Recent Research in Science and Technology 2010, 2(3): 32–37
ISSN: 2076-5061
www.recent-science.com

ENTOMOLOGY & TOXICOLOGICAL SCIENCES

BIOCHEMICAL ALTERATIONS IN THE HAEMOLYMPH OF SILKWORM [*BOMBYX MORI* (L).(LEPIDOPTERA: BOMBYCIDAE)] FED WITH MULBERRY LEAVES ENRICHED WITH INDIAN BEAN (*DOLICHOS LABLAB*)

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Abstract

A study was carried out to evaluate the effect of Indian bean *(Dolichos lablab)* supplementation on silkworm. Finely powdered *Dolichos lablab* was dissolved in distilled water and diluted to 2.5 %, 5 %, 7.5 % and 10 % concentrations. Fresh mulberry leaves (*Morus alba* L.) were sprayed by each concentration and were fed to silkworms, from 3rd to 5th instar, five feedings/day. Group 1 larvae received mulberry leaves sprayed with distilled water and served as control, group 2 larvae received 2.5% *Dolichos lablab* sprayed mulberry leaves, group 3 larvae received 5 % *Dolichos lablab* sprayed mulberry leaves, group 4 larvae received 7.5 % *Dolichos lablab* sprayed mulberry leaves and group 5 larvae received 10 *% Dolichos lablab* sprayed mulberry leaves. Silkworm larvae fed on *Morus alba.* L. (mulberry) leaves enriched with 7.5 % concentrations of *Dolichos lablab*, significantly gained more Cocoon weight, Shell weight and Shell/cocoon ratio as compared to those fed on normal MR_2 mulberry leaves. Hence, 7.5% dose was fixed as an effective dose. Further, same study was conducted to find out the biochemical changes in the haemolymph occurred in the first day of Vth instar larvae. There was a significant increase in the haemolymph glucose, cholesterol, urea, total protein, aspartate transaminase, alanine transaminase and alkaline phosphatase. But haemolymph uric acid was significantly decreased. The results suggest that coadministration of *Dolichos lablab* with mulberry leaves at a concentration of 7.5% has enhanced the biochemical reaction involved in the silk production in the silkworm.

Key Words: *Morus alba* L., *Dolichos lablab, Bombyx mori* L., Haemolymph

Introduction

The *Bombyx mori* L (silkworm) is a phytophagous lepidopteran insect that is monophagous feeder on *Morus alba* L (mulberry leaves). Scientists have tried alternative hosts for the rearing of silkworm, but they were not cost effective. So they used some nutrients, minerals and vitamins as food supplements. They found positive impact of supplements on the silkworm growth and silk production. Ravikumar [1] emphasized that the quality and the nutritional status of mulberry has a great influence on the silkworm growth, silk yield and disease resistance. Silkworm requires specific essential sugars, amino acids, proteins and vitamins for its normal growth [2]. Javed and Gondal [3] have also reported that silkworm fed with nitrogen and ascorbic acid supplemented mulberry leaves showed higher growth and lower mortality. The effect of vitamin supplementation on the growth of silkworm has been investigated by many researchers [4, 5, 6]. *Dolichos lablab* (*D. lablab*) is a leguminous plant, found in India, is a seasonal dicotyledonous legume. It is commonly called as Indian bean. For the fulfillment of need of dietary proteins, the population of the subtropics, being predominantly vegetarian, looks to legumes like *D. lablab* as it is having more protein content. It is also called as poor man's bean as it is cheap when compared to other beans.

Extracts of *D. lablab* seeds were found to be have mitogenic properties [7]. Although the effects of nitrogen, vitamin and salts supplementation on the growth of silkworm have been investigated by many researchers, the effect of mulberry leaves enriched with *D. lablab* was not investigated. So, the present study was aimed to find out the effective dose of *D. lablab* application to mulberry leaves on cocoon weight, shell weight and shell/cocoon ratio of silkworm. Till now no authors focused on biochemical changes of silkworm during food supplementations. By using the effective dose, further biochemical studies were done in the haemolymph of first day of fifth instar larvae of *Bombyx mori* and an ultimate aim to find out whether the change in concentration of

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biochemicals have impact on the growth and silk production of silkworm.

Materials and methods

The eggs of silkworm L NB4, D2 (local Bivoltine) race were collected from farmers' training centre at Jayankodapattiam, Tamilnadu, India. The eggs were placed at ambient temperature of 25±2º C and relative humidity of 70 to 80 % in an incubator for hatching. After hatching, larvae were isolated from stock culture. The larvae were divided into 5 experimental groups including controls (distilled water control), each group consisting of 10 larvae. The larvae were reared in card board boxes measuring 22 X 15X 5 cm^3 covered with polythene sheet and placed in an iron stand with ant wells. The larvae were subjected to the following treatments. *D. lablab* was purchased from the local market surrounding Chidambaram, Tamil nadu, India, identified and authenticated from the Department of Botany, Annamalai University. Shade dried and powdered using mortar. Finely powdered *D. lablab* was dissolved in distilled water and diluted to 2.5 %, 5 %, 7.5 % and 10 % concentrations. Fresh mulberry leaves were sprayed by each concentration and then dried in air for 10 min. The supplementary leaves were fed to silkworms, five feedings/day. Group 1 larvae received mulberry leaves sprayed with distilled water and served as control, group 2 larvae received 2.5% *D. lablab* sprayed mulberry leaves, group 3 larvae received 5 % *D. lablab* sprayed mulberry leaves, group 4 larvae received 7.5 % *D. lablab* sprayed mulberry leaves and group 5 larvae received 10 % *D. lablab* sprayed mulberry leaves, respectively. And they were maintained up to cocoon. Cocoon weight, shell weight, pupa weight and shell cocoon weight ratio were determined for all groups.

The same protocol was repeated only with 7.5% *D. lablab* sprayed mulberry leaves and control larvae received mulberry leaves sprayed with distilled water for the estimation of biochemical parameters. On first day of V^{th} instar, 10 larvae were selected randomly from each group. The larval haemolymph was taken with a cut through one of the prolegs. From each larva, 0.5ml haemolymph was extracted and pooled haemolymph for each treatment used to biochemical measurement. To avoid the activity of prophenol oxidase followed by melanization of haemolymph, 1mg phenylthiourea was added to the samples [8]. Then they were centrifuged for 10min in 10000 rpm [9]. The supernatant was transferred to new tubes and was kept in -20 °C until the beginning of the experiments.

All the biochemical estimations were done in ERBA Smartlab, fully auto analyzer. Glucose estimations in the haemolymph were done by GOD-POD based end-point method by the kit of Span Diagnostics Ltd., Surat. The total cholesterol in haemolymph was estimated based on the method of Richmond [10]. The principles of this method are based on hydrolysis of cholesterol esters by cholesterol oxidase, cholesterol esterase and peroxidase. The concentration of urea was determined by measuring ammonia produced from urea, using a commercial urea assay kit (Span Diagnostics Ltd., Surat). Uric acid contents in the haemolymph were determined using uricase as described by Valovage and Brooks [11]. The serum total protein was estimated by Biuret's method [12]. Aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) were assayed using the diagnostic kit based on the method of Reitman and Frankel [13]. Alkaline phosphatase (ALP, EC 3.1.2.3.1) was estimated using the diagnostic kit based on Kind and King's method [14, 15].

Satistical analysis

Data were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available statistics software package (SPSS® for Windows, V. 16.0, Chicago, USA). Results were presented as means ±SD. p values < 0.05 were regarded as statistically significant.

Results and discussion

Table 1 shows the effects of various concentrations of *D. lablab* supplementation with mulberry on the cocoon weight, shell weight and shell/cocoon ratio of silkworm. There is a significant raise in the cocoon weight, shell weight and shell/cocoon ratio of the larvae fed with *D. lablab* supplemented mulberry when compared with control groups. This may be due to the increased protein content of the mulberry supplemented with *D. lablab.* This is in agreement with the work done by Javed [3] and Islam et al. [16] regarding the increased cocoon weight, shell weight and shell/cocoon ratio when silkworm treated with various combination urea, ascorbic acid and nickel chloride. There is no significance between the 7.5% and 10% dose of *D. lablab* with respect to cocoon weight and shell weight but there is a significant difference with respect to shell/cocoon ratio. This may be due to the somewhat decreased in cocoon weight in the 10% dose. So we can fix the effective dose as 7.5%.

Table 1. Effects of various concentrations of *Dolichos lablab* supplementation with mulberry on the Cocoon weight, Shell weight and Shell/cocoon ratio of *Bombyx mori.*

Group	**Cocoon weight (g)**	**Shell weight (g)**	**Shell/cocoon ratio**
Control	1.85 ± 0.03^{a}	0.29 ± 0.005^{a}	16.07 ± 0.30^{a}
Mulburry + 2.5% *Dolichos lablab*	1.95 ± 0.05^{b}	0.32 ± 0.007^{b}	16.24 ± 0.37^{a}
Mulburry + 5% *Dolichos lablab*	2.31 ± 0.02^{c}	0.38 ± 0.002^{c}	16.56 ± 0.12^{b}
Mulburry + 7.5% *Dolichos lablab*	2.54 ± 0.03^{d}	0.47 ± 0.005^{d}	18.49 ± 0.20^{c}
Mulburry + 10% *Dolichos lablab*	2.52 ± 0.06^{d}	0.47 ± 0.012^{d}	18.97 ± 0.48^{d}

Values are means ± S.D. for 10 larvae in each group. $^{a-d}$Values not sharing a common superscript letter within each coloumn differ significantly at $P < 0.05$ (DMRT).

Table 2 shows the effects of 7.5% *D. lablab* supplementation with mulberry on the haemolymph glucose and cholesterol levels of silkworm. There is a significant increase in the haemolymph glucose in the *D. lablab* supplemented group when compared with control group supplemented with distilled water. Increase of glucose indicates full fed state and stress free condition as decrease in glucose in haemolymph is seen in starvation and outside stress or a response to suppress the stress. *D. lablab* could enhance the feeding of larvae by favorable effects on leaves or decrease the antifeedant characteristics. Etebari and Matindoost [17, 18] have demonstrated that even starvation could cause the reduction of many biochemical compounds of haemolymph such as glucose. Haemolymph cholesterol level in *D. lablab* groups showed a significant increase when compared with control groups. Needless to say that the lipids are important source of energy for insects. Decrease of some compounds, including glucose and cholesterol to a physiological stress could be attributed to the interruption in absorption system. Normal cholesterol in haemolymph indicates that there is no interruption in absorption system when mulberry leaves enriched with *D. Lablab*, Furthermore, increased cholesterol level may indicate the effective activation of absorption system. Which inturn enhance the growth and more silk production.

Table 2. Effects of 7.5% concentrations of *Dolichos lablab* supplementation with mulberry on the haemolymph glucose and cholesterol of Bombyx mori.

Group	Glucose (mg/dl)	Cholesterol (mg/dl)
Control	13.09 ± 0.70^{a}	30.36 ± 1.38^{a}
Mulburry + 7.5% *Dolichos lablab*	15.10 ± 0.18^{b}	38.36 ± 2.05^{b}

Values are means ± s.d. for 10 larvae in each group. $^{a, b}$Values not sharing a common superscript letter within each coloumn differ significantly at $P < 0.05$ (DMRT).

Table 3 shows the effects of 7.5% *D. lablab* supplementation with mulberry on the haemolymph urea, uric acid and total protein levels of silkworm. There is a significant elevation in the haemolymph urea in *D. lablab* supplemented group when compared with control group. From the standpoint of urea formation within the insects, the enzyme such as arginase in several tissues might be induced by a diet with higher protein content such as an artificial diet, *D. lablab* is also having more protein content. Changes in liver arginase activity according to dietary protein intake are well documented in mammals [9]. Preliminary studies on fat body arginase in silkworm larvae indicate that mitochondrial arginase activity per individual insect is higher in the larvae reared on an artificial diet than in those reared on fresh mulberry leaves (unpublished data). There is a significant decrease in the haemolymph uric acid of the larvae fed with *D. lablab* supplemented mulberry when compared with control groups. Generally, there is an adverse correlation between the amount of protein and uric acid in haemolymph of silkworm and those larvae with lowered protein have elevated uric acid. One of the important factors for measuring uric acid and urea was to know the nitrogen catabolism procedure in silkworm, because *D. lablab* is having more nitrogen content. Uric acid decrease in this group of larvae is representative of decrease in some metabolisms specially protein catabolism activity in them. There is a significant elevation in the haemolymph total protein in *D. lablab*

supplemented group when compared with control group. Studies on protein metabolism are considered most important in silkworm physiology because of its vital role in the determination of chemical characteristics of silk proteins [20]. In general, the breakdown of proteins dominates over their synthesis due to enhanced proteolytic activity [21]. Maintenance of proteins in a highly organized state requires an active and continuous supply of energy. From the present study as *D. lablab* is having more protein content, the larvae groups may be supplied with uninterrupted energy.

Table 3. Effects of 7.5% concentrations of *Dolichos lablab* supplementation with mulberry on the haemolymph urea, uric acid and total protein of *Bombyx mori*.

Group	Urea (mg/ml)	Uric acid (mg/dl)	Total protein (g/dl)
Control	8.11 ± 0.35^{a}	2.72 ± 0.14^{a}	6.28 ± 0.28^{a}
Mulburry + 7.5% *Dolichos lablab*	7.55 ± 0.41^{b}	1.81 ± 0.10^{b}	8.26 ± 0.44^{b}

Values are means ± s.d. for 10 larvae in each group. $^{a, b}$Values not sharing a common superscript letter within each coloumn differ significantly at $P < 0.05$ (DMRT).

Table 4 shows the effects of 7.5% *D. lablab* supplementation with mulberry on the haemolymph AST, ALT and ALP levels of silkworm. There is a significant increase in the AST, ALT and ALP activities in the haemolymph of *D. lablab* supplemented group when compared with control group. The transaminases are the important components of amino acid catabolism, which is mainly involved in transferring an amino group from one amino acid to another keto acid, thus forming another amino acid. The aspartate and alanine aminotransferases which serve as a strategic link between the carbohydrate and protein metabolism [22]. Elevation in the activities of both AST and ALT enzymes in the hemolymph of silkworm supplemented with *D. lablab* indicated an active transportation of amino acids which provide keto acid to serve as precursors in the synthesis of essential constituents for the synthesis of silk. The alkaline phosphatase is a set of hydrolytic enzymes that hydrolyze phosphomonoesters under the alkaline condition. The activity of these enzymes is related to the physiological situation of silkworms and reflects the absorption, digestion and positive transportation of nutrients in the midgut. Different stress and disease causes considerable decrease in the activity of ALP [23]. In the present study ALP level of activity increased significantly indicates the better physiological situation and reflects the absorption, digestion and positive transportation of nutrients in the midgut. And also it indicates stressfree and disease free condition of the silkworm, which indicates the silkworm are in healthyway for the synthesis of silk. It is also reported that treatment with 20-hydroxy ecdyson did increase ALP activity in all tissues except the midgut [24].

Table 4. Effects of 7.5% concentrations of *Dolichos lablab* supplementation with mulberry on the haemolymph AST, ALT and ALP of *Bombyx mori*.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	390.22 ± 18.21^{a}	212.08 ± 10.22^{a}	19.17 ± 0.96^{a}
Mulburry + 7.5% *Dolichos lablab*	440.02 ± 23.62^{b}	249.72 ± 13.40^{b}	24.16 ± 1.30^{b}

Values are means ± s.d. for 10 larvae in each group. $^{a, b}$Values not sharing a common superscript letter within each coloumn differ significantly at $P < 0.05$ (DMRT).

It may be concluded that supplementing *D. lablab* in mulberry as food for silkworm has different effects on its biochemicals. *D. lablab* showed considerable change in all biomolecules, which indicates the more utilization and turnover of carbohydrates, lipids and proteins. This may be responsible for healthy, diseasefree and stressfree growth of the silkworm. Therefore this supplementation can be incorporated to the sericulture farms.

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