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# Influence of Bioactive Phytojuvenoid on the silk producing Potential of Multivoltine Mulberry Silkworm (Bombyx mori Linn.)

**Roli Srivastava, V.B. Upadhyay**

**ABSTRACT**
The application of phytojuvenoid on *Bombyx mori* larvae has been proved to be significance in the sericulture industry. The thickness of cocoon shell increased with the increasing number of larval treatment from single to triple in 10, 20 and 30% phytojuvenoid concentration and the thickness of cocoon shell (in mm) was highest (0.836±0.05) in 30% phytojuvenoid concentration - triple treated larvae. % shell ratio of *Bombyx mori* was significantly influenced due to variation in the phytojuvenoid concentration. Maximum shell ratio (15.50±0.04) was recorded in case of 30% phytojuvenoid concentration at triple treated larvae. But at higher concentration (40%) both are decreased. A control set was always maintained with each set of experiment. The phytojuvenoid influences the level of protein in the larvae and caused beneficial effect on the productivity of cocoon and the life pattern of silkworm.

**Keywords:** Phytojuvenoid, silk producing Potential, Larvae, *Bombyx mori*, larval treatment.

## 1. Introduction

The Indian sericulture industry depends mainly on multivoltine races. India is now on the threshold of vitalizing the silk industry with greater emphasis on quality as well as quantity. The continued efforts for the improvement of cocoon characters of domesticated silkworm were aimed at increased quality silk production. The main object of silkworm rearing is to produce qualitatively and quantitatively superior cocoons which in turn will have a direct bearing on the raw silk production. 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], relative humidity [3], photoperiod [4], artificial diet [5], X-rays [6] etc on the performance of silkworm. The Magnetization of eggs influences silk producing potential [7, 8] and incubation period of eggs [9, 10] and larval performance [11]. The phytoecdysteroid has been noticed to influence the development, growth, silk producing and reproductive potential of *B. mori* [12-16]. The juvenile hormone analogue also has been noticed to influence the reproductive potential of *Bombyx mori* [17]. Ever since the discovery of juvenile hormone by Carroll Williams (1956) from *Hyalophora cecropia*, a new approach has captured worldwide attention in the control of growth and development in insects. The process of growth and development, in insect, 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 pattern of insect development can be regulated artificially by the mimics or analogues of these circulating hormones. The JH analogues and mimics have been reported to have some hormonal influence on the growth of *Bombyx mori* and cocoon production. The exogenous application of JH delays larval maturation and increases the silk yield. The indigenously available products showing JH activity, on the growth of silkworm and silk yield, function as strategic defense tool for the plants against insects by acting as either the feeding deterrent or through developmental disruption in insects.

The response of silkworm to very small quantities of these 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 [18]. 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* [19, 20]. 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 [21]. Some plants like *Pinus longifolia, Abies balsamea, Psoralea corylifolia* and *Azadirachta indica* act on *Bombyx mori* larvae as bioactive juvenoid compounds [22]. In the present study *Pinus* was taken for experiment due to its good availability. To observe the influence of bioactive phytojuvenoid on the performance of *Bombyx mori*, the experiments were performed with different concentrations of phytojuvenoid with respect to the treatment of 3$^{rd}$, 4$^{th}$ and 5$^{th}$ instar larvae.

**1.1 Seed cocoons:** The seed cocoons (pupa enclosed in silken case) of multivoltine mulberry silkworm *(Bombyx mori* nistari), a native of West Bengal in India, 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 [23] 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 moths emerged generally in the morning at around 4 A.M. The tray, in which seed cocoons were kept, was suddenly illuminated by light in the morning at 4 O'clock on 9$^{th}$ and 10$^{th}$ day of spinning.
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*. and Jolly [23, 24].
**1.2 Copulation:** Moths have a tendency to pair immediately after emergence, therefore, the female moths required to copulate with the male moths, were allowed their mates for copulation. 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 by holding the female moths between the thumb and middle finger gently and pushing the male away by the fore finger. The male moths were discarded while the female moths were allowed to egg laying.

**1.3 Oviposition:** The gravid females laid eggs on the sheet of paper in the dark condition at 26 ± 1 $^0$C and 80 ± 5% RH. The egg laying moths were covered by open plastic cellules to prevent intermixing of egg masses deposited by different moths. After 24 hours of egg laying, the female moths were individually examined for their disease freeness. The females were crushed individually in mortar with pestles and blood smears were examined by microscope under 15 x 45 magnifications for the detection of bacterial and protozoan pathogens.

**1.4 Incubation of eggs and hatching:** The disease free layings (D.F.L's), thus prepared, were treated with 2% formaline for 15 minute to increase the adhesiveness of eggs on the paper sheet and surface disinfection. Thereafter, the egg sheets with eggs laid on were thoroughly washed with running water to remove formaline and the eggs were dried in shade. The dried eggs were transferred to the incubator for hatching.

**1.5 Rearing of Larvae:** After two consecutive days of hatching, the silkworm larvae were collected with the help of feather of birds and reared to maintain a stock culture in the silkworm laboratory at 26 ± 1$^0$C and 80 ± 5% RH and 12 ± 1 hours light a day. Four feedings of the small pieces of fresh and clean leaves of *Morus alba* were given to the larvae and care was taken that food always remained in excess 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. Now small mountages were provided to the ripe worms. The ripe worms soon begin the mounting which was completed within three days. Thus, sufficient number of cocoons was obtained from the silkworm larvae reared in our laboratory.

**2. 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 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.

**2.2 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.

**2.3 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.
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. All the data obtained were analyzed statistically by two-way ANOVA and Post- hoc test.

**2.4 Thickness of cocoon shell:** For determining the shell thickness, the cocoon was cut length wise and the thickness of shell was measured with the help of screw gauze. The thickness of 30 cocoons shell (three batches of 10 cocoon shells in each batch) was recorded for each replicate. Three replicates of each experiment were made.

**2.5 Shell ratio:** To estimate the shell ratio, the weight of 20 cocoons and 20 shells from each batch were recorded separately on the fifth day of spinning. Three replicates of each experiment were made. The shell ratio percentage was calculated based on at least 20 cocoons and 20 cocoon shells taken at random from the good cocoon lot.

$$\text{Shell ratio (\%)} = \frac{\text{Weight of cocoon shells}}{\text{Weight of cocoon}} \times 100$$

**(Ghosh, 1987)**

**3. Results**

**3.1 Thickness of cocoon shell –** The data given in table-1a clearly indicates that the phytojuvenoid concentration and the number of larval treatment influenced the thickness of cocoon shell. With the increasing number of larval treatment with 10, 20 and 30% phytojuvenoid concentration, the thickness of cocoon shell increased gradually and reached to the maximum level of 0.836± 0.05 mm in case of triple treated larvae with 30% phytojuvenoid concentration. In case of larval treatment with 40% phytojuvenoid concentration, the thickness of cocoon shell increased in single treated larvae but further increase in the number of the larval treatment caused decline in the thickness of cocoon shell which reached to the minimum level of 0.758±0.01 mm in triple treated larvae. The trend of increase in the thickness of cocoon shell 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 phytojuvenoid concentration and number of larval treatment did not cause significant influence on the thickness of cocoon shell of *Bombyx mori*. The Post– hoc test (table-1b) indicates that there was no significant group difference in the thickness of cocoon shell in any of the group combination.

**Table 1a:** Effect of phytojuvenoid treatment on the thickness of cocoon shell (mm) of *Bombyx mori*.

| | | 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** | **0.77** | **0.784** | **0.792** | **0.796** | **0.798** | |
| **(V)** | ±0.06 | ±0.05 | ±0.03 | ±0.02 | ±0.04 | |
| **Double** | **0.774** | **0.769** | **0.804** | **0.816** | **0.776** | **3.66*** |
| **(IV-V)** | ±0.06 | ±0.04 | ±0.01 | ±0.03 | ±0.03 | |
| **Triple** | **0.774** | **0.816** | **0.820** | **0.836** | **0.758** | |
| **(III-V)** | ±0.06 | ±0.02 | ±0.03 | ±0.05 | ±0.01 | |

**$F_2$-ratio =0.69*** **$n_2$ = 2**

*** Non significant**

Each value represents mean ± S.E. of three replicates $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are the mean values of thickness of cocoon shell (mm) in control, 10, 20, 30 and 40 % phytojuvenoid concentration respectively.

**3.2 Shell ratio –** The data presented in table-2a shows that variation in the phytojuvenoid concentration and the number of larval treatment influenced the shell ratio. With the increasing number of larval treatment with 10, 20 and 30% phytojuvenoid concentration the shell ratio increased gradually and reached to the maximum level of 15.50±0.04 in case of triple treated larvae with 30% phytojuvenoid concentration. In case of larval treatment with 40% phytojuvenoid concentration the shell ratio increased in single treated larvae but further increase in the number of larval treatment caused decline in the shell ratio which reached to the minimum level of 10.82±0.09% in triple treated larvae. The trend of increase in the shell ratio 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 ($P_1 < 0.05$) influenced on the shell ratio while number of larval treatment has no significant influence on it. The Post–hoc test (table-2b) shows significant group difference in the shell ratio in between control and 20%, control and 30% and 10 and 30 % in case of single treated larvae. In the double treated larvae group difference in the shell ratio in between control and 20%, control and 30%. 20 and 40%, and 30 and 40 % was noticed. In the triple treated larvae the significant group difference in the shell ratio were recorded in between all group combinations except in between 10 and 20%, 10 and 30% and

20 and 30% phytojuvenoid concentration.

**Table 1b:** Post - hoc test showing effect of phytojuvenoid treatment on the thickness of cocoon shell (mm) in *Bombyx mori*

| Mean difference in between groups | stage of treatment | | |
|---|---|---|---|
| | Single | Double | Triple |
| $X_1$~$X_2$ | **0.010** | **0.022** | **0.042** |
| $X_1$~$X_3$ | **0.018** | **0.030** | **0.046** |
| $X_1$~$X_4$ | **0.022** | **0.002** | **0.062** |
| $X_1$~$X_5$ | **0.024** | **0.002** | **0.016** |
| $X_1$~$X_3$ | **0.002** | **0.008** | **0.004** |
| $X_1$~$X_4$ | **0.010** | **0.020** | **0.020** |
| $X_1$~$X_5$ | **0.010** | **0.020** | **0.020** |
| $X_1$~$X_4$ | **0.004** | **0.012** | **0.016** |
| $X_1$~$X_5$ | **0.012** | **0.028** | **0.062** |
| $X_1$~$X_5$ | **0.002** | **0.040** | **0.078** |

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

$= 5.05\sqrt{\frac{0.007}{3}}$

$= 0.149$

**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 cocoon shell thickness (mm) in *Bombyx mori* in control, 10, 20, 30 and 40 per cent phytojuvenoid concentration respectively.

**Table 2a:** Effect of phytojuvenoid treatment on the shell ratio (%) in *Bombyx mori*.

| Phytojuvenoid concentration (%) | | | | | | |
|---|---|---|---|---|---|---|
| **Stage of treatment** | **Control** | | | | | **$F_1$-ratio** |
| **(Larval instar)** | **$X_1$** | **$X_2$** | **$X_3$** | **$X_4$** | **$X_5$** | **$n_1$ =4** |
| **Single** | 12.52 | 13.15 | 13.91 | 14.67 | 13.65 | |
| (V) | ±0.01 | ±0.05 | ±0.04 | ±0.07 | ±0.09 | |
| **Double** | 12.52 | 13.68 | 14.56 | 14.98 | 12.45 | 6.25* |
| (IV-V) | ±0.07 | ±0.03 | 0.04 | ±0.02 | ±0.01 | |
| **Triple** | 12.52 | 14.20 | 14.95 | 15.50 | 10.82 | |
| (III-V) | ±0.07 | ±0.11 | ±0.09 | ±0.04 | ±0.09 | |

**$F_2$-ratio = 0.0064**** **$n_2$ = 2**

***$P_1$< 0.01** **** Non significant**

Each value represents mean ± S.E. of three replicates $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are the mean values of shell ratio (%) in control, 10, 20, 30 and 40 % phytojuvenoid concentration respectively.

**Table 2b:** Post - hoc test showing effect of phytojuvenoid treatment on the shell ratio ( %) in *Bombyx mori*.

| Mean difference in between groups | stage of treatment | | |
|---|---|---|---|
| | Single | Double | Triple |
| $X_1$~$X_2$ | 0.63 | 1.16 | *1.68 |
| $X_1$~$X_3$ | *1.39 | *2.04 | *2.43 |
| $X_1$~$X_4$ | *2.15 | *2.46 | *3.48 |
| $X_1$~$X_5$ | 1.13 | 0.07 | *1.70 |
| $X_1$~$X_3$ | 0.76 | 0.88 | 0.75 |
| $X_1$~$X_4$ | *1.52 | 1.30 | 1.30 |

| | | | |
|---|---|---|---|
| $X_1 \sim X_5$ | 0.50 | 1.22 | *3.38 |
| $X_1 \sim X_4$ | 0.76 | 0.42 | 0.55 |
| $X_1 \sim X_5$ | 0.25 | *2.11 | *4.13 |
| $X_1 \sim X_5$ | 1.02 | *2.53 | *4.68 |

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

$= 5.05\sqrt{\frac{0.658}{3}}$

$= 1.39$

**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 shell ratio (%) in *Bombyx mori* in control, 10, 20, 30 and 40 per cent phytojuvenoid concentration respectively.

## 4. Discussion

**4.1 Thickness of cocoon shell:** The thickness of cocoon shell was influenced due to variations in the phytojuvenoid concentration and the number of larval treatments of *Bombyx mori* larvae. With the increasing number of larval treatment in 10, 20 and 30% phytojuvenoid concentration, the thickness of cocoon shell increased and reached to the maximum level in case of 30% phytojuvenoid concentration at triple treated larvae. The ellipsoidal cocoon has the least thickness at its two ends [26, 27]. The spinneret positions in three races of *Bombyx mori* viz., N140×C140, N124×C124 and Okusa X Kojiki, has been noticed to affect directly the unevenness of a cocoon shell in thickness [28]. A silkworm larva can built consecutive two, three or four cocoons, which are thinner than a normal compact cocoon for the limited raw materials in its body [29]. It may be referred that the treatment of larvae with low phytojuvenoid concentration caused an increase in the cellular activity of silk gland resulting in the increased thickness of cocoon shell; whereas, higher concentration may cause stress response, lessening the thickness of cocoon.

**4.2 Shell ratio:** The variation in the phytojuvenoid concentration and number of larval treatment of *Bombyx mori* influenced the shell ratio. The maximum level of shell ratio was noticed at 30% phytojuvenoid concentration – triple treated larvae (Table – 1a). The seasonal variation influenced the production of cocoon and shell ratio [30] and the reduced amount of food intake by the parasitized silkworm larvae reduced the silk production of *Bombyx mori* [31]. The plant growth regulators significantly increased the shell ratio *of Bombyx mori* [32] and temperature variation also influenced the shell ratio in *Bombyx mori* [33]. Similarly, variation in the photoperiod regime has also been reported to cause considerable variation in the silk producing potential of *Bombyx mori* [33]. The exposure of silkworm larvae in the magnetic field of 3500 gauss caused an increase in the silk yield due to the increasing protein level in the silk gland [6]. Magnetization of eggs considerably influenced the silk producing potential of *Bombyx mori* [10]. The administration of plant growth hormone Indloe-3- acetic acid increased the shell ratio [34]. Methoprene and fenoxycarb treated *Bombyx mori* showed increased shell percentage [35]. JHA Lobomin at various age, hours and season affected the shell ratio [36]. JHA R394 at different hours of treatment increased the shell ratio [20]. Twenty four juvenile hormone mimicking compounds showed improvement in the shell ratio [22]. BPE epoxide treatment on the *Bombyx mori* significantly increased the shell ratio [37]. R394 has no significant difference in the shell ratio except that in the 48h treatment in PM x NB4D2 [38]. The phytoecdysteroid were administered at different ages of $5^{th}$ instar larvae of *Bombyx mori*, which influenced the shell ratio [39]. The combined administration of JH and PE increased the shell ratio [40]. Mulberry leaves treated with chemical, biofertilizers and their mixture increased shell ratio of *B. mori* cocoon [41].

In the present investigation, the shell ratio increased with the increase in phytojuvenoid concentration up to 30%. The treatment of larvae with phytojuvenoid, extended the larval period and larvae consumed more food which is utilized in the synthesis of more protein increasing both the cocoon weight and cocoon shell weight. Thus, shell ratio increased at low concentrations while a higher phytojuvenoid concentration caused stress response and the shell ratio decreased.

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