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Roli Srivastava
Department of Silkworm laboratory, Department of Zoology, D.D.U. Gorakhpur University Gorakhpur-273009, India.

VB Upadhyay
Silkworm laboratory, Department of Zoology, D.D.U. Gorakhpur University Gorakhpur-273009, India.

Correspondence:
Roli Srivastava
Silkworm laboratory, Department of Zoology, D.D.U. Gorakhpur University Gorakhpur-273009, India.

Biochemical constituents of multivoltine mulberry silkworm (*Bombyx mori* Linn.) Influenced by Phytojuvenoid compound

Roli Srivastava, VB Upadhyay

Abstract
The topical application of phytojuvenoid on *Bombyx mori* larvae has been proved to be of biotechnological significance in the sericulture industry. Total DNA content in the fat body of *Bombyx mori* larvae at the initial and final stage of spinning was significantly ($P_1 < 0.01$) influenced due to change in the phytojuvenoid concentration. Higher level of the total DNA content was recorded in case of 30% phytojuvenoid concentration – triple treatment of larvae at initial and final stage of spinning. The phytojuvenoid influences the level of DNA in the larvae and caused some beneficial effect on the life pattern of silkworm and the productivity of cocoon.

Keywords: Phytojuvenoid, DNA content, Fat body, Larvae, Bombyx mori

1. Introduction
The silkworms, *Bombyx mori* is the only fully domesticated insect whose survival depends entirely on breeding and rearing by humans. The main trends in 5,000 years of sericulture have been to increase the quality and quantity of silk production by improving the productivity of *Bombyx mori* strains. The historical and social importance of silk production, the industrial and commercial use of silk and the tremendous number of empirical observations and applications all over the world finally contributed to the promotion of the silkworm as the laboratory animal for basic research in biology. The success in increasing the silk productivity by strain selection underlines the existence of multifactorial control of silk production. Comparative biometric studies have attempted to identify which of the silk gland parameters is the target; four parameters show high correlation with silk productivity of different strain of *B.mori*. They are number of silk gland cells, silk gland weight, the DNA and the RNA content [1]. In order to increase, the production of silk, efforts have been made to study effect of the Magnetization eggs influences silk producing potential [2] and incubation period of eggs [3] and larval performance [4]. The phytoecdysteroid and the juvenile hormone analogue also has been noticed to influence the development, growth, biochemical constituents, silk producing and reproductive potential of *B. mori* [5-8]. 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 [9]. 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* [10]. 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 [11]. The aim of present contribution is to give to the entomologist a picture of the most recent progress and trends in the field of silk production.

2. Material and Method
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 5cm) under the ideal rearing conditions 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 Krishnaswamy *et al*. (1973) [12]. 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 formalin 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 number of cocoons was 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 soxlet 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.

2.1.1. 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.

2.1.2. 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.

2.1.3. 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.

2.1. 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 biochemical constituent like DNA in the fat body of larvae at the initial and final stage of spinning, following method was adopted.

2.2. The estimation of DNA was performed according to Schneider (1957) [13], by using the diphenylamine regents. For measuring the DNA content, took 0.10 gm fat body from fifth instar larval stage and pupae. The tissues thus obtained were homogenized separately in 5% T.C.A at and centrifuged at 5000 rpm for 20 minutes. And then took 1 ml of supernatant and added 1.0 ml distilled water and 4 ml of freshly prepared diphenylamine regent (1gm diphenylamine, 100 ml of glacial acetic acid and 2.5 ml conc.H_2SO_4). Now the reaction mixture was kept in boiling water bath for 10 minutes. The blue colour developed, was measured at 600 nm. Standard curve were drawn using different concentrations of calf thymus DNA as standard. DNA had been expressed as µg/mg tissue. Six replicates of experiment were made.

Similarly, other series of experiments were performed with silkworm larvae, treated with 20, 30 and 40% phytojuvenoid concentrations separately with a set of control. All the data obtained were analyzed statistically by two-way ANOVA and Post- hoc test.

3. Results

3.1. Total DNA Content in the Fat Body of Larvae at the Initial Stage of Spinning

The data given in table-1a clearly indicates that the phytojuvenoid concentration and number of larval treatment influenced the total DNA content in the fat body of larvae at the initial stage of spinning. With the increasing number of larval treatment with 10, 20 and 30% phytojuvenoid concentration, total DNA content in the fat body of larvae at the initial stage of spinning increased gradually and reached to the highest level of 1.25±0.04 µg/mg in case of triple treated larvae with 30% phytojuvenoid concentration. In case of the larval treatment with 40% phytojuvenoid concentration, the total DNA content in the fat body of larvae at the initial stage of spinning increased in single treated larvae but further increase in the number of larval treatment caused decline in the total DNA content in the fat body of larvae at the initial stage of spinning which reached to the lowest level of 0.87±0.03 µg/mg in triple treated larvae. The trend of increase in the total DNA content in the fat body of larvae at the initial stage of spinning was almost of same fashion in 10, 20 and 30% phytojuvenoid concentration in relation to the number of larval treatment.

Table 1a: Effect of phytojuvenoid treatment on the total DNA content (µg/mg) in the fat body of *Bombyx mori* larvae at the initial 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	0.91	0.93	0.98	1.05	0.98	
(V)	±0.03	±0.02	±1.05	±0.01	±0.03	
Double	0.91	0.98	1.07	1.16	0.93	7.37*
(IV-V)	±0.03	±0.02	±0.04	±0.06	±0.02	
Triple	0.91	1.06	1.15	1.25	0.87	
(III-V)	±0.03	±0.01	±0.04	±0.04	±0.03	

F_2-ratio=1.7442** n_2=2 $P_1 < 0.01$ ** Non significant

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

X_1, X_2, X3, X4 and X_5 are the mean values of the total DNA content (µg/mg) in the fat body in control, 10, 20, 30 and 40% phytojuvenoid concentration respectively.

Two-way ANOVA indicates that variation in the phytojuvenoid concentration significantly ($P_1 < 0.01$) influenced the total DNA content while number of larval treatment has no significant influence on the total DNA content in the fat body of larvae at the initial stage of spinning. The Post–hoc test (table-1b) indicates significant group difference in the total DNA content in the fat body of larvae at the initial stage of spinning in between control and 10% and 10 and 30% in single treated larvae. In the double treated larvae significant group difference in the total DNA content was observed in all the group combinations except in between control and 10%, control and 40% and 10 and 40% phytojuvenoid concentration. In the triple treated larvae significant group difference in the total DNA content was noticed in between all the group combinations except in control and 40% phytojuvenoid concentration.

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

Mean difference in between groups	Stage of treatment		
	Single	**Double**	**Triple**
X_1~X_2	0.02	0.07	*0.15
X_1~X_3	0.07	*0.16	*0.24
X_1~X_4	*0.14	*0.25	*0.34
X_1~X_5	0.07	0.02	0.04
X_2~X_3	0.05	*0.09	*0.09
X_2~X_4	*0.12	*0.18	*0.19
X_2~X_5	0.05	0.05	*0.19
X_3~X_4	0.07	*0.09	*0.10
X_3~X_5	0.00	*0.14	*0.23
X_4~X_5	0.07	*0.28	*0.38

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

$= 6.10\sqrt{\frac{0.004}{6}}$

$= 0.08$

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 DNA content in the fat body of *Bombyx mori* larvae in control,10, 20, 30 and 40 per cent phytojuvenoid concentration respectively.

3.2. Total DNA content in the fat body of larvae at the final stage of spinning

The data presented in the table-2a that the phytojuvenoid concentration and number of larval treatment influenced the total DNA 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, total DNA content in the fat body of larvae at the final stage of spinning increased gradually and reached to the maximum level of 0.98± 0.02 µg/mg in case of triple treated larvae with 30% phytojuvenoid concentration. In case of larval treatment with 40% phytojuvenoid concentration, the total DNA content in the fat body of larvae at the final stage of spinning increased in single treated larvae but further increase in the number of larval treatment caused decline in the total DNA content in the fat body of larvae at the final stage of spinning which reached to the minimum level of 0.81±0.05 µg/mg in triple treated larvae. The trend of increase in the total DNA content in the fat body of larvae 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.

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

Stage of treatment (Larval instar)	Phytojuvenoid concentration (%)					
	Control X_1	**10 X_2**	**20 X_3**	**30 X_4**	**40 X_5**	**F_1-ratio n_1 =4**
Single	0.85	0.87	0.88	0.92	0.87	
(V)	±0.04	±0.07	±0.06	±0.04	±0.02	
Double	0.85	0.90	0.91	0.95	0.83	8.82*
(IV-V)	±0.04	±0.03	±0.01	±0.02	±0.03	
Triple	0.85	0.93	0.94	0.98	0.81	
(III-V)	±0.04	±0.03	±0.06	±0.02	±0.05	

F_2-ratio = 1.0000** 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 DNA content (µg/mg) in the fat body in control, 10, 20, 30 and 40 % phytojuvenoid concentration Respectively.

Two-way ANOVA indicates that variation in phytojuvenoid concentration significantly ($P_1 < 0.01$) influenced on the total DNA content but the number of larval treatment did not cause significant influence on the total DNA content in the fat body of larvae at final stage of spinning. The Post–hoc test (table-2b) shows significant group difference in the total DNA content in the fat body of larvae at the final stage of spinning in between control and 30% in the single treated larvae and the control and 20%, control and 30%, 10 and 40%, 20 and 40 %, and 30 and 40% in the double treated larvae. In triple treated larvae significant group difference in the total DNA content in between control and 10%, control and 20%, control and 30%, 10 and 40%, 20 and 40% and 30 and 40% phytojuvenoid concentrations was observed.

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

Mean difference in between groups	stage of treatment		
	Single	Double	Triple
X_1~X_2	0.02	0.05	*0.08
X_1~X_3	0.03	*0.06	*0.09
X_1~X_4	*0.07	*0.10	*0.13
X_1~X_5	0.02	0.02	0.04
X_2~X_3	0.01	0.01	0.01
X_2~X_4	0.05	0.05	0.05
X_2~X_5	0.00	*0.07	*0.12
X_3~X_4	0.04	0.04	0.04
X_3~X_5	0.01	*0.08	*0.13
X_4~X_5	0.05	*0.12	*0.17

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

$= 6.10\sqrt{\frac{0.001}{6}}$

$= 0.06$

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 the total DNA content in the fat body of *Bombyx mori* larvae in control, 10, 20, 30 and 40 per cent phytojuvenoid concentration respectively.

4. Discussion

The level of DNA content in the fat body of larvae at the initial and final stage of spinning was influenced due to variation in the number of larval treatment and phytojuvenoid concentration in *Bombyx mori* larvae. The DNA content in the fat body increased with the increasing number of larval treatment from single to triple in 10, 20 and 30% phytojuvenoid concentration while in 40% phytojuvenoid concentration, the DNA content reached to the minimum level in case of triple treated larvae. An increase in the DNA content took place during the development of *Pharate* adult [14]. Higher DNA/RNA ratio was noticed in the larval muscles, cuticle and the fat body of silkworm [15]. An initial increase in the level of RNA and DNA ratio was noticed in the fat body during early days of vth instar larvae of *Philosamia cyntheia ricini* [16]. It is well known that the resistance to biotic and abiotic constraints is governed by polygene with complex inheritance patterns and with lot of environmental influences [17]. New tools like molecular markers can be effectively applied with conventional breeding strategies and the genes for the resistance can be discovered [18]. Methoprene and fenoxycarb enhanced the fibroin and sericine which activate the silk gland to synthesize more DNA and RNA [19]. Varietal characterization of four morphologically different strains of eri silkworm (*Samia cynthia ricini*) i.e. yellow (Y), yellow spotted (Ys), green (G) and green spotted (Gs), through biochemical and molecular techniques, the genotypes belonging to different groups will constitute promising parents for hybridization in silk improvement programme [20] and the Jaccard similarity matrix ranged from 0.72 to 1.00, indicating low genetic variation among the Jatropha accessions. This maybe mainly attributed to the fact that *J. curcas* is an introduced plant species in Malaysia and comes from the same source or due to the low number of markers used [21].

The variation in the phytojuvenoid concentration and number of larval treatment influenced the DNA content in fat body tissues of larvae in the initial and final stage of spinning. Thus, it is concluded that the phytojuvenoid enhanced the larval duration and the larvae consume more mulberry leaves, leading to an increase in the fibroin and sricine present in the food causing stimulatory effects on the silk gland to synthesize more DNA through the replication at low concentration of phytojuvenoid. The higher concentration of the phytojuvenoid seems to cause stress response causing decline in the nucleic acids content.

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