BIOCHEMICAL STUDIES ON TOTAL PROTEIN, CARBOHYDRATE AND LIPIDS CONTENT LEVEL DURING THE INFECTION BY FUNGI WHITE MUSCARDINE DISEASE OF SILKWORM, *BOMBYX MORI* L.

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ABSTRACT: The changes of protein, carbohydrate and lipids (pcl) metabolism were studied in haemolymph and whole larval tissues during the 5th instar larval development on 1st, 2nd, 3rd, 4th and 5th day after inoculation with fungi *Beauveria bassiana* (white muscardine) of conidial suspension,. The concentration level of pcl showed a similar trend of increasing in multivoltine and bivoltine silkworm breeds of control batches and reducing level was noticed in the infected batches, due to effect of fungal infection in both the breeds. The progressive concentration was found to be day by day reach peak level in healthy/control breeds, the bio-molecules are correlated to the degree of their absorption, inter-conversion and utilization in control batches. The biochemical results presumed on the basis of these major changes in protein, carbohydrate and lipid content level in infected batches causes for high toxicity of the silkworm larvae showing significant variation in their concentration. Two different varieties of silkworm breeds multivoltine 2000H and bivoltine CSR6 were utilized and bio-physiological status were discussed in relation to the 5th instar larvae under inoculated and control conditions.

KEY WORDS: *Beauveria bassiana*, Haemolymph and whole larval tissues, Protein (P), Carbohydrate(C), Lipids (L), 5th instar silkworm larvae.

The silkworm, *Bombyx mori* is a manophagous and holometabolous lepidopteron insect and its growth, development and metabolism mainly depends on its nutritional requirements and environmental conditions. Generally insects acquire infection, parasitized or become diseased in many routes, they manifest there conditions in variety form of actions, appearances and signs. The manifestations known as symptoms are most useful in the detection or diagnosis of disease (Steinhanus, 1963). The silkworm larvae accumulate large quantity of fuel reserves in various tissues, and it is endowed with a unique bio-chemical adaptation to conserve nutritional resources available during the active larval stage. The lepidopteron insects maintain relatively higher level of heamolypmh sugar compared to other insects (Roeder, 1953). Haemolymph sugar levels in the larvae of *B. mori* have been worked by many entomologists (Hemmingsen, 1924; Weing & Joachin, 1936; Kawana, 1937). Carbohydrates and proteins are very essential for adult larva and pupal development and are obtained from the fat body and haemolymph stored during the 5th instar larval stage. The concentration of bio-molecules like proteins, carbohydrates, lipids, amino acids, nucleic acid, enzymes etc., may vary significantly during the life cycle of all living organisms. The carbohydrates are stored in the fat body as glycogen, which is converted into simple sugar and trehalose before it is released in to the haemolymph for its utilization. Carbohydrate plays a polysaccharides and free sugars of a tissue and its size dynamics is a good indication of metabolic status of the tissue. The quantitative variation of these bio-molecules in the body of insects depends upon

the nutritional status of the food and their utilization during growth and metamorphosis stated by (Nagata & Yashitake, 1989). The major sources of bio-molecules are carbohydrate, protein and lipids which play a pertinent role in the development and morphogenesis in almost all the intermediary metabolic pathways of insects (Wyatt, 1978). The haemolymph proteins serve as the important source for other tissue proteins as well as reserve energy in the adult during starvation (Buck, 1953). Carbohydrate is a major part of the total caloric intake of the organism and serves as a temporary store of glucose. Lipids are important constituents of cuticle and help in acylation of glucose-6-phosphate during chitin synthesis (Wyatt, 1967). Lipids serve as a source of metabolic energy and essential for structural components of cells. The lipid in the fat body is an energy reserve, which can be mobilized rapidly during starvation, oogenesis, embryogenesis and moulting and is used to sustain continuous muscular activity (Gilbert & Chino, 1974). The multiplication of pathogen in the host system is often reflected by specific metabolic variations along with gradual changes in the infected tissues and susceptibility to a disease differs according to the physiological status of the host. Studies on the changes in various biochemical constituents in haemolymph and whole tissues in relation to entomo-pathogenic fungi of *B. bassiana* (white muscardine) is very scanty and several biochemical work reports indicates only on viral and bacteria disease. There are only two literatures available regarding biochemical changes in silkworm haemolymph infection by fungi (Mallikarjuna et al., 2002). In the present study, an investigation was conducted to understand the patho-physilogical changes of major biochemical quantitative in protein, carbohydrates and lipids activity in haemolymph and whole larval tissues were examined in the identified resistant multivoltine silkworm breed 2000H and susceptible bivoltine breed CSR6 under inoculated/treated and control/healthy conditions during different days of progressive infection.

MATERIAL AND METHODS

The entomopathogenic fungi of *Beauveria bassiana* (white muscardine) was cultured on potato dextrose agar medium and isolated. The conidial suspension of fungus prepared, inoculation was done on newly ecdysed 4th moult-out larvae of 5th instar per cutaneous. Dosage of 1x10^{6}conidial suspension/ml/100 larvae sprayed on the body of multivoltine 2000H and bivoltine CSR6 breeds. Treated batches were kept under temperature 25±1°C and humidity 90±95%, along with control batches of same breeds larvae reared under normal temperature and humidity without any inoculation.

Sample collection method: The haemolymph or blood and whole larval tissues were collected after 24h of inoculation 1st, 2nd, 3rd, 4th and 5th day (24, 48, 72, 96 and 120 h) from the silkworm breeds of 2000H and CSR6. Three samples of five larvae each randomly selected every day from both in infected/inoculated and healthy/control batches. Haemolymph was collected by cutting of thoracic-legs in pre-chilled eppendrof tubes which contained a few crystals of phenyl thiourea (PTU) followed by (Joy & Gopinathan, 1995) and immediately frozen at -80˚C, the same larvae were dissected gut wall debris was removed and crushed thoroughly with the help of sterilized mortar and pestle and stored at -80˚C till processed for analysis of various organic compounds.

Estimation process: 1000ul of haemolymph was homogenized with 5ml of 15% Trichloroacetic acid (TCA) and 250mg larval tissues was homogenized with 5ml of 15% TCA individually and centrifuged at 6000rpm for 20 minutes. The supernatant was transferred to another test tube individually for the estimation of carbohydrates. The residues were washed twice with cold 80% acetone followed by cold diethyl ether and finally suspended in 5ml of 1N NaOH (Sodium hydroxide pellets) solution for tissues and haemolymph respectively and again centrifuged, supernatant was used for protein estimation.

Protein estimation method: Soluble total Protein was estimated according to the methods of Lowry et al., (1951) by Folin-phenol methods, using BSA as standard. For 100ul samples of haemolymph and tissues were taken separately, to that 5ml of alkaline copper-sulphate reagent C was added, [**Reagent A:** 2% sodium carbonate mixed with 50ml of 1N NaOH solution. **Reagent B:** 5ml of 0.5% copper sulphate mixed with 5ml of 1% sodium potassium tartrate. **Reagent C:** prepared by (50:1 ratio) 100ml of Reagent A and 2ml of Reagent B] contents were mixed well after 10 minutes, 1:1 ratio of 0.5ml folin-phenol reagent was added and tubes were shaken well. The blank sample contained 100ul of distilled water, 5ml of alkaline copper reagent and 0.5ml folin-phenol reagent. The colour intensity (light blue) was read at 660nm in a spectrophotometer against blank after 30minutes. The protein content was recorded from the standard curve prepared by bovine serum albumin (BSA) (10-100ug). The total protein content values were expressed as mg protein/ml of haemolymph and for whole tissues mg/g. wet wt. of tissue.

Carbohydrate estimation method: Total Carbohydrate was followed Carroll et al. (1956) by Anthrone method and Glucose was used as standard. For 100ul each supernatant haemolymph and whole tissues sample taken individually in a test tube, to that 4ml of anthrone reagent (200mg of anthrone powder dissolved in 72 % of 100ml of sulphuric acid) solution was added and contents mixed well. All tubes kept in a boiled how water bath for 10 min, then tubes were cooled at room temperature. The developed colour intensity (light green) was read at 620nm in a spectrophotometer using a blank sample. The total carbohydrates present in sample was expressed as mg/ml of haemolymph and for tissues expressed as mg/gm of wet wt. of tissues.

Lipid estimation method: Lipids were carried out gravimetrically of Folch's et al. (1957) by chloroform and methanol mixture. For 1000ul of haemolymph in 10ml of chloroform methanol mixture (2:1 ratio**)** was added and homogenized with the help of mortar, pestle and transferred to test tube. 250mg of crushed tissues was thoroughly macerated with 10ml of chloroform methanol mixture, and transferred to test tube, kept it over night under normal room temperature and next day morning centrifuged at 5000rpm for 20 min. Then 4ml of 0.9% Sodium chloride solution were added all tubes and vortexing for few seconds and centrifuged again at low speed at 2500rpm for 10 min to separate the two phases of upper and lower layer. After centrifugation, remove the upper siphoning solution (white) and lower chloroform phase (yellow) containing lipid was collected into a pre-weighed plastic vial. The lipid fraction was air dried and the weight of the vials was noted, the differences between in initial and final weight of the plastic vials were recorded and quantity of lipid content was expressed as mg/ml of haemolymph and mg/g wet wt. for tissues.

RESULTS

Biochemical changes brought out by the two varieties of samples, haemolymph and whole larval tissues and changes in the haemolymph are mainly caused by diseases, the basic components of protein, synthesis involving in the regulation of ionic balance for energy rescuing operations. Variation in the content of soluble total protein, carbohydrates and lipids in haemolymph and whole larval tissues during 5th instars development in the multivoltine and bivoltine silkworm breeds were studied. Significant difference in protein, carbohydrate, and lipids concentration between healthy and infected/diseased was recorded. As the disease progresses haemolymph and larval tissues pcl content was disturbed and significant variations was noticed in infected batches. Although in case of healthy/control larvae of pcl increased steadily as the age of the larvae increases their protein, carbohydrate and lipids respectively. The inoculated batches of protein content level in the haemolymph was found maximum (63.00mg/ml) on third day in 2000H followed by 57.46 mg/ml in CSR6 and decreased gradually towards the end of 4th and 5th day 56.50 to 52.25 mg/ml and 52.32 to 49.46 mg/ml due to muscardine infection. In the control batches of 2000H, protein level increased simultaneously day by day 64.29 to 81.35 mg/ml from 1st to 5th day, and 59.58 to 78.00 mg/ml in CSR6 Similarly, the larval tissues protein content ranged from 43.42 to 57.90mg/gm in 2000H to 40.23 to 55.30 mg/gm in CSR6 in the control batches. Whereas treated batches of 2000H showed 40.10 to 38.60 mg/g followed by 34.75 to 33.31 mg/gm in CSR6, Table 1.

Total carbohydrates or sugar content level in the haemolymph showed normal in the 1st day was found 22.70 and 20.42mg/ml and decreased gradually towards the end of 5th day 21.50 mg/ml in inoculated batches, same trend was observed in the larval tissues. Significant variation was also noticed in the control batches carbohydrates level of concentration gradually increased day by day both in haemolymph and whole tissues and same trend was also exhibited in lipids profiles, Table 2.

The treated batches lipid concentration level in the haemolymph was 56.70 and 55.35 mg/ml in 2000H followed by 53.47 and 51.45 mg/ml in CSR6 breed. Higher concentration level was found 67.18 to 77.58 mg/ml and 59.00 to 73.25 mg/ml under both the control breeds respectively. Similarly the whole tissues of lipid content level ranged from 36.76 to 42.26 mg/g up to 3rd day and simultaneously decreased their level to 35.00 at the end of 5th day in inoculated batches and gradually increased 39.44 to 53.50 mg/g under the control of 2000H multivoltine breed. Same trend was observed in CSR6 larval tissues under inoculated and control batches, Table 3.

The results presumed on the basis of these major changes in protein, carbohydrates and lipids content level showed a similar trend of reducing and increasing in infected batches in both the breeds, and it was found to be progressive concentration day by day reach peak level in health/control breeds values have been presented in the Table 1, 2 and 3.

The change of biochemical constituents in the tissues reflects the physiological status at different stages of development of an insect. The increase and decrease in the pcl content of heamolymph and whole larval tissues form the 1st day of 5th instars up to 5th day after inoculated/treated batches due to infection by white muscardine and pathogen of fungi utilized the host organic profiles for its growth and development. Whereas in the control batches the pcl level gradually ranged day by day due to by accumulation of proteins that are transported to other

tissues through the haemolymph for further physiological activities in the larva, the active secretion of protein by other tissues like fat bodies.

The patho-biochemical progressive variation in the infected and control batches have been presented in the Graphs. 1, 2, 3, 4, 5 and 6**.** The changes in the concentration of these bio-molecules are correlated to the degree of their absorption, inter-conversion and utilization in the control batches and cause for high toxicity of the silkworm larvae under infected batches.

DISCUSSION

Proteins are one of the important macromolecule organic substances and their role is compensatory mechanisms, especially during the stress (infection) conditions in silkworm which occupies the pivotal role both in structural and dynamic aspects of living systems. The total protein content consists of structural and soluble proteins involved in the architecture and metabolism of cells respectively. In the present study, biochemical changes after inoculation of fungi *Beauveria bassiana* showed major changes in the total protein, carbohydrate and lipid content in the haemolymph and whole larval tissues during the course of white muscardine infection. The physiological anomalies and infection by pathogens are responsible for altered metabolism in any organisms. Fluctuation in blood protein concentrations during metamorphosis are known since many years (Heller, 1924). The carbohydrates and lipids both constitute important source of energy during larval development and increased physical activities (Chino & Gilbert, 1965). Pathogenic infections are reported to induce several biochemical and physiological alterations in insect tissues (Martignoni, 1964; Shigematsu & Noguchi, 1969). The decrease of protein, carbohydrate and lipids content level under infection condition, as well as it was increased simultaneously in the control conditions. The increase in protein content from first day to third day clearly indicated that the digestive activities are high during the early part of 5th instars development, which results in increased accumulation of proteins that are then transported to other tissues through the haemolymph for further physiological activities in the healthy larva (Horie et al., 1982). The study of bio-chemical constituents was elevated by under control conditions, where in the worms were healthy and not subjected to infection by fungus *Beauveria bassiana*. Carbohydrates serve as main source of energy of insect species (Chino & Gilbert, 1965), as energy plays a vital force in the biological system, a break down of organic constituents mainly carbohydrates is required to meet the energy under stress condition (Manohar Reddy, 2004). The decreased pcl level in haemolymph and tissues can be attributed to the excessive utilization of carbohydrates to meet the demand of energy of fungi *Beuveria bassiana* infection. The percentage level of protein, carbohydrate and lipids variation in the silkworm larvae under infected and control have been presented in the Graphs. 1, 2, 3, 4, 5 and 6**.** The infection by fungi white muscardine seems to be a reflection of stepped-up demand for energy in the host to combat the disease as a natural response and maximum significant reduction of total protein, carbohydrate and lipids concentrations in the whole tissues were observed in the infected/inoculated batches. This decrease was suggested to be due to reduction in the level of pcl in the haemolymph of diseased larvae which in-turn affects the formation of silk protein in the glands.

The infected larval protein, carbohydrate and lipid content level was decreased significantly in the haemolymph and whole larval tissues cause under stress condition to meet the energy demands or due to increased synthesis. Similarly the

carbohydrate content might have been actively mobilized towards glucose under stress to provide maximum energy. The total lipid content also increased as the age of the larvae increased both in control and decreased under inoculated batches of both the breeds. Fungi might have stimulated the protein, carbohydrate and lipid utilization in order to meet requirements of toxic stress, where as the pcl content level was gradually increased under healthy/control batches, data have been presented in the Tables 1, 2 and 3.

In conclusion of this results of the study clearly indicated that, white muscardine caused a severe disturbance in the protein metabolism, that cause for decrease in major organic compounds of protein, carbohydrate and lipids contents under highly toxic relation by fungus produced ammonium and magnesium oxalate has a toxins impact on silkworm, The degradation products may in-turn be fed into tricarboxylic acid (TCA) cycle through the amino-transferase system to cope up with the high energy demands augmented during stress conditions reported by (Nath et al., 1997). Protein depletion in tissues may constitute a role of compensatory mechanism under the influence of toxins of chemical reactions. The higher and lower level of organic compounds in silkworm breeds may be due to its susceptible and resistant nature also their polygenic characters, resistant breed feeding activity is normal compared to susceptible breed after infection.

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LITERATURE CITED

Buck, J. B. 1953. Physical properties and chemical composition of insect blood. Insect physiology (Ed. K. W. Roeder), John Willey and Sons Inc., New York.

Carroll, N. V.; Longely, N. V. & Roe, J. H. 1956. J. Biol. Chem., 220-583.

Chino, H. & Gilbert, L. I. 1965. Lipid release and transport in insect. Biochem. Bio Phys. Acta., 98: 94-110.

Folch, J. M., Less, P. & Stane-Stanely, G. H. 1957. A simple methods for the isolation and purification of total lipids from animal tissues. J. Bio. Chem., 226: 497-509.

Gilbert, L. I. & chino, P. 1974. Transport of lipids in insects. J. Lipid Res., 15: 439-456.

Hemmingsen, A. M. 1924. Sband. Arch. Physiol., 45: 204.

Horie, Y., Watanabe, K. & Sakamoto, E. 1982. Evidence of stepwise digestion of protein in the digestive system of the silkworm, *Bombyx mori*. App. Ent. Zool., 17: 358-363.

Heller, J. 1924. Compt. Rend. Soc. Biol., 90: 1360.

Kawana, Z. 1937. Japan J. Zool., 7: 203-273.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. 1951. Protein measurement with Folin Phenol reagent. J. Bio. Chem., 193: 265-275.

Mallikarjuna, M., Balavenkatasubbaiah, M., Nataraju, B. & Thiagarajan, V. 2002. Effect of systemic fungicides on total haemocyte count and haemolymph biochemical changes in silkworm, *Bombyx mori* L. infected with *Beauveria bassiana*. Int. J. Entomol., 5 (2): 189-194.

Manohar Reddy, B. 2004. Metabolic modulations in various tissues of pre spinning silkworm *Bombyx mori* (L) Larvae during grassarie (BmNPV) disease. Ph.D. Thesis, Sri Venkateswara University, Tirupathi, India.

Martignoni, E. 1964. Pathophysilogy in the insect. Ann. Rev. Entomol., Pp. 179-206.

Nagata, M. & Yashitake, N. 1989. Protein metabolism in the larval development of silkworm, *Bombyx mori*. Protein reutilization at the IV moult. J. Seric. Jpn., 58 (3): 221-233.

Nath, B. S., Suresh, A., Varma, B. M. & Kumar, R. P. S. 1997. Changes in protein metabolism in haemolymph and fat body of the silkworm, *Bombyx mori* (Lepidoptera: *Bombycidae*) in response to organophosphorus insecticides toxicity. Ecotoxicol. Environ. Saf., 36: 169-173.

Shigematsu, H. & Noguhi, A. 1969. Biochemical studies on the multiplication of a nuclear polyhedrosis virus in the silkworm, *Bombyx mori*. II. Protein synthesis in the larval tissue after infection. J. Invertebr. Pathol., 14: 301-307.

Steinhause, E. A. (ed). 1963. Insect Pathology, Vols. 1 and 2. Academic Press, New York. Pp. 661-689.

Roeder, K. D. (ed) 1953. Insect Physiology. John. Wiley & Sons, Inc., New York. Pp. 147-190.

Weing, K. & Joachim, J. 1936. Biochem. Zeitschr., 285: 98.

Wyatt, G. R. 1967. The biochemistry of sugar and polysaccharides in insects. Adv. Insect Physiol., 4: 287-360.

Wyatt, G. R. & Pan, M. L. 1978. Insect plasma protein. Ann. Rav. Entomol., 47: 779-817.

Table 1. Protein concentration in the breeds of 2000H and CSR6.

Larval days	Treated He(2H) (mg/ml)	Control He (2H) (mg/ml)	Treated Lt(2H) (mg/g)	Control Lt(2H) (mg/g)	Treated He (C6) (mg/ml)	Control He (C6) (mg/ml)	Treated Lt(C6) (mg/g)	Control Lt(C6) (mg/g)
1st	55.17	64.29	40.10	43.42	50.41	59.58	34.75	40.23
2nd	59.95	69.42	44.57	46.55	53.29	65.17	37.85	44.57
3rd	63.00	73.00	49.50	51.88	57.46	69.52	40.95	49.88
4th	56.50	78.49	42.27	53.45	52.32	73.25	36.72	52.73
5th	52.25	81.35	38.60	57.90	49.46	78.00	33.31	55.30

Table 2. Carbohydrate level in the breeds of 2000H and CSR6.

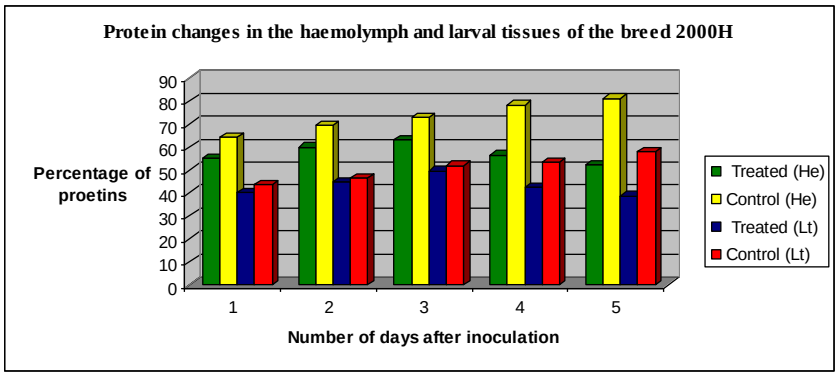
Larval days	Treated He(2H) (mg/ml)	Control He(2H) (mg/ml)	Treated Lt (2H) (mg/g)	Control Lt (2H) (mg/g)	Treated He(C6) (mg/ml)	Control He(C6) (mg/ml)	Treated Lt(C6) (mg/g)	Control Lt(C6) (mg/g)
1st	22.70	28.92	19.89	22.42	20.42	26.52	17.30	21.38
2nd	27.30	33.97	22.73	25.73	24.29	30.92	21.26	24.35
3rd	32.60	41.26	25.88	29.36	29.31	35.41	25.00	27.50
4th	26.88	47.92	21.19	32.68	25.10	39.70	21.25	31.94
5th	21.50	52.64	18.75	35.71	21.50	43.75	18.05	34.15

Table 3. Lipid content in the breeds of 2000H and CSR6.

Larval days	Treated He(2H) (mg/ml)	Control He (2H) (mg/ml)	Treated Lt (2H) (mg/g)	Control Lt (2H) (mg/g)	Treated He(C6) (mg/ml)	Control He(C6) (mg/ml)	Treated Lt (mg/g)	Control Lt(C6) (mg/g)
1st	56.70	67.18	36.76	39.44	52.47	59.00	33.69	36.57
2nd	59.44	69.36	39.57	43.02	55.60	62.35	37.33	41.15
3rd	63.35	73.85	42.26	48.23	61.19	66.57	40.92	45.78
4th	58.00	75.43	37.35	50.73	57.78	70.29	35.73	49.30
5th	55.35	77.58	35.00	53.50	51.45	73.25	32.00	51.10

He- haemolymph and Lt- larval tissues. (2H), (C6) indicates breeds name

Biochemical Progress changes in the infected and control silkworm *Bombyx mori* L.



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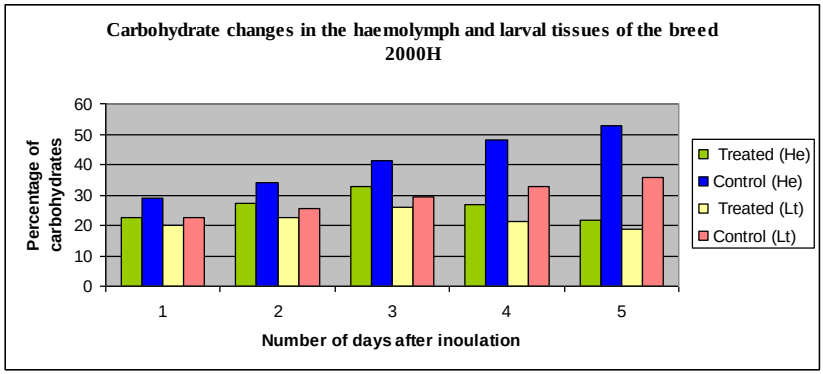

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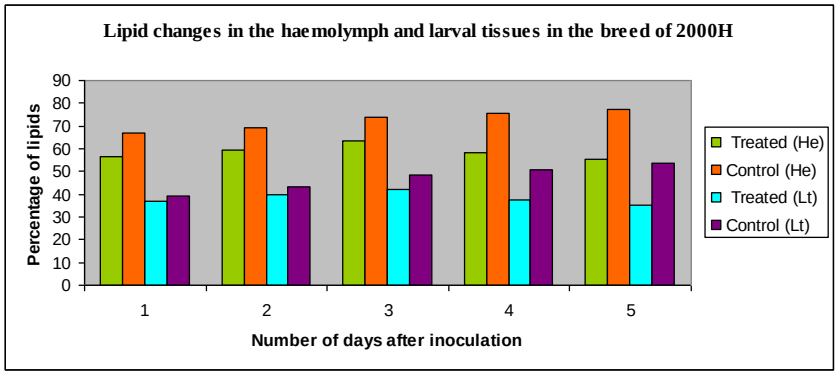

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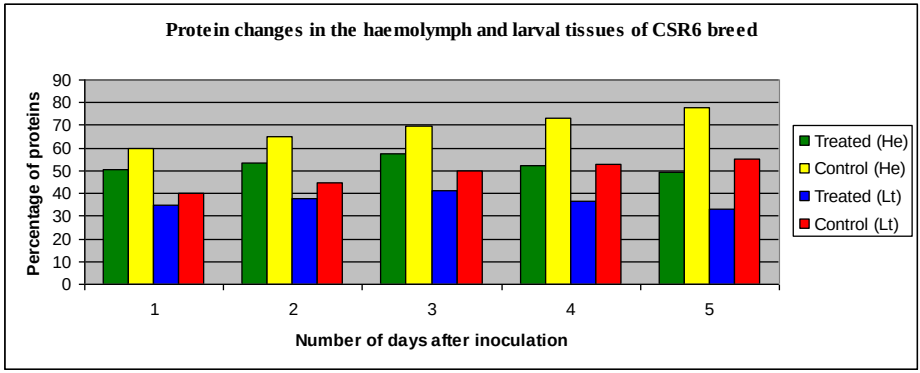

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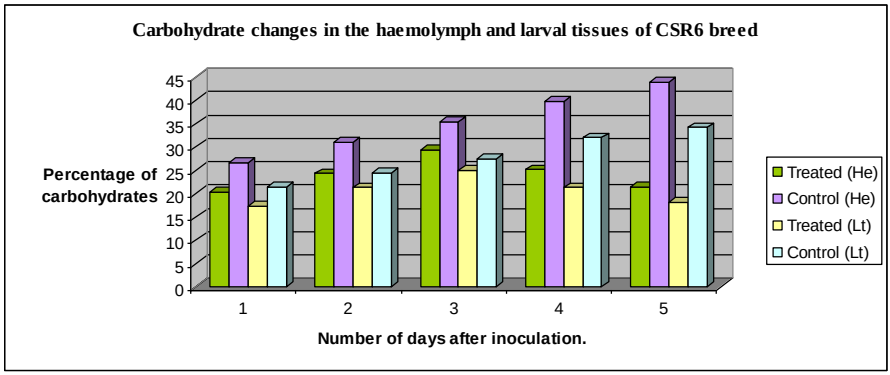

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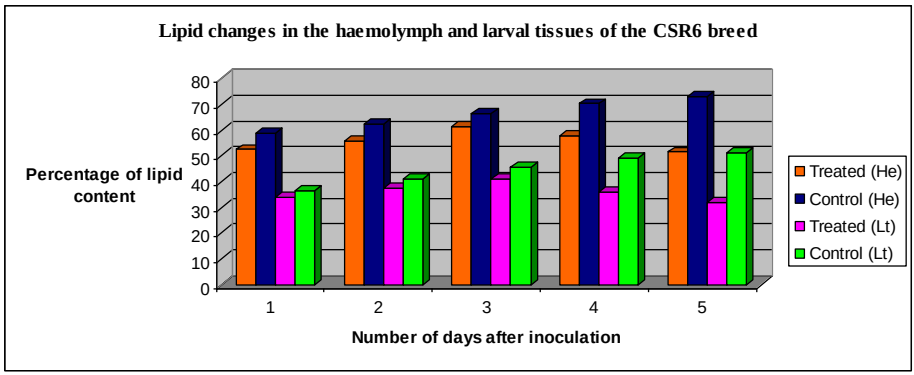

Graphs 6.