body and muscle of the fifth instar *Bombyx mori* was observed in T9 (195.0 ±7.07mg/mL), both T8 and T9 (190 ±13.13mg/g), T8 (245.0 ±49.49mg/g) and T9 (152.5 ±16.6mg/g) respectively, and for the maximum protein content, the respective values were in T9 (158.0 ±31.11mg/mL), T9 (172.0 ±11.31mg/g), T8 (166.0 ±76.36mg/g) and T9 (195.0 ± 86.26mg/g). The maximum lipid, trehalose and glycogen in haemolymph was observed in T8 (5.96

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±1.40mg/mL), T3 (7.66 ±1.25mg/mL) and T5 (1.30 ±2.51μg/mg/mL) respectively, and for maximum content in fat body, the respective values were observed in T6 (16.87 ±1.20mg/mL), T9 (7.91 ±0.24mg/mL) and T8 (11.87 ±0.10mg/mL) Overall results of the present study emphasized that supplemented micronutrients in T9 ($CuSO_4$ 15Kg/ha + $ZnSO_4$ 15Kg/ha + $FeSO_4$ 30Kg/ha) followed by T8 ($CuSO_4$ 10Kg/ha + $ZnSO_4$ 10Kg/ha + $FeSO_4$ 20Kg/ha) positively influenced the biochemical characteristics of *Bombyx mori* fifth instar larvae.

Keywords: *Bombyx mori* larva; micronutrients; biochemical; haemolymph; silk gland; fat body; muscle.

1. INTRODUCTION

Silkworm nutrition refers to the substances required by the silkworm for its growth and metabolic functions which are obtained from ingested food of mulberry/supplemented diet, and other nutritional components are being synthesized itself through various biochemical pathways including proteinaceous silk fibre of commercial interest [1]. Mulberry is a robust, perennial deep-rooted high biomass producing foliage crop, being the sole source of nourishment from which the mulberry silkworm, *Bombyx mori* derives nearly 70% of protein for silk synthesis. Mulberry leaves provide proteins, vitamins and other nutrients from which silk proteins are synthesized. Quality and quantity of mulberry leaves along with environmental factors affect production of raw silk spun by larvae before pupation in the form of cocoons [2]. The growth rate and development of silkworm larvae and subsequent silk production are greatly influenced by the quality and nutritional constituents of mulberry leaves [3,4].

The quality of mulberry leaves plays an important role in the success of the sericulture industry and directs its economics [5]. Improved nutrient management coupled with improved agronomic management technologies substantially increase the harvest index of mulberry. However, owing to the increasing demands for silk, stress has to be laid on the further improvement of leaf productivity both in terms of quality and quantity. Enrichment of the mulberry leaves is one of the strategies by which cocoon and silk productivity can be increased and the quality can be enhanced and maintained by fortifying nutrient supplement. Feeding of nutritionally enriched leaves showed better growth and development of silkworms as well as improve the economic value of cocoons [3]. Supplementation of various micronutrients to mulberry plants have had a remarkable change in sericulture [6]. Micronutrients play a major role in several metabolic activities responsible for protein, sugar and enzyme synthesis leading to better quality mulberry leaf production. The body tissue of *Bombyx mori* larval instars, especially, the fifth instar accumulates large quantity of carbohydrates, proteins, lipids and glycogen during development, due to the reflection of efficient consumption and utilization of nutrient biocompounds of mulberry leaves [7]. Therefore, in the present study, the soil application of micronutrients in mulberry plants, and its effect on the biochemical characteristics of the fifth instar of *Bombyx mori* was determined.

2. MATERIALS AND METHODS

The field experiment was conducted in the year 2012 under irrigated conditions at Poovancode village, Kanyakumari district, Tamil Nadu, India (8.3031° N, 77.2881° E) at an elevation/altitude of 29m above sea level. The experimental plot was free from other plants and received direct sunlight exposure. MR_2 (Mildew Resistant Variety –2) mulberry plant (*Morus alba*) developed by the Sericulture Department, Govt. of Tamil Nadu experimental station, Coonoor, Tamil Nadu, India was selected for the experiments, and were planted at 90x60cm spacing. Prior to the commencement of the experiment, mulberry plants were pruned, followed by ploughing. Farm yard manure was applied at the rate of 20t/ha/year, and single dose of nitrogen, phosphorous and potash at 120:120:60kg/ha/year was incorporated in the soil uniformly by hoeing. Irrigation was provided at five days interval depending upon the climatic conditions. Micronutrients were added to the soil after twenty days of pruning. The experimental plot was protected from plant pests, and the diseased/affected parts of the plant were removed periodically. The field experiment was laid out in a randomized block design with twelve treatments and the treatments were replicated thrice. Each treatment was supplemented with the desired quantity of the respective micronutrient in single or in combination which are as follows.

T0 - Control (mulberry plants which did not receive micronutrients supplementation)
T1 - $FeSO_4$ 10Kg/ha
T2 - Zn SO_4 5Kg/ha
T3 - Cu SO_4 5Kg/ha
T4 - $CuSO_4$ 5Kg/ha + $ZnSO_4$ 5Kg/ha
T5 - $CuSO_4$ 5Kg/ha + $FeSO_4$ 10Kg/ha
T6 - $FeSO_4$ 10Kg/ha + $ZnSO_4$ 5Kg/ha
T7 - $CuSO_4$ 5Kg/ha + $ZnSO_4$ 5Kg/ha + $FeSO_4$ 10Kg/ha
T8 - $CuSO_4$ 10Kg/ha + $ZnSO_4$ 10Kg/ha + $FeSO_4$ 20Kg/ha
T9 - $CuSO_4$ 15Kg/ha + $ZnSO_4$ 15Kg/ha + $FeSO_4$ 30Kg/ha
T10 - $CuSO_4$ 20Kg/ha + $ZnSO_4$ 20Kg/ha + $FeSO_4$ 40Kg/ha
T11 - $CuSO_4$ 25Kg/ha + $ZnSO_4$ 25Kg/ha + $FeSO_4$ 25 Kg/ha

Twenty early *Bombyx mori* fifth instar larvae in plastic trays were used for each replicate of the respective treatment, and were fed with the 5-6 fully grown mature mulberry leaves (T0 to T11) twice a day (morning and evening).

2.1 Biochemical Analysis

The present investigation was carried on PM×CSR_2 hybrid variety of *Bombyx mori* procured from Government Sericulture Training Centre, Konam, Nagercoil, Kanyakumari Tamil Nadu, India. The rearing of silkworm commenced when the mulberry plants were 45 days old. Since the experiments required continuous maintenance of the test species, the silkworms were reared in a rearing room in accordance with the procedure of Krishnaswami [8]. Biochemical analysis with regard to carbohydrates, proteins, lipids [9-11], trehalose [12] and glycogen [13] present in the body parts of the control and treated fifth instar larvae of *Bombyx mori* were estimated by isolation of tissues from haemolymph, silk gland, fat body and muscle. The prolegs of the fifth instar were cut, and haemolymph was collected in eppendorf tubes containing a speck of phenyl thio urea. The larvae were dissected, and the silk glands on both the sides were carefully removed with the help of forceps, cut into pieces, and 5g was taken, and homogenated in 10mL distilled water with the help of mortar and pestle. Fat body was removed carefully by using forceps, and was washed in distilled water. For muscle, the dorsal side of the skin was scrapped away from the fat body, and was washed in distilled water. All the isolated tissues were stored in phosphate buffer solution with a pH of 7.4 and kept in 5°C for biochemical estimation. The obtained data were statistically analysed using student’s ‘t’ test.

3. RESULTS

The control group (T0) silkworm’s haemolymph had a carbohydrate content of 90.0 ±0.0mg/mL, and the maximum and minimum carbohydrate content was recorded in T9 (195.0 ±7.07mg/mL) and T1 (90.0 ±0.0 mg/mL) respectively. The carbohydrate content in the silk gland of control was 115.0 ±21.21mg/g and both T8 and T9 showed a maximum content of 190.0 ±13.13mg/g, while both T2 and T11 showed a minimum content of 110.0 ±0.0mg/g respectively. The fat body control group recorded 155.0 ±63.63mg/g of carbohydrate content, and T8 and T3 showed the maximum and minimum content with respective values of 245.0 ±49.49mg/g and 165.0 ±49.49mg/g. Control muscle had a carbohydrate content of 105.0 ±49.47mg/g, and T9 showed the maximum content (152.5 ±16.6mg/g) while T1 showed the minimum content (110.0 ±70.71mg/g) (Table 1; Fig. 1).

The protein content in the haemolymph of control was 102.0 ±19.79mg/mL, and T9 showed the maximum content (158.0 ±31.11mg/mL), while T1 showed the minimum content (114.0 ±25.45mg/mL). The control silk gland had a protein content of 102.0 ±5.65mg/g, and T9 and T3 recorded the maximum and minimum content with respective values of 172.0 ±11.31mg/g and 114.0 ±25.45mg/g. The fat body control group recorded a protein content of 101.5 ±6.30mg/g, and T8 showed a maximum protein content of 166.0 ±76.36mg/g, while T2 and T1 showed the minimum content with respective values of 102.0 ±5.65mg/g and 102.0 ±8.48mg/g. Control muscle had a protein content of 114.0 ±14.14mg/g, and the maximum and minimum content was present in T9 (195.0 ± 86.26mg/g) and T1 (120.0 ±22.62mg/g) respectively (Table 2; Fig. 2).

Lipid, trehalose and glycogen in haemolymph was found to be 4.01 ±0.60, 6.91 ±0.14 and 1.24 ±1.79mg/mL respectively in control. Maximum and minimum level of lipid, trehalose and glycogen in haemolymph was observed in T8 and T5 (5.96 ±1.40 and 5.21 ±1.10mg/mL), T3 and T4 (7.66 ±1.25 and 6.94 ±1.91mg/mL), and T5 and T2 (1.30 ±2.51 and 1.25 ±4.10mg/mL) respectively. Lipid, trehalose and glycogen in fat body was found to be 13.97 ±0.70, 5.45 ±0.24 and 6.50 ±0.09mg/g respectively in control. Maximum and minimum level of lipid, trehalose and glycogen in fat body was observed in T6 and T2 (16.87 ±1.20 and 14.17 ±1.20mg/mL), T9 and T4 (7.91 ±0.24 and 5.78 ±0.07mg/mL), and T8 and T1 (11.87 ±0.10 and 6.70 ±0.31mg/mL) respectively (Table 3; Fig. 3).

4. DISCUSSION

Carbohydrates, proteins and lipids play an important role in the biochemical process underlying growth and development of insects [14-16]. The late stage silkworm larvae accumulate higher carbohydrates when compared to young age worms. Silkworm requires certain essential sugars, proteins, amino acids, fatty acids, vitamins and micronutrients for its growth and higher production of good quality silk [17]. It has been reported that enrichment of mulberry leaves with supplementary compounds enhances the silk productivity. Magnesium, calcium, phosphorus, iron, manganese, zinc and copper are the essential micronutrients required by silkworms. The fifth instar larvae of silkworm assumes lot of significance because in this stage, the silk gland develops rapidly and all the biochemical constituents, *viz*., glycogen, trehalose, proteins and lipids reach the peak level. Carbohydrates are the major component in the food of all the living organisms which either directly or indirectly are used as the source of energy for all vital activities. The capacity to accumulate carbohydrates was more in PM×CSR_2, and the accumulated sugars might get converted to glycogen [18], and later used as a source of energy for better growth of the larvae. Therefore, the level of carbohydrates during larval development reveals the degree of utilization of carbohydrates which are the major sources of energy in the body, for growth and development of the larva that might ultimately determine the quality and quantity of silk production.

Table 1. Effect of micronutrients supplemented leaves on carbohydrate content of *Bombyx mori*

Treatment	Haemolymph (mg/mL)	Silk gland (mg/g)	Fat body (mg/g)	Muscle (mg/g)
T0	90.0 ±0.0	115.0 ±21.21	155.0 ±63.63	105.0 ±49.47
T1	90.0 ±0.0	115.0 ±21.21	170.0 ±0.0*	110.0 ±70.71
T2	100.0 ±14.14*	110.0 ±0.0	170.0 ±14.14*	130.0 ±48.99*
T3	130.0 ±84.85*	115.0 ±21.21	165.0 ±49.49	130.0 ±98.94*
T4	115.0 ±35.35*	130.0 ±56.56*	185.0 ±35.35*	125.0 ±77.71*
T5	95.45 ±21.21	115.0 ±7.07	180.0 ±70.71*	137.5 ±60.10*
T6	100.24 ±6.24*	155.0 ±21.21*	210.0 ±0.0*	132.5 ±60.10*
T7	115.0 ±21.21*	140.0 ±42.42*	215.0 ±21.21*	147.5 ±81.31*
T8	140.0 ±0.0*	190.0 ±13.13*	245.0 ±49.49*	150.0 ±17.27*
T9	195.0 ±7.07*	190.0 ±13.13*	235.0 ±28.28*	152.5 ±16.60*
T10	180.0 ±28.28*	135.0 ±49.49*	193.0 ±70.71*	135.0 ±7.07*
T11	125.0 ±49.49*	110.0 ±0.0	170.0 ±84.85*	136.5 ±65.76*

**Significant @ P≤0.05 (t-test)*

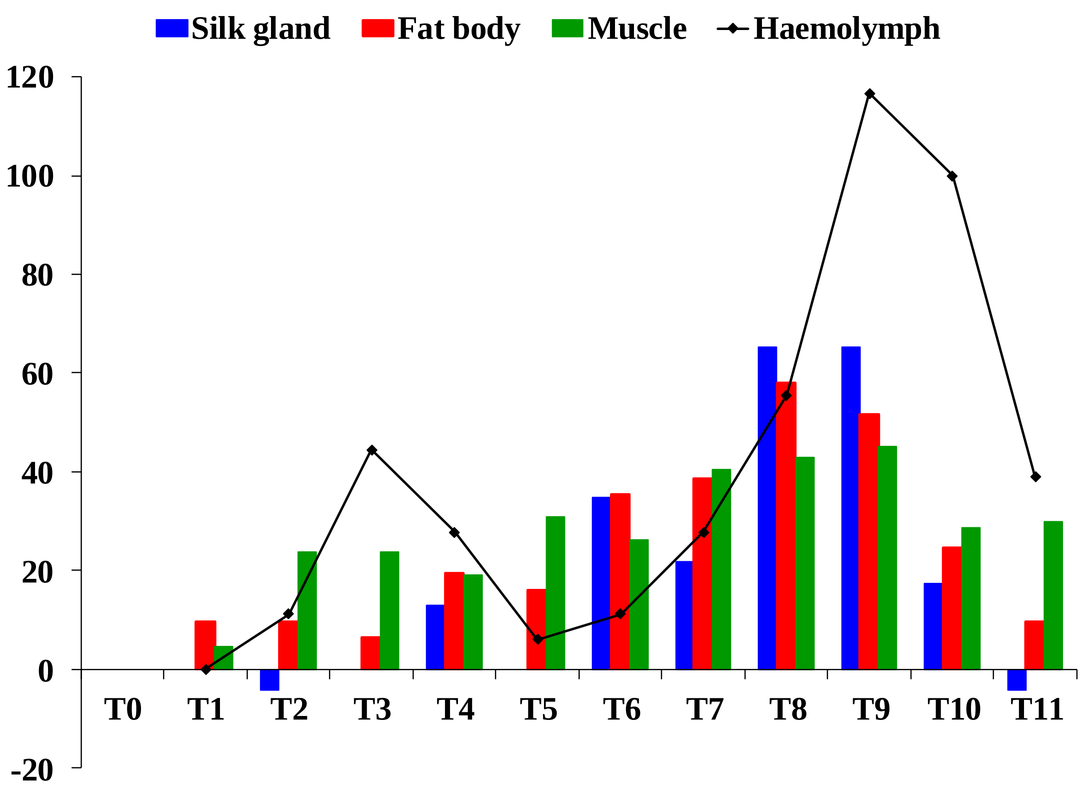

Fig. 1. Percent deviation over control values on carbohydrate content of *Bombyx mori*

Table 2. Effect of micronutrients supplemented leaves on protein content of *Bombyx mori*

Treatment	Haemolymph (mg/mL)	Silk gland (mg/g)	Fat body (mg/g)	Muscle (mg/g)
T0	102.0 ±19.79	102.0 ±5.65	101.5 ±6.30	114.0 ±14.14
T1	114.0 ±25.45*	125.0 ±55.15*	102.0 ±8.48	120.0 ±22.62
T2	125.0 ±18.38*	120.0 ±50.91*	102.0 ±5.65	134.0 ±14.14*
T3	118.0 ±39.59*	114.0 ±25.45*	118.0 ±19.79*	133.0 ±1.414*
T4	134.0 ±14.14*	116.5 ±40.30*	122.0 ±31.11*	128.0 ±22.62*
T5	146.0 ±56.56*	140.0 ±11.31*	129.0 ±7.07*	136.0 ±0.0*
T6	144.0 ±56.56*	126.0 ±14.14*	112.0 ±22.62*	138.0 ±8.48*
T7	144.0 ±16.97*	142.0 ±53.74*	147.0 ±12.72*	150.5 ±19.09*
T8	156.0 ±0.0*	150.0 ±8.48*	166.0 ±76.36*	154.0 ±25.45*
T9	158.0 ±31.11*	172.0 ±11.31*	158.0 ±53.74*	195.0 ±86.26*
T10	148.0 ±45.25*	162.0 ±25.45*	158.0 ±36.76*	144.0 ±16.97*
T11	147.0 ±12.72*	146.0 ±14.14*	144.0 ±39.59*	141.0 ±39.59*

**Significant @ P≤0.05 (t-test)*

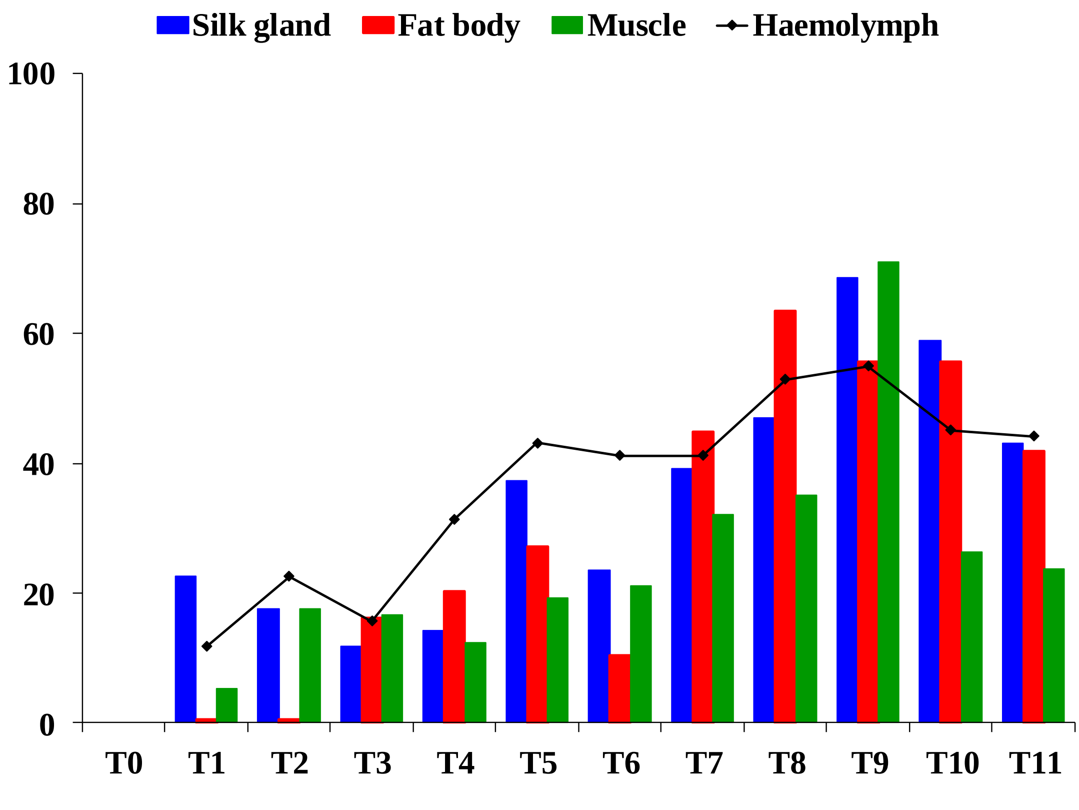

Fig. 2. Percent deviation over control values on protein content of *Bombyx mori*

Proteins in the haemolymph and fat body may provide the raw material for the synthesis of silk protein. The estimation of total protein in different parts of the larvae increased due to supplementation of enriched leaves fed to silkworm. Further, the increase of protein in the haemolymph of silkworm observed may be due to the supplementation of enriched leaves to silkworm, and this clearly indicated the influence of dietary protein on the increase in haemolymph protein during fifth instar since this instar was considered as prime feeding stage of the silkworm larva wherein about 80-85% of the total leaf is consumed [19]. Further, Krishnaswami [8] observed that increase in the protein concentration in the silkworm body after the fourth moult was due to regular feeding, and substantial increase in the body weight by the time the larva attains the spinning stage. The results of the present study demonstrated perturbations in protein content of the silkworm in different tissues, *viz.*, haemolymph, silk gland, fat body and muscle, when fed on mulberry leaves supplemented with micronutrients. Protein is the main component that produces tissue and organs of the silkworm. It is established that silk protein produced in the silk gland of *Bombyx mori* is mostly synthesized from the amino acids present in the mulberry leaf proteins [20]. Protein metabolism is an important process in building up of body i.e., in the development of muscles, tissues etc. The skeletal muscle of silk worm is known to synthesize and store about 258 proteins [21], that play a vital role in larval locomotion and body movements apart from larval growth and development [22,23]. Nutritive value of different proteins for the silkworm vary largely, and these differences seem to depend on qualitative and quantitative amino acid composition of proteins [24]. Zinc ions entering into the haemolymph, silk gland cells, fat body, and into the muscle cells, initiate the protein elevations depending upon the tissue, and because of the changes in the protein elevations, the body size of the silkworm show distinct variations.

Table 3. Effect of micronutrients supplemented leaves on lipid content of *Bombyx mori*

Treatment	Haemolymph (mg/mL)			Fat body (mg/mL)		
	Lipid	Trehalose	Glycogen	Lipid	Trehalose	Glycogen
T0	4.01 ±0.60	6.91 ±0.14	1.24 ±1.79	13.97 ±0.70	5.45 ±0.24	6.50 ±0.09
T1	5.74 ±0.90*	7.03 ±0.18	1.27 ±2.00	14.36 ±0.40	5.90 ±1.92	6.70 ±0.31
T2	5.82 ±1.80*	7.23 ±0.28	1.25 ±4.10	14.17 ±1.20	6.83 ±0.12*	6.79 ±0.47
T3	5.41 ±1.60*	7.66 ±1.25*	1.26 ±3.25	15.34 ±1.70*	5.91 ±0.14	10.56 ±0.07*
T4	5.36 ±0.90*	6.94 ±1.91	1.28 ±1.64	16.24 ±1.80*	5.78 ±0.07	10.72 ±0.01*
T5	5.21 ±1.10*	7.32 ±1.36	1.30 ±2.51	16.78 ±1.80*	6.60 ±0.13*	11.26 ±0.39*
T6	5.52 ±1.60*	7.37 ±1.47	1.26 ±1.54	16.87 ±1.20*	7.20 ±0.09*	11.53 ±0.24*
T7	5.63 ±1.30*	7.13 ±1.54	1.24 ±1.23	16.34 ±0.90*	6.69 ±0.16*	10.68 ±0.07*
T8	5.96 ±1.40*	7.41 ±1.72	1.28 ±3.33	14.89 ±0.20*	7.45 ±0.14*	11.87 ±0.10*
T9	5.92 ±1.70*	7.41 ±1.11	1.27 ±1.53	15.04 ±2.10	7.91 ±0.24*	11.23 ±0.08*
T10	5.89 ±2.20*	7.21 ±0.98	1.27 ±1.19	15.45 ±1.60*	6.84 ±0.07*	11.03 ±0.26*
T11	5.41 ±1.90*	7.32 ±1.90	1.26 ±3.28	15.22 ±1.70	6.72 ±0.10*	10.90 ±0.38*

**Significant @ P≤0.05 (t-test)*

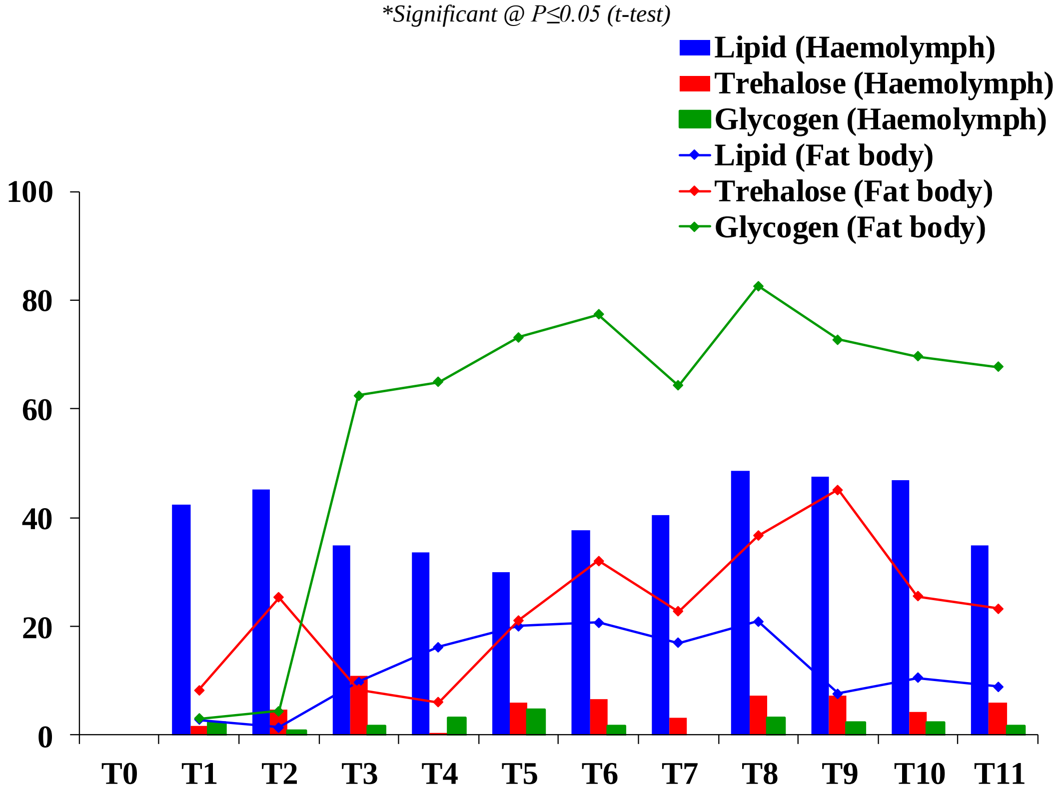

Fig. 3. Percent deviation over control values on lipid content of *Bombyx mori*

The biochemical composition of silkworm haemolymph is an important factor for silkworm development and growth [25-27]. Haemolymph of *Bombyx mori* is the chief circulating fluid and transport medium for about 298 proteins, involved in larval growth, ecdysis, metamorphosis, silk production, chitin and haemocyte formation, growth of salivary glands and reproduction [28]. Haemolymph is the only extracellular fluid of insects with diverse functions and a reservoir for most of the biochemicals that are required for every physiological activity of the insect body, and changes in the composition of haemolymph, reflects on the physiological, morphogenic and biochemical transformations taking place in the insect tissues [29], which in turn reflects on the economic characters of *Bombyx mori*. Considerable increase of haemolymph trehalose content was observed in all the treatments. Trehalose is one of the most important carbohydrolases in insects occurring in the gut, flight muscles, fat bodies, labial glands, haemolymph and also in the silk glands of silkworm. It causes the breakdown of trehalose into glucose for internal supply, chitin synthesis, muscular activity during flight, cocoon formation and other metabolic process [30]. Trehalose plays an important role in energy supply to an insect [16], and midgut trehalose serves as an indicator of energy reserves resulting from the availability of carbohydrate nutrients.

Feeding mulberry leaves supplemented with micronutrients increases total lipids of the fat body in the silkworm. Padmaja [31] reported that feeding mulberry leaves supplemented with manganese, zinc and copper sulphate increased the total lipids of the fat body in the Eri silkworm, *Philosamia ricini*, and it was nickel chloride in the case of the bivoltine silkworm, *Bombyx mori* [32]. The insect fat body plays a key role in the metabolism similar to that of the mammalian liver and adipose tissues. In *Bombyx mori*, it synthesizes and stores over 177 proteins that are involved in its growth and metabolism [33]. In the present study, larva treated with copper, zinc and iron resulted in a significant increase of total lipids of the fat body which might possibly be due to the stimulatory effect of micronutrient mixture of copper, zinc and iron at a required concentration on the synthetic activity of fat body, and also by the possible mechanism of action of these micronutrients along with other nutrients on the biochemical contents of the silkworm. The present study correlated with the findings of Bhattacharya and Kaliwal [34-36] who reported that micronutrients stimulate the enzyme activity which influences the metabolic process thereby increasing the biochemical contents of the fat body and haemolymph of *Bombyx mori*.

The amount of trehalose present in the fat body is directly related to the glycogen content of the tissue, and trehalose production in insect fat body is influenced by a number of endogenous organic and inorganic factors [37]. Satake et al. [38] showed that the quality of the food taken by the larvae would have had considerable effect on the haemolymph glucose. This study showed an increase in the haemolymph trehalose than fat body trehalose which might possibly be due to the conversion of glycogen into trehalose and its subsequent release into the haemolymph by the fat body. Moreover, the larvae treated with copper and zinc showed higher level of haemolymph trehalose content, and the synergetic effect of copper, zinc and iron in the present study, may have a role in activating the trehalose synthase activity of fat body. Therefore, from this study it may be inferred that the increase in the fat body glycogen and haemolymph trehalose in micronutrient treated groups, with copper, zinc and iron at required combination may be utilized as additional sources of fuel or energy required during the pupal and adult transformation. Simex and Kodrik [39] reported that the glycogen content in the fat body, body wall and silk gland are the free carbohydrates in the haemolymph which change significantly during the last larval instar and metamorphosis in silkworms. The glycogen content in the fat body reach its maximum level before spinning. This was true with the present findings too. The glycogen level in all the treatments was much higher when compared with haemolymph. Similar increase in the fat body glycogen content has been reported after supplementing the feed with potassium and magnesium sulphates in the bivoltine silkworm [40]. In the present study, the increase in the fat body glycogen may possibly be due to the stimulatory effect on the amylase activity of the midgut resulting in increased production of carbohydrates as suggested by Pant and Morris [41].

5. CONCLUSION

The present study indicated that the biochemical parameters of the fifth instar larvae increased when fed with mulberry leaves supplemented with micronutrients. The biochemical analysis showed an increase with regard to the carbohydrate and protein content in haemolymph, silk gland, fat body and muscle, and the lipid, trehalose and glycogen content in the haemolymph and fat body of the fifth larval instar of *Bombyx mori*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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