International Journal of Fauna and Biological Studies
Available online at www.faunajournal.com

I J F B S International Journal of Fauna And Biological Studies

ISSN 2347-2677
IJFBS 2014; 1 (3): 21-26

Received: 07-10-2013
Accepted: 02-11-2013

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Application of phytojuvenoid enhances the protein level in the multivoltine mulberry silkworm (*Bombyx mori* Linn.)

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ABSTRACT
The application of phytojuvenoid on the total protein level of *Bombyx mori* was studied. Variation in the phytojuvenoid concentration significantly (P_1<0.01) influenced the total protein level in the fat body and haemolymph of larvae at final stage of spinning. The maximum level of total protein content was recorded in case of 30% phytojuvenoid concentration – triple treated (III^{th} - IV^{th}) larvae while it was lowest in 40% phytojuvenoid concentration – triple treated larvae at final stage of spinning. The phytojuvenoid influences the level of protein in the larvae and caused some beneficial effect on the life pattern of silkworm and the productivity of cocoon.

Keywords: Phytojuvenoid, Protein content, Fat body, Haemolymph, Larvae, *Bombyx mori.*

1. Introduction
In India, sericulture has been an attractive cash crop. It is well known for its low investment and quick and high return which make it an ideal industry fitting well into the socio-economic frame of India. Sericulture has become one of the most important cottage industries in a number of countries. The cottage industries occupy a special importance in developing country's economy. Nistari is a resistant variety of multivoltine mulberry silkworm (*Bombyx mori*) which contributes up to a great extent in the commercial production of cocoon. The efforts are being made to evolve new technologies that are effective, labour saving and eco-friendly. In order to increase, the production of silk, efforts have been made to study effect of ecological factor (1), temperature (2) etc. on the performance of silkworm. The Magnetization of eggs influences silk producing potential (3) and incubation period of eggs (4) and larval performance (5). In insects, the process of growth and development is regulated by circulating hormones viz., prothoracicotropic hormone (PTTH), juvenile hormone (JH) and ecdysone, which directly and indirectly manifest the phenomenon of moulting and metamorphosis. The response of silkworm to very small quantities of phytojuvenoids or its analogues may extend the larval maturation events and influence the spinning process. The synchronized maturation of larvae and simultaneous spinning of cocoon is very important in the sericulture industry. However, the response to such treatment varies depending on the dosage of compounds showing duration and number of applications (6). The phytoecdysteroid has been noticed to influence the development, growth, silk producing and reproductive potential of *B. mori* (7-11). The more food ingested during this period gets converted and it turn contributes to silk protein. Delay in moulting is probably due to the inhibitory action of JH on ecdysone synthesis in *B. mori* (12). JH is claimed to inhibit protein synthesis in early treated larvae with later on region protein synthesis resulting in bigger silk gland and the result is improvement of cocoon shell weight (13). The phytojuvenoid caused beneficial effect on the reproductive and commercial traits and the life pattern of silkworm (14-16). Keeping this in view, an attempt has been made to study the topical effect of bioactive phytojuvenoid on the improvement in the commercial parameters in the monphagous insect (*Bombyx mori*), which is the aim of the present investigation.

2. Materials and Methods
The seed cocoons (pupa enclosed in silken case) of multivoltine mulberry silkworm *(Bombyx mori* nistari) were obtained from the silkworm grainage, Directorate of sericulture,

Behraich Uttar Pradesh and were maintained in the plywood trays (23 x 20 x 5 cm) under the ideal rearing conditions (17) in the silkworm laboratory, Department of Zoology D.D.U. Gorakhpur University, Gorakhpur. The temperature and relative humidity were maintained at 26±1 ^{0}C and 80±5% RH respectively till the emergence of moths from the seed cocoons. The newly emerged moths were quickly picked up and kept sex-wise in separate trays to avoid copulation. The male moths were smaller in size but more active than the female moths which were comparatively larger and less active. The whole grainage operation was performed as per description given by (17). Moths have a tendency to pair immediately after emergence, therefore, Sufficient pairs, each containing one male and one female from newly emerged moths were allowed to mate at 26±1 ^{0}C and 80±5% RH in 12 hour/day dim light condition. After four hours of mating, the paired moths were detached manually. The female moths were allowed for egg laying. After 24 hours of egg laying, the female moths were individually examined for their disease freeness and after formaline treatment the dried eggs were transferred to the incubator for hatching. After hatching, the silkworm larvae were reared on the fresh and clean leaves of *Morus alba* given as food in the rearing trays. These larvae were taken for the purpose of experiments. After completion of fifth instar, the ripe worms ceased feeding and ready for spinning. Small mountages were provided to the ripe worms and thus, sufficient numbers of cocoons were obtained from the silkworm larvae reared in our laboratory.

2.1. Design of experiment

For extraction of phytojuvenoid the needle of *Pinus* were collected, washed thoroughly with distilled water and dried in incubator at 37 ^{0}C. The dried materials were powdered separately with the help of mechanical device. Further, 50 gm powder was subjected to extraction separately through soxhlet apparatus with 250 ml distilled water for 40 hours. After 40 hours of extraction a little amount of concentrated solution of plant extract was obtained. The concentrated solution was dried and 6.45 gm material was obtained in powdered form. The dried powder thus obtained, was dissolved in distilled water as 5 gm in 25 ml water and used this solution for further experiment, as 100% concentration of phytojuvenoid. For further experiment the suitable narrow ranges of *Pinus* phytojuvenoid concentrations viz. 10, 20, 30 and 40% were taken. Thus, four phytojuvenoid concentrations were applied topically by spraying as 1 ml on to 100 larvae separately. Three sets of experiments were designed viz., single, double and triple treatment of larvae.

2.1a. Single treatment of larvae

Single treatment of larvae was performed at the initial stage of fifth instar larvae just after fourth moulting. One hundred larvae of fifth instar at the initial stage were taken out from the BOD incubator and treated with one ml of 10% concentrated solution of *Pinus* needle extract by sprayer.

2.1b. Double treatment of larvae

Double treatment of larvae was started from the initial stage of fourth instar larvae. In the first treatment, one hundred larvae of fourth instar were treated by 1 ml of 10% concentrated solution of *Pinus* needle extract by spraying. The treated larvae were then transferred in BOD incubator for rearing and development. Further, similar second treatment for the same larvae was given at the initial stage of fifth instar larvae. Thus, in double treatment, fourth and fifth instar larvae were treated.

2.1c. Triple treatment larvae

For triple treatment, the third instar larvae in the initial stage were separated from BOD incubator. In the first treatment one hundred, third instar larvae, were treated by 1 ml of 10% concentrated solution of *Pinus* needle extract by sprayer and kept in BOD for rearing. The second treatment of same larvae was done just after third moulting i.e. at the initial stage of fourth instar larvae and transferred in BOD incubator for rearing. Third treatment was given at the initial stage of fifth instar i.e. just after fourth moulting of the same treated larvae as earlier. Thus, in the triple treatment third, fourth and fifth instar larvae were treated.

Similar experiments were performed by 20, 30 and 40% concentrations of phytojuvenoid obtained from *Pinus* needle extract. A control set was always maintained with each set of experiment.

To observe the effect of phytojuvenoid at various stages of *Bombyx mori* larvae on certain biochemical constituents like total protein contents in the haemolymph and fat body of larvae at the final stage of spinning, following methods were adopted.

2.2. Total protein content

For the estimation of total protein content from silk gland, haemolymph and fat body of larvae, the fifth instar larvae were dissected in distilled water at the final stage of spinning and 0.01 gm and 0.5 ml (0.62 mg) tissue of fat body and haemolymph were taken respectively, Protein content was estimated according to Lowery *et al*. (1951) as modified by Singh and Agarwal (1989). In above tissues added 4.0 ml of 10% T.C.A. and prepared the homogenate separately. The homogenate, thus obtained, was centrifuged at the top speed (20,000 rpm) for 10 minutes. Further, the supernatant was discarded, washed the precipitate with 5% T.C.A., again centrifuged for 10 minutes and discarded the supernatant. The precipitate was again washed with 10% T.C.A., centrifuged and discarded the supernatant. The precipitate, thus obtained, was dissolved in 4 ml of 10 N NaoH. Now in 1 ml of diluted supernatant, 0.5 ml, of freshly prepared alkaline copper solution (Reagent C) was added. Reagent C was prepared by the addition of 50.9 ml, reagent A (2% sodium carbonate in 0.1 N NaoH) and 1 ml Reagent B (1% of sodium potassium tartrate, 0.5% copper sulphate, mixed in 1:1 ratio at the time of experiment), The reaction mixture was kept for 10 minutes at room temperature, then 0.5 ml of folin ciocalteau reagent (diluted 1:2 ratio with distilled water at the time of experiment) was added and mixed thoroughly. Thirty minutes after this the blue colour developed which was measured at 600 nm. Six replicates of each experiment were made. Standard curves were prepared with different concentrations of Bovin serum albumen. The value of protein has been expressed as µg/mg of respective tissues.

All the data obtained by the experiment were analyzed statistically by two-way ANOVA and Post- hoc test.

3. Result

3.1 Total protein content in the fat body of larvae at the final stage of spinning

The data presented in the table-1a indicates that the phytojuvenoid concentration and number of larval treatment influenced total protein content in the fat body of larvae at the final stage of spinning. With the increasing number of larval

treatment with 10, 20 and 30% phytojuvenoid concentration, the total protein content in the fat body at final stage of spinning increased gradually and reached to the maximum level of 14.56±0.32 µg/mg in case of triple treated larvae with 30% phytojuvenoid concentration. In case of larval treatment with 40% phytojuvenoid concentration, the total protein content in the fat body of larvae at final stage of spinning increased in single treated larvae but further increase in the number of larval treatment caused decline in the total protein content in the fat body at final stage of spinning which reached to the minimum level of 12.08±0.63 µg/mg in triple treated larvae.

The trend of increase in the total protein content in the fat body at final stage of spinning was almost of same fashion in 10, 20 and 30% phytojuvenoid concentration in relation to the number of larval treatment. Two-way ANOVA indicates that variation in the phytojuvenoid concentration significantly ($P1<0.01$) influenced the total protein content in the fat body at final stage of spinning. The Post–hoc test (table-1b) shows significant group difference in the total protein content in the fat body of larvae at final stage of spinning in between all group combinations except in control and 10%, 10 and 40 % and 20 and 30% in single treated larvae. In case of double treatment of larvae significant group difference in the total protein content in the fat body in between all group combinations was noticed except in control and 40% and 20 and 30% phytojuvenoid concentration. In case of triple treatment of larvae significant group difference in the protein content was noticed in between all group combinations.

Table 1a: Effect of phytojuvenoid treatment on the total protein content (µg/mg) in the fat body of *Bombyx mori* larvae at the final stage of spinning.

	Phytojuvenoid concentration (%)					
Stage of treatment (Larval instar)	**Control X_1**	**10 X_2**	**20 X_3**	**30 X_4**	**40 X_5**	**F_1-ratio n_1 =4**
Single (V)	12.65 ±0.24	12.76 ±0.13	13.22 ±0.17	13.85 ±0.26	13.07 ±0.29	
Double (IV-V)	12.65 ±0.24	13.08 ±0.26	13.53 ±0.97	14.23 ±0.28	12.52 ±0.95	**10.63***
Triple (III-V)	12.65 ±0.24	13.41 ±0.19	13.85 ±0.61	14.56 ±0.32	12.08 ±0.63	

F_2-ratio = 0.3808** n_2=2

*P_1< 0.01, ** Non-significant

Each value represents mean ± S.E. of six replicates, X_1, X_2, X_3, X_4 and X_5 are the mean values of the total protein content (µg/mg) in the fat body in control, 10, 20, 30 and 40 % phytojuvenoid concentrations respectively.

Table 1b: Post - hoc test showing effect of phytojuvenoid treatment on the total protein content in the fat body of *Bombyx mori* larvae at the final stage of spinning.

Mean difference in between	**Stage of treatment**		
Group	**Single**	**Double**	**Triple**
X1~X2	0.11	*1.43	*1.71
X1~X3	*0.57	*0.88	*1.20
X1~X4	*1.20	*1.58	*1.91
X1~X5	*0.42	0.12	*0.57
X2~X3	*0.46	*0.45	*0.44
X2~X4	*1.09	*1.15	*1.15
X2~X5	0.31	*0.56	*1.33
X3~X4	*0.63	*0.70	*0.71
X3~X5	0.15	0.01	*1.77
X4~X5	*0.78	*1.71	*2.48

Honesty Significant difference (HSD) $= q\sqrt{\frac{MS\ within}{n}}$

$= 6.10\sqrt{\frac{0.132}{6}}$

$= 0.37$

MS=Mean square value of ANOVA table

q = studentized range static

n = No. of replicates

***** = shows significant group difference

X_1, X_2, X_3, X_4 and X_5 are the mean values of total protein content in the fat body of *Bombyx mori* in control,10,20, 30 and 40 per cent phytojuvenoid concentrations respectively.

3.2. Total protein content in the haemolymph of the larvae at the final stage of spinning

The data given in the Table 2a clearly indicates that the phytojuvenoid concentration and number of larval treatment influenced the total protein content in the haemolymph of larvae at the final stage of spinning. With the increasing number of larval treatment with 10, 20 and 30% phytojuvenoid concentration, the total protein content in the haemolymph of the larvae at the final stage of spinning increased gradually and reached to the maximum level of 23.85±0.03 µg/mg in case of triple treated larvae with 30% phytojuvenoid concentration. In case of larval treatment with 40% phytojuvenoid concentration, the total protein content in the haemolymph of the larvae at final stage of spinning increased in single treated larvae but further increase in the number of the larval treatment caused decline in the total protein content in the haemolymph of the larvae at final stage of spinning which reached to the minimum level of 20.90±0.78 µg/mg in triple treated larvae. The trend of increase in the total protein content in the haemolymph of the larvae at the final stage of spinning was almost same in 10, 20 and 30% phytojuvenoid concentration in relation to the number of larval treatment.

Two-way ANOVA indicates that variation in the phytojuvenoid concentration significantly ($P_1 < 0.01$) influenced the total protein content in the haemolymph of larvae at the final stage of spinning. The Post–hoc test (Table 2b) shows significant group difference in the total protein content in the haemolymph of the larvae at the final stage of spinning in between control and 20%, control and 30% and 30 and 40% at single treated larvae. In case of double treated larvae significant group difference was recorded in between 10 and 40% phytojuvenoid concentration was observed. In the triple treated larvae significant group difference in the protein content in between control and 10%, 10 and 40% and 30 and 40% phytojuvenoid concentration.

Table 2a: Effect of phytojuvenoid treatment on the total protein content (µg/mg) in the haemolymph of *Bombyx mori* larvae at the final stage of spinning.

Phytojuvenoid concentration (%)						
Stage of treatment	**Control**	**10**	**20**	**30**	**40**	**F1-ratio**
(Larval instar)	**X1**	**X2**	**X3**	**X4**	**X5**	**n1 =4**
Single (V)	21.58±1.05	21.76±0.82	22.81±0.66	23.16±0.08	21.75±0.99	
Double (IV-V)	21.58±1.05	22.06±0.95	23.14±0.64	23.51±0.08	21.40±0.86	23.27*
Triple (III-V)	21.58±1.05	22.42±0.73	23.56±0.57	23.85±0.03	20.90±0.78	

F_2-ratio = 0.6594** **n_2**=2

***P_1**< 0.01, ** Non-significant

Each value represents mean ± S.E. of six replicates

X_1, X_2, X_3, X_4 and X_5 are the mean values of the total protein content (µg/mg) in the haemolymoph in control, 10, 20, 30 and 40 % phytojuvenoid concentrations respectively.

Table 2b: Post - hoc test showing effect of phytojuvenoid treatment on the total protein content in the haemolymph *Bombyx mori* larvae at final stage of spinning.

Mean difference in between	**stage of treatment**		
groups	**single**	**double**	**triple**
X1~X2	0.33	0.32	*1.03
X1~X3	*0.41	0.24	0.10
X1~X4	*0.46	0.20	0.15
X1~X5	0.09	0.27	0.30
X2~X3	0.08	0.08	0.08
X2~X4	0.13	0.12	0.12
X2~X5	0.24	*1.05	*1.73
X3~X4	0.05	0.04	0.04
X3~X5	0.32	0.03	0.03
X4~X5	*0.37	0.07	*1.81

Honesty Significant difference (HSD) $= q\sqrt{\frac{MS\ within}{n}}$

$= 6.10\sqrt{\frac{0.118}{6}}$

$= 0.35$

MS=Mean square value of ANOVA Table, **q** = studentized range static, **n**= No. of replicates, ***** = shows significant group difference

X_1, X_2, X_3, X_4 and X_5 are mean values of total protein content in the haemolymph of *Bombyx mori control,10*, 20, 30, and 40 per cent phytojuvenoid concentrations respectively.

4. Discussion

The change in the phytojuvenoid concentration and number of larval treatment of *Bombyx mori* influenced the total protein content in the fat body of *Bombyx mori* at the final stage of spinning. With the increase in number of larval treatment from single to triple, the protein content in the fat body at the final stage of spinning increased in 10, 20 and 30% concentration while in 40% concentration, the declining tendency in the protein content was noticed up to triple treatment of larvae. The highest level of protein content was noticed to be 20.33±0.12 µg/mg in the fat body obtained from the triple treated larvae in 30% concentration (Table 1a). The fat body plays an important role in the protein synthesis (20-22) and high level of protein content has also been found in the larval fat body of *Dixippus morossus* (23). Some of the protein, originally synthesized in the fat body, was isolated from the 5th instar larvae of *Bombyx mori* (24-26). In the pupal stage of grain moth *(Sitotroga cerealella)* four major proteins were lost from the haemolymph and sequestered by the fat body, where they serve as a store of amino acids to be used in the development of adult (27,28). The cold adaptation in the 5th instar and during the period of spinning is closely associated with the increase in RNA/DNA ratio of the tissue during the earlier and latter days of spinning and the accumulation of protein in the fat body (29). The treatment of *Bombyx mori* eggs at 3000 gauss- 96 hours exposure in the static magnetic field caused an increase in the protein content in the fat body of larvae and pupae (30)**.** JHA isolated from Bemchi (*Psoralea corylifolia*) increased the total protein content significantly in the fat body of *Bombyx mori* (31). The total protein content in the fat body of *Bombyx mori* increased significantly with increasing the strength of magnetic field from 1000-3000 Gauss but in 4000G it was decreased and the maximum was observed in 3000G (32).

The level of protein content in the haemolymph of *Bombyx mori* at the final stage of spinning is influenced due to variation in the phytojuvenoid concentration and number of larval treatment. The total protein content in the haemolymph at the final stage of spinning increased with the increasing number of larval treatment from single to triple in case of 10, 20 and 30% concentration. The protein content was noticed to be increased slightly in single treatment of larvae under 40% concentration but further increase in the exposure duration up to triple treatment caused regular decline in the level of protein content. The biological function of Three crystal structures of a lipoprotein (Bmlp7), a member of the 30 kDa lipoprotein family from mulberry silkworm (*Bombyx mori* L.) haemolymph, is unknown, but its structural homology to sugar-binding proteins suggests that, in analogy to other 30 kDa haemolymph lipoproteins, it could play a role as an anti-apoptotic factor or function in the immune response of the insect to fungal infections (33).The change in the protein metabolism during insect development has been studied with reference to the changes in amino acid spectrum, net synthesis of protein and kinetics of certain enzymes associated with the synthesis and degradation of amino acids and protein (34). The decreased protein content in the haemolymph was recorded in *Rhodnius prolixus* at high temperature regimes (35). The protein content in the haemolymph towards the end of the last instar was attributed to the increased rate of protein biosynthesis in the fat body of *Gallaria mellonella* (36). The variation in the protein and related components in the haemolymph during insect development is directly related to the spinning process (37) while an increased protein level in the haemolymph due to cold adaptation of ectoderm has also been noticed (38). The application of magnetic field caused an increase in the enzyme (Carboxymutase and Catalase) activities in the biological system (39). The total protein content increased in the haemolymph of larvae and pupae due to magnetization of *Bombyx mori* eggs in magnetic strength of 3000 gauss for 96 hours exposure (30)**.** JHA isolated from Bemchi (*Psoralea corylifolia*) significantly increased the total protein content in the haemolymph of *Bombyx mori* (31**)**.The total protein content in the haemolymph of *Bombyx mori* increased significantly with increasing the strength of magnetic field from 1000- 3000 Gauss but in 4000G it was decreased. The maximum was observed in 3000G (32). Phytoecdysteroids increased the total protein content in the haemolymph of fifth instar of *Bombyx mori* at the final stage of spinning (40).

As discussed, a number of factors influenced the utilization and synthesis of protein and the level of total protein content in different tissues of silkworm at varying stages of the development. In last phase of 5th instar when silk gland is ready for synthesizing the silk fibers, the posterior silk gland cells synthesized large amount of fibroin. Such high protein synthetic activity implies coordinated functioning of all the elements of the cells machinery devoted to fibroin assembling and maturation. The variation in the number of larval treatment and phytojuvenoid concentration, extended the larval period and the larvae feed more mulberry leaves during extended period, causing such physiological and biochemical changes which may have influenced the synthesis and utilization of protein level in the fat body and haemolymph of *Bombyx mori* larvae. But 40% concentration generated stress response, causing general decline in the rate of protein synthesis.

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