International Journal of Entomology Research
ISSN: 2455-4758; Impact Factor: RJIF 5.24
www.entomologyjournals.com
Volume 2; Issue 2; March 2017; Page No. 31-35

Effect of Phytojuvenoid hormone on biochemical performance of 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. Total RNA content in the silk gland of larvae at the initial and final stage of spinning significantly influenced due to variation in the phytojuvenoid concentration and number of larval treatment. The total RNA content in the silk gland increased with the increasing number of larval treatment up to triple treatment of larvae in 10, 20 and 30% phytojuvenoid concentration which reached to the maximum level of 1.38 ±0.05 µg/mg in initial and 1.08±0.09 µg/mg in the 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 may also be helpful to device improvement in the rearing of *Bombyx mori* on industrial scale to boost up the production of silk.

Keywords: phytojuvenoid, RNA content, silk gland, larvae, *bombyx mori*

1. 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 (*Bombyx 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, 7, 8]. The juvenile hormone analogue also has been noticed to influence the reproductive potential and biochemical constituents of *Bombyx mori* [9, 10]. 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 [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]. In the present study *Pinus longifolia* was taken for experiment due to its good availability and containing juvenile compound.

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) [14]. 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 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.

2.2 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.

2.3 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.

2.4 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 silk gland of larvae at the initial stage of spinning, following methods were adopted:

2.5 Nucleic Acid
The estimation of RNA was performed according to Schneider (1957) [15] by using the orcinol regents. For measuring the RNA content took 1.0 gm silk gland from the V^{th} instar larval stage and pupae. The tissues, thus obtained were homogenized separately in 5% TCA and centrifuged at 5000 rpm for 20 minutes. Took one ml of supernatant and added 2ml of distilled water and 3.0 ml of orcinol regent (1 gm orcinol, 100ml concentrated HCl and 0.5 gm Ferric Chloride).The reaction mixture was kept in boiling water-bath for 20 minutes. The greenish blue-colour developed was measured at 660nm. Standard curves were drawn using different concentrations of yeast RNA as standard. RNA has been expressed as µg/mg tissue. Six replicates of each 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 RNA content in the silk gland of larvae at the initial stage of spinning
The data given in table1a clearly indicates that the phytojuvenoid concentration and number of larval treatment influenced the total RNA 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 RNA content in the silk gland of larvae at the initial stage of spinning increased gradually and reached to the highest level of 1.38±0.05 µg/mg in case of triple treated larvae with 30% phytojuvenoid concentration. In case of larval treatment with 40% phytojuvenoid concentration, the total RNA 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 RNA content in the silk gland of larvae at the initial stage of spinning which reached to the minimum level of 1.05±0.02 µg/mg in triple treated larvae. The trend of increase in the total RNA content in the silk gland of larvae at the initial stage of spinning was almost of same pattern 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 ($P_1 < 0.05$) influenced the total RNA content but the number of larval treatment did not cause significant influence on the total RNA content in the silk gland of larvae at the initial stage of spinning. The Post–hoc test (table-1b) shows significant group difference in the total RNA content in the silk gland of larvae at the initial stage of spinning in between control and 20%, control and 30%, control and 40%, 10 and 30% and 20 and 30 % in single treated larvae. In case of double treatment of larvae significant group difference was noticed in between all the group combinations except in control and 10%, control and 40% and 10 and 20%. In triple treatment of larvae significant group difference was recorded in between all group combinations except in 10 and 20% phytojuvenoid concentration.

3.2 Total RNA content in the silk gland of larvae at the final stage of spinning
The data obtained from table-2a clearly indicates that the phytojuvenoid concentration and number of larval treatment influenced the total RNA 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 RNA content in the haemolymph of larvae at the final stage of spinning increased gradually and reached to the maximum level of 1.08±0.09 µg/mg in case of triple treated larvae with 30% phytojuvenoid concentration. In case of the larval treatment with 40% phytojuvenoid concentration, the total RNA content in the haemolymph 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 RNA content in the haemolymph of larvae at the final stage of spinning which reached to the minimum level of 0.88±0.04µg/mg in triple treated larvae. The trend of increase in the total RNA content in the haemolymph of 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 RNA content but the number of larval treatment did not cause significant influence on the total RNA content in the silk gland at the final stage of spinning. The Post–hoc test (table-2b) shows significant group difference in the total RNA content in the silk gland at final stage of spinning in between control and 30%, 10 and 30% and 30 and 40% in single treated larvae. In the double treated larvae significant group difference in the total RNA content was noticed in between all the group combinations except in control and 40% and 10 and 20%. In triple treated larvae significant group difference in the total RNA content was recorded in between allthe group the combinations except in between control and 40%, 10 and 20% and 20 and 30% phytojuvenoid concentration.

Table 1a: Effect of phytojuvenoid treatment on the total RNA content (µg/mg) in the silk gland of *Bombyx mori* larvae at the initial stage of spinning.

Stage of treatment	Control	Phytojuvenoid concentration (%)				F1-ratio
		10	20	30	40	
(Larval instar)	X1	X2	X3	X4	X5	n1 =4
Single (V)	1.18 ±0.03	1.21 ±0.04	1.24 ±0.08	1.32 ±0.02	1.26 ±0.06	5.28*
Double (IV-V)	1.18 ±0.03	1.24 ±0.05	1.27 ±0.03	1.35 ±0.07	1.15 ±0.04	
Triple (III-V)	1.18 ± 0.03	1.27 ±0.09	1.30 ±0.07	1.38 ±0.05	1.05 ±0.02	

F_2-ratio = 0.0136** n_2=2

$P_1 < 0.05$ **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 RNA content (µg/mg) in the silk gland in control, 10, 20, 30 and 40 % phytojuvenoid concentration respectively.

Table 1b: Post - hoc test showing effect of phytojuvenoid treatment on the total RNA content 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
X1~X2	0.03	0.06	*0.11
X1~X3	*1.06	*0.09	*0.12
X1~X4	*1.14	*0.17	*0.20
X1~X5	*0.08	0.03	*0.13
X2~X3	0.03	0.03	0.03
X2~X4	*0.11	*0.11	*0.11
X2~X5	0.05	*0.09	*0.22
X3~X4	*0.08	*0.08	*0.08
X3~X5	0.02	*0.12	*0.25
X4~X5	0.06	*0.20	*0.32

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

$= 6.10\sqrt{\frac{0.003}{6}}$

$= 0.07$

Where;

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, X4 and X_5 are the mean values of the total RNA content in the silk gland of *Bombyx mori* larvae in control,10, 20, 30 and 40 per cent phytojuvenoid concentration respectively.

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

Stage of treatment	Control	Phytojuvenoid concentration (%)				F1-ratio
		10	20	30	40	
(Larval instar)	X1	X2	X3	X4	X5	n1 =4
Single (V)	0.93 ±0.03	0.96 ±0.05	0.98 ±0.08	1.02 ±0.07	0.95 ±0.02	11.58*
Double (IV-V)	0.93 ±0.03	0.99 ±0.08	1.01 ±0.06	1.05 ±0.04	0.91 ±0.03	
Triple (III-V)	0.93 ±0.03	1.02 ±0.02	1.04 ±0.05	1.08 ±0.09	0.88 ±0.04	

F_2-ratio =0.7295** 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 RNA content (µg/mg) in the silk gland in control, 10, 20, 30 and 40 % phytojuvenoid concentration respectively.

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

Mean difference in between groups	stage of treatment		
	Single	Double	Triple
X1~X2	0.03	*0.06	*0.09
X1~X3	0.05	*0.08	*0.11
X1~X4	*0.09	*0.12	*0.15
X1~X5	0.02	0.02	0.05
X2~X3	0.02	0.02	0.02
X2~X4	*0.06	*0.06	*0.06
X2~X5	0.01	*0.08	*0.14
X3~X4	0.04	*0.06	0.04
X3~X5	0.03	*0.10	*0.16
X4~X5	*0.13	*0.14	*0.20

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

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

$= 0.06$

Where;

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, X4 and X_5 are the mean values of the total RNA content in the silk gland of *Bombyx mori* larvae in control,10, 20, 30 and 40 per cent phytojuvenoid concentration respectively.

4. Discussion

The level of RNA content in the silk gland of *Bombyx mori* was influenced due to variation in the phytojuvenoid concentration and number of larval treatment at the initial and final stage of spinning. The maximum level of RNA content at the initial and final stage of spinning was noticed in case of 30% phytojuvenoid concentration with triple treatment of larvae while minimum at the initial and final stage of spinning was noticed in case of 40% phytojuvenoid concentration(Table 1a and 2a). The wet weight and amount of RNA and protein of silk gland in *Bombyx mori*, during spinning period, decreased while during thermal acclimation, the amount of RNA in the silk gland decreased sharply [16]. Total protein content and RNA: DNA ratio of silk gland of eri silkworm on the onset of v^{th} instar, in comparison to their levels at the end of v^{th} instar, was probably in response to the resumption of exponential growth of silk gland during the v^{th} instar [17, 18]. An increase in the RNA content of silk gland in the early stage of the v^{th} instar larvae in *Antheraea pernyi* has also been noticed [19]. The nucleic acid content (RNA and DNA) was increased in silk gland of larvae in the magnetic field up to 3500 gauss [20]. Methoprene and fenoxycarb enhanced the fibroin and sericine which activate the silk gland to synthesize more DNA and RNA [21]. 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 [22]. 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 [23].

Thus, variation in the phytojuvenoid concentration and number of larval treatment influenced the DNA and RNA content in different tissues of larvae in the initial and final stage of spinning. 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 and RNA through the replication and transcription at low concentration of phytojuvenoid. The higher concentration of the phytojuvenoid seems to cause stress response causing decline in the nucleic acids content.

5. References

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