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The Effect of Phytojuvenoid on Larvae of the Multivoltine Mulberry Silkworm (*Bombyx mori* Linn.)

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Abstract: The topical application of phytojuvenoid on *Bombyx mori* larvae has been proved to be of biotechnological significance in the sericulture industry. Variation in the phytojuvenoid concentration significantly ($P_1 < 0.01$) influenced the total protein level in the silk gland of larvae at the initial stage of spinning. The maximum level of total protein content was recorded in case of 30% phytojuvenoid concentration at triple treated (III^{th} - IV^{th}) larvae while it was lowest in 40% phytojuvenoid concentration at triple treated larvae at the initial 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.

Key words: Protein Content · Silk Gland · Initial stage of spinning

INTRODUCTION

Sericulture, or silk farming, is the rearing of silkworms for the production of silk. Although there are several commercial species of silkworms, *Bombyx mori* is the most widely used and intensively studied silkworm. Nistari is a resistant variety of multivoltine mulberry silkworm *B. mori* which contributes up to a great extent in the commercial production of cocoon. In order to increase, the production of silk, efforts have been made to study effect of ecological factor [1], photoperiod [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]. The phytoecdysteroid has been noticed to influence the development, growth, silk producing and reproductive potential of *B. mori* [6-10]. The juvenile hormone analogue also has been noticed to influence the reproductive potential and biochemical constituents of *B. mori* [11, 12]. 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 [13]. 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* [14]. 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 [15]. In the present study *Pinus longifolia* was taken for experiment due to its good availability and containing juvenile compound.

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 5cm) under the ideal rearing conditions [16] in the silkworm laboratory, Department of Zoology D.D.U. Gorakhpur University, Gorakhpur. The temperature and relative humidity were maintained at 26±1°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*. [16]. 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°C and 80±5% RH in 12 hour / day dim light condition. After four

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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 number of cocoons was obtained from the silkworm larvae reared in our laboratory.

Design of Experiment: For extraction of phytojuvenoid the needle of *Pinus* were collected, washed thoroughly with distilled water and dried in incubator at 37°C. The dried materials were powdered separately with the help of mechanical device. Further, 50 g powder was subjected to extraction separately through soxlet apparatus with 250 ml distilled water for 40 h. After 40 h of extraction a little amount of concentrated solution of plant extract was obtained. The concentrated solution was dried and 6.45 g material was obtained in powdered form. The dried powder thus obtained, was dissolved in distilled water as 5 g 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.

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.

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.

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 instars of *B. mori* larvae on certain biochemical constituents like total protein contents in the silk gland of larvae at the initial stage of spinning, following methods were adopted:

Total Protein Content: For the estimation of total protein content from silk gland of larvae, the fifth instar larvae were dissected in distilled water at the initial stage of spinning and 1.0 g tissue of silk gland was taken and protein content was estimated according to Lowery *et al.* [17] as modified by Singh and Agarwal [1989]. In above tissue 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 ciocalten 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.

RESULTS

It is clear from the data given in the Table-A that the phytojuvenoid concentration and number of larval treatment influenced the total protein content in the silk gland of larvae at the initial stage of spinning. With the increasing number of larval treatment with 10, 20 and 30% phytojuvenoid concentration, the total protein content in the silk gland of larvae at the initial stage of spinning increased gradually and reached to the maximum level of 12.45±0.25 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 silk gland 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 protein content in the silk gland of larvae at the initial stage of spinning which reached to the minimum level of 9.75±0.21 µg/mg in triple treated larvae. The trend of increase in the total protein content in the silk gland 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.Two-way ANOVA indicates that variation in phytojuvenoid concentration significantly ($P_1 < 0.01$) influenced the total protein content in the silk gland of larvae at the initial stage of spinning. The Post–hoc test (Table- B) shows significant group difference in the total protein content in the silk gland in single treated larvae in between all group combinations except in control and 10%, control and 40% and 10 and 40% phytojuvenoid concentration. In the double treated larvae significant group difference in the total protein content was noticed in between all group combinations except in control and 40% and in the triple treated larvae significant group difference in the total protein content was recorded in between all group combinations except in control and 10% and 10 and 20% phytojuvenoid concentration.

DISSCUSION

The change in the phytojuvenoid concentration and number of larval treatment of *Bombyx mori* influenced the total protein content in the silk gland of *B. mori* at the initial of spinning. With the increase in number of larval treatment from single to triple, the protein content in the silk gland at the initial 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 most rapid protein metabolism was noticed in the silk gland of silkworm [19] and the rate of increase in the total protein content in the haemolymph of *B. mori* was rapid in the larvae than in any other stage [20]. Thirty per cent of the silk protein mass, in *Rhynchosciara americana* was derived from the free amino acids and protein of the haemolymph while the rest was synthesized by the salivary gland during the

Table A: Effect of phytojuvenoid treatment on the total protein content (µg/mg) in the silk gland of *Bombyx mori* larvae at the initial stage of spinning

	Phytojuvenoid concentration (%)					
Stage of treatment	Control	10	20	30	40	F_1-ratio
(Larval instar)	X_1	X_2	X_3	X_4	X_5	n_1 =4
Single	10.60	10.73	11.27	11.98	10.79	
(V)	±0.23	±0.12	±0.11	±0.13	±0.15	
Double	10.60	10.98	11.46	12.18	10.57	13.47*
(IV-V)	±0.23	±0.15	±0.14	±0.23	±0.12	
Triple	10.60	11.26	11.80	12.45	9.75	
(III-V)	±0.23	±0.13	±0.12	±0.25	±0.21	

F_2-ratio = 0.1160** 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 silk gland in control, 10, 20, 30 and 40% phytojuvenoid concentration respectively.

Table B: Post - hoc test showing effect of phytojuvenoid treatment on the total protein content (µg/mg) in the silk gland 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	0.13	*0.38	0.34
X_1~X_3	*0.67	*0.86	*1.20
X_1~X_4	*1.38	*1.58	*1.85
X_1~X_5	0.19	0.03	*0.85
X_2~X_3	*0.54	*0.48	0.54
X_2~X_4	*1.25	*1.20	*1.19
X_2~X_5	0.06	*0.41	*1.51
X_3~X_4	*0.71	*0.72	*0.65
X_3~X_5	*0.48	*0.89	*2.05
X_4~X_5	*1.19	*1.63	*2.70

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

$= 6.10v \frac{0.121}{6}$

$= 0.35$

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 the mean values of total protein content in the silk gland of *Bombyx mori* larvae in control, 10, 20, 30 and 40 per cent phytojuvenoid concentration respectively

spinning process [21]. The silk protein secretion is controlled by the brain hormones, secreted by some of the median neurosecretory cells while starvation caused inhibition in the protein synthesis in *B. mori* and silk gland started synthesis of silk protein at about 10 days of embryonic life and this continue till the beginning of the spinning of cocoon [22]. In 4th and 5th instar larvae, 2-3% and 97-98% of assimilated protein was utilized respectively in the silk protein synthesis [23]. Both the size of gland and its RNA content explains the extra production of silk [24]. Special food intake after the fourth ecdysis, the exponential growth of the silk gland is accompanied by a parallel increase in the cellular content of DNA, RNA and proteins [25] while treatment of the silkworm larvae in 3500 gauss magnetic field caused an increase in the silk protein [26]. The total protein content, present in the silk gland of *B. mori* declined notably with the increasing cold storage treatment of eggs but the pre refrigeration period has no considerable impact on the total protein level in the silk gland of *B. mori* [27]. Methoprene and fenoxycarb significantly enhanced the fibroin and sericin of the silk ([28]. A bivoltine silkworm hybrid, KA x NB4D2 was treated with the juvenoid R394 (Ethyl-9 cyclohexyl-3,7-dimethyl- 2,4-nonadienoate) at a dose of 0.039 nl/larva at 24, 48, 72 and 96 h of 5th instar, the result indicated that the highest content of protein in the silk gland was in the larvae treated at 72 h. [29] and JHA isolated from Bemchi (*Psoralea corylifolia*) significantly increased the total protein content in the posterior silk gland of *B. mori* [30]. Under the influence of ultrasound, the protein metabolism is stimulated and achieved greater turnover of silk proteins in *B. mori* [31]. The total protein content in the silk gland of *B. 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]. The total protein content in the silk gland of *B. mori* increased significantly with increasing the phytojuvenoid concentration from 10- 30% but in 40% it was decreased and the maximum was observed in 30% at triple treated larvae [33].

As discussed, a number of factors influenced the utilization and synthesis of protein and the level of total protein content in silk gland tissue 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 silk gland of *B. mori* larvae. But 40% concentration generated stress response, causing general decline in the rate of protein synthesis.

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