Effect of Phytoecdysteroid on Incubation Period and Per Cent Fertilized Egg of Multivoltine Mulberry Silkworm Bombyx mori Linn.

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Abstract: Effect of phytoecdysteroid on incubation period and per cent fertilized egg of multivoltine mulberry silkworm Bombyx mori was studied. Variation in the phytoecdysteroid concentration and number of larval treatment(treated the larvae) influenced the incubation period and per cent fertilized egg of Bombyx mori eggs. In 40, 50 and 60% phytoecdysteroid concentration, the incubation period decreased slowly and slowly, in single(treated the Vth instar larvae onlyone times just before two days of larval spinning) and double treatment(in first treatment treated the IVth instar larvae just before two days of IVth moult further, second treatment for the same larvae was given to the Vth instar just before two days of spinning). Seventy per cent phytoecdysteroid concentration in all the treatment caused considerable increase in the incubation period of eggs. The minimum incubation period was noticed (7.95 ± 0.180) days in case of double treatment by 60% phytoecdysteroid concentration. Maximum per cent fertilized egg was noticed (94.75 ± 2.03) in case of double treatment by 60% phytoecdysteroid concentration. Experiments were performed by different concentration of phytoecdysteroid obtained from Achyranthes leaf extract. A control set was always maintained with each set of experiment. Number of larval treatment and variation in the phytoecdysteroid concentration did not cause significant effect on the incubation period of egg as the economy of Bombyx mori. Two-way ANOVA indicates that number of larval treatment significantly (P < 0.01) influenced the percentage of fertilized egg. Phytoecdysteroid treatment, if applied tactfully, may be useful for boosting up the sericulture industry as well as the economy of silkworm rearing.

Key words: Bioactive Hormone · IVth Instar · Larvae · Eggs · Moultin

INTRODUCTION

The silk industry has developed as a popular cottage industry providing self employment to more than ten million rural persons in the unorganized sector. It is well known for its low investment and quick and high return which make it an ideal industry fitting well in to the socio-economic frame of India. An analysis of the international trends in the silk production suggests that sericulture has better prospects for growth in the developing countries than in the developed countries. The efforts are being made to evolve new technologies that are cast effective, labour saving and ecofriendly. In order to increase the production of silk efforts have been made to study the effect of temperature [1], relative humidity [2], photoperiod [3], artificial diet [4], X rays [5] etc on the performance of silkworm. The magnetization of egg influences silk producing potential [6,7] and incubation period of eggs[8,9]. Ecdysone and juvenile hormone are the two major circulating hormones in insects which control the growth and development in insects. The plant that produce insect moulting hormone termed phytoecdysteroids (PES), functions as strategic defenses for plants against insects by acting as either feeding deterrents or through developmental disruption in insects. The response of silkworm to the small quantities of these phytoecdysteroids or its analogues may hasten the larval moulting events and also influenced the spinning process of the several silkworm larvae may prove to be very much useful for the management of rearing program and economics of silkworm industry.

Ecdysteroids play key role in moulting and metamorphosis in insects. Many plants are known to contain ecysosteroids with high moulting hormone activity. The plant like Achyranthes aspera (Lat jeera) and Cassia tora (Choti chakwar) have been identified to
have phytoecdysteroids [10]. Keeping this in view, an attempt has been made to investigate the influence of phytoecdysteroid on incubation period of multivoltine mulberry silkworm (*B. mori*).

**MATERIALS AND METHODS**

**Seed Cocoon:** 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 Behraich, Directorate of Sericulture Uttar Pradesh and , maintained in the plywood trays (23 x 20 x 5 cm) under the ideal rearing conditions [11] 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 moth emerged generally in the morning at around 4 am. The tray in which the seed cocoons were kept was suddenly illuminated by light in the morning at 4 o’clock on 9th and 10th day of spinning. The seed cocoons were quickly picked up and kept sexwise in separate trays to avoid copulation. The newly emerged moths from seed cocoon were quickly examined by microscope under 15 x 45 magnifications for the detection of bacterial and protozoan pathogens.

The disease free layings (D.F.L’”), thus prepared, were treated with 2% formalin for 15 min to increase the adhesiveness of egg of the paper sheet and surface disinfection. Thereafter, the egg sheets with egg laid on were thoroughly washed with running water to remove formalin and eggs were dried in shade. The dried eggs, thus obtained, were taken for phytoecdysteroid treatment under various experimental conditions.

**Experimental Design:** To observe the influence of bioactive phytoecdysteroid hormone on the performance of *B. mori*, the experiments were performed with different concentrations of phytoecdysteroid hormone with respect to the treatment of IIIrd, IVth and Vth instar larvae. For extraction of phytoecdysteroid, the leaves of *Achyranthes aspera* were collected, washed thoroughly with distilled water and dried in incubator at 37°C. The dried leaves were powdered with the help of mechanical device. Further, 50 g powder, thus obtained was subjected to extraction through soxlet apparatus with 250 ml distilled water for 40 h. After 40 h of extraction a little amount of concentrated solution was obtained which was dried and 6.75 g powdered material was obtained. The dried powder was dissolved in distilled water as 5 g in 25 ml water and used this solution for further experiment as 100% concentration of phytoecdysteroid. For further experiment the suitable narrow range of *Achyranthes* phytoecdysteroid concentration viz; 40, 50, 60 and 70 % were taken. Thus, four phytoecdysteroid concentrations were applied topically by spraying as 10 ml on 100 g mulberry leaves and the larvae were fed on the treated leaves. Three set of experiments were designed viz single, double and triple treatment of larvae.

**Single Treatment:** Single treatment of larvae was performed with the Vth instar larvae just before two days of the beginning of larval spinning. 100 larvae were taken out from the Biological Oxygen Demand(BOD) incubator and the mulberry leaf, treated with 40% concentration of *Achyranthes* leaf extract, was given as food. Further, the treated larvae were given normal mulberry leaves as food.

**Double Treatment:** Double treatment of larvae was started from the final stage of IVth instar larvace. In the first treatment, 100 larvae of IVth instar were treated just before two days of IVth moulting, by providing treated mulberry leaf with 40% solution of *Achyranthes* leaf extract. The treated larvae were then transferred in BOD incubator for further rearing and development. Further, second treatment for the same larvae was given at the final stage of Vth instar ie just before two days of spinning.
Triple Treatment: For triple treatment, the third instar larvae just before III\textsuperscript{a} moult were separated from BOD incubator. In the first treatment 100 larvae of III\textsuperscript{a} instar were treated by providing extract treated mulberry leaf and kept in BOD incubator for rearing. The second treatment of same larvae was done just before two days of IV\textsuperscript{th} moult at the final stage of IV\textsuperscript{th} instar larvae and transferred in BOD incubator for rearing. Third treatment was given to V\textsuperscript{th} instar larvae, two days before the start of spinning. Thus, in the triple treatment III\textsuperscript{a}, IV\textsuperscript{th} and V\textsuperscript{th} instar larvae were treated. Similar experiments were performed by 50, 60 and 70% concentration of phytoecdysteroid obtained from Achyranthes leaf extract. A control set was always maintained with each set of experiment.

Incubation Period of Egg: The eggs (resulted from treatments) were transferred chronically to BOD incubator maintained at the optimum condition of 26 ± 1°C temperature, 80 ± 5% RH and 12 h of photoperiod a day. The time required for the incubation before the hatching of larvae was calculated for each set of experiment separately. The average of eggs was counted from 8 pm on the day of decoupling. Three replicates of 10 laying, in each replicate, were made. The average time taken for incubation by the eggs of different experimental conditions was calculated by taking the mean value of the data obtained.

Percent Fertilized Eggs: For determining the effect of phytoecdysteroid on per cent fertilized eggs, obtained from the moths of treated larvae were considered and the eggs thus obtained, were incubated at optimum conditions of temperature relative humidity and photoperiod of 26 ± 1°C, 80 ± 5% and 12 ± 1 h, respectively. At the head pigmentation stage the eggs were counted. Thirty layings (3 batches of 10 laying in each batch) were counted for each replicate. The percent of the fertilized eggs was calculated as follows:

\[
\text{Percent fertilized egg} = \frac{\text{No of eggs fertilized}}{\text{Total No. of egg}} \times 100
\]

*Eggs were counted at the head pigmentation stage, i.e. eggs having black spots are fertilized eggs.

RESULTS

Incubation Period of Egg: The data presented in Table 1a shows that change in the phytoecdysteroid concentration and the number of larval treatment influenced the incubation period of egg. With the increasing number of larval treatment by phytoecdysteroid from one to two times, the incubation period of egg decreased in case of 40, 50 and 60% phytoecdysteroid treatment but triple treatment caused notable increase in the incubation period in all the above concentrations. 70% phytoecdysteroid treatment caused notable increase in the incubation period of egg with the increase in number of treatment from single to triple. The trend of decrease in the incubation period of egg with the increasing number of treatment has been recorded to be almost same in case of 40, 50 and 60% phytoecdysteroid treatment. The minimum incubation period was noticed to be 7.95 ± 0.180 days in case of double treatment by 60% phytoecdysteroid concentration and the maximum 10.20 ± 0.746 days was recorded in case of triple treatment by 70% phytoecdysteroid concentration.

Two-way ANOVA indicates that number of larval treatment and variation in the phytoecdysteroid concentration did not cause significant effect on the incubation period of egg of *Bombyx mori*. The Post–hoc test (Table 1b) shows significant group difference in the incubation period of eggs in the double treatment of larvae, in between control and 60%, 40 and 60% and 60 and 70% concentration of phytoecdysteroid treatment.

<table>
<thead>
<tr>
<th>Number of treatment (Larval instar)</th>
<th>Phytoecdysteroid concentration (%)</th>
<th>F\textsubscript{1} ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control(X\textsubscript{1})</td>
<td>40(X\textsubscript{2})</td>
</tr>
<tr>
<td>Single (V)</td>
<td>9.50±0.272</td>
<td>9.35±0.413</td>
</tr>
<tr>
<td>Double (IV-V)</td>
<td>9.50±0.272</td>
<td>9.20±0.510</td>
</tr>
<tr>
<td>Triple (III-V)</td>
<td>9.50±0.272</td>
<td>9.65±0.381</td>
</tr>
</tbody>
</table>

F\textsubscript{1} ratio = 4.07* n\textsubscript{1} = 2
*Non-significant

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

X\textsubscript{1}, X\textsubscript{2}, X\textsubscript{3}, X\textsubscript{4} and X\textsubscript{5} are the mean values of the incubation period (days) in control, 40%, 50%,60% and 70% phytoecdysteroid concentration respectively.
Table 1 b: Post-hoc test showing group difference in the effect of phytoecdysteroid treatment on the incubation period (days) of *Bombyx mori*

<table>
<thead>
<tr>
<th>Mean difference in between groups</th>
<th>Number of treatment</th>
<th>Double</th>
<th>Triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1–X2</td>
<td>0.15</td>
<td>0.30</td>
<td>0.15</td>
</tr>
<tr>
<td>X1–X3</td>
<td>0.28</td>
<td>0.53</td>
<td>0.20</td>
</tr>
<tr>
<td>X1–X4</td>
<td>0.75</td>
<td>*1.55</td>
<td>0.37</td>
</tr>
<tr>
<td>X1–X5</td>
<td>0.15</td>
<td>0.50</td>
<td>0.70</td>
</tr>
<tr>
<td>X2–X3</td>
<td>0.13</td>
<td>0.23</td>
<td>0.05</td>
</tr>
<tr>
<td>X2–X4</td>
<td>0.60</td>
<td>*1.25</td>
<td>0.22</td>
</tr>
<tr>
<td>X2–X5</td>
<td>0.30</td>
<td>0.80</td>
<td>0.55</td>
</tr>
<tr>
<td>X3–X4</td>
<td>0.47</td>
<td>1.02</td>
<td>0.17</td>
</tr>
<tr>
<td>X3–X5</td>
<td>0.43</td>
<td>1.03</td>
<td>0.50</td>
</tr>
<tr>
<td>X4–X5</td>
<td>0.90</td>
<td>*2.05</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Honestly significant difference (HSD) = \( \sqrt{MS_{within}/q} \)

\( n = \sqrt{0.1442/5.05} \)

\( q = 1.11 \)

MS = Mean square values of ANOVA table

q = Studentized range static

n = Number of replicates

* Showing significant group difference

X1, X2, X3, X4, and X5 are the mean values of the incubation period (days) in control, 40%, 50%, 60% and 70% phytoecdysteroid concentration respectively.

Table 2 a: Effect of phytoecdysteroid treatment on the percent fertilized eggs of *Bombyx mori*

<table>
<thead>
<tr>
<th>Number of treatment</th>
<th>Phytoecdysteroid concentration (%)</th>
<th>F1 ratio</th>
<th>n1 = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Larval instar)</td>
<td>Control(X1) 40(X2) 50(X3) 60(X4) 70(X5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single (V)</td>
<td>88.25±1.98 89.00±2.24 90.00±2.16 92.75±2.16 84.50±2.01</td>
<td>3.71*</td>
<td></td>
</tr>
<tr>
<td>Double (IV-V)</td>
<td>88.25±1.98 90.50±2.36 92.20±1.98 94.75±2.03 82.00±2.04</td>
<td>11.38**</td>
<td></td>
</tr>
<tr>
<td>Triple (III-V)</td>
<td>88.25±1.98 86.50±2.11 84.75±2.48 82.50±2.47 76.75±1.74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( F_1 \) ratio = 11.38** n2 = 2

*Non-significant **P < 0.01

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

X1, X2, X3, X4, and X5 are the mean values of the percent fertilized eggs in control, 40%, 50%, 60% and 70% phytoecdysteroid concentration respectively.

Table 2 b: Post-hoc test showing group difference in the effect of phytoecdysteroid treatment on the percent fertilized eggs of *Bombyx mori*

<table>
<thead>
<tr>
<th>Mean difference in between groups</th>
<th>Number of treatment</th>
<th>Double</th>
<th>Triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1–X2</td>
<td>0.75</td>
<td>2.25</td>
<td>1.75</td>
</tr>
<tr>
<td>X1–X3</td>
<td>1.75</td>
<td>3.95</td>
<td>3.50</td>
</tr>
<tr>
<td>X1–X4</td>
<td>4.50</td>
<td>6.50</td>
<td>5.75</td>
</tr>
<tr>
<td>X1–X5</td>
<td>3.75</td>
<td>6.25</td>
<td>*11.50</td>
</tr>
<tr>
<td>X2–X3</td>
<td>1.00</td>
<td>1.70</td>
<td>1.75</td>
</tr>
<tr>
<td>X2–X4</td>
<td>3.75</td>
<td>4.25</td>
<td>4.00</td>
</tr>
<tr>
<td>X2–X5</td>
<td>4.50</td>
<td>*8.50</td>
<td>*9.75</td>
</tr>
<tr>
<td>X3–X4</td>
<td>2.75</td>
<td>2.55</td>
<td>2.25</td>
</tr>
<tr>
<td>X3–X5</td>
<td>5.50</td>
<td>*10.20</td>
<td>*8.00</td>
</tr>
<tr>
<td>X4–X5</td>
<td>*8.25</td>
<td>*12.75</td>
<td>5.75</td>
</tr>
</tbody>
</table>

Honestly significant difference (HSD) = \( \sqrt{MS_{within}/q} \)

\( n = \sqrt{6.7896/7.60} \)

MS = Mean square values of ANOVA table

q = Studentized range static

n = Number of replicates

* Showing significant group difference

X1, X2, X3, X4, and X5 are the mean values of the percent fertilized eggs in control, 40%, 50%, 60% and 70% phytoecdysteroid concentration respectively.
**Per Cent Fertilized Egg:** The data presented in table 2a shows that change in the phytoecdysteroid concentration and the number of larval treatment influenced the percentage of fertilized egg. With the increasing number of larval treatment from one to two times, the percentage of fertilized eggs increased in case of 40, 50 and 60% phytoecdysteroid treatment but triple treatment caused notable decline in the percentage of fertilized eggs in all the above concentrations. Seventy percentage phytoecdysteroid treatment caused notable decline in the percentage fertilized egg with the increase in number of treatment from single to triple. The trend of increase in the percentage of fertilized egg with the increasing number of treatment has been recorded to be almost same in case of 40, 50 and 60% phytoecdysteroid treatment. The maximum percentage of fertilized egg was noticed to be 94.75 ± 2.03 in case of double treatment by 60% phytoecdysteroid concentration and was minimum 76.75 ± 1.74 in case of triple treatment by 70% phytoecdysteroid concentration. Two-way ANOVA indicates that number of larval treatment significantly ($P_2 < 0.01$) influenced the percentage of fertilized egg. The Post-hoc test (Table 2b) shows significant group difference in the percentage of fertilized eggs in between 60 and 70% concentration of phytoecdysteroid concentration in case of single treatment of larvae. In the double treatment of larvae, significant group difference in the percent fertilized egg was determined in between 40 and 70%, 50 and 70% and 60 and 70% concentration of phytoecdysteroid treatment. In the triple treatment of larvae, significant group difference in percent fertilized egg was noticed in between control and 70%, 40 and 70% and 50 and 70% concentration of phytoecdysteroid treatment.

**DISCUSSION**

**Incubation Period:** Variation in the phytoecdysteroid concentration and number of larval treatment influenced the incubation period of *Bombyx mori* eggs. In 40, 50 and 60% phytoecdysteroid concentration, the incubation period decreased slowly and slowly, in single and double treatment. 70% phytoecdysteroid concentration in all the treatment stages caused considerable increase in the incubation period of eggs. Thus, the trend of variation of the incubation period in 40, 50 and 60% phytoecdysteroid concentration is almost of similar pattern in single and double treatment of larvae while in 70% phytoecdysteroid concentration, the trend of variation with the change in number of treatment is different. The mating duration has been noticed to affect the incubation period of *Bombyx mori* eggs [12]. The role of temperature and relative humidity on oviposition and incubation of eggs was reported by several workers [13-15]. The activity of juvenile hormones considerably influenced the incubation period of *Bombyx mori* eggs [16]. The low magnetic field acts on enzyme molecules and produces conformational changes in enzyme which is concerned for the activation of enzyme system, whereas, at higher strength of magnetic field, the change brought about the negative hence the enzyme get inhibited [17]. The functional significance of sequence homology can be illustrate by cytochrome, an iron containing mitochondrial protein that transfer electron during biological oxidation in eukaryotic cells [18] and the magnetic field below 0.03 T caused an increase in the oxidation rate due to enhance reducing power of cytochrome C in cell [19]. The present investigation shows positive correlation between the reproductive potential and larval treatment with phytoecdysteroids. The exogenous hormones influenced only the choriogenic follicle cell [20] supported by the fact that more eggs were clustered within a limited region along with a single ovariole [21]. Eggs containing different amounts of ecdysteroids showed different level of embryonic development and maternal ecdysteroids appear to be required at different tiers for fertilization, embryogenesis and hatching of the silkworm larvae [22]. Most ecdysone 20-monoxygenase activity was associated with microsomes and that there was little intrinsic mitochondrial ecdysone 20-monoxygenase [23].

**Per Cent Fertilized Egg:** The variation in the phytoecdysteroid concentration and number of larval treatment caused notable influence on the per cent fertilized eggs. With the increasing number of larval treatment from one to two times, the hatchability increased from 40 to 60% concentration of phytoecdysteroid but a considerable decline was noticed in triple treatment in all the concentration. Maximum per cent fertilized eggs were noticed in double treatment with 60% phytoecdysteroid concentration. Maternal ecdysteroids appear to be required at different tiers for fertilization and embryogenesis in the silkworm [22]. Developmental response and fertility under various conditions have studied in different insects [24, 25]. Yolk proteins of the silkworm, *B. mori* are important sources of nutrition and energy during embryo development [26]. Temperature stress caused delay and poor hatching, depression of eggs and death of fully formed larvae inside egg [27]. Large and heavy eggs resembling the giant egg can be induced by the administration of
20- hydroxyecdysone in to normal female pupae [28]. In insects, the steroid hormone 20- hydroxyecdysone (20 E) plays a major role in activating vitellogenesis, a process required for egg development [29]. The occurrence of fertilized and unfertilized eggs is not affected by periodicity of egg laying [30]. At higher temperature (30 °C and above) both fecundity and fertility are severely affected [31]. KK-42 is a novel agent to suppress the ecdysteroid accumulation in eggs and 80% eggs containing less than 10 µg free ecdysteroids/g eggs were not fertilized [18].

Thus, it is concluded that incubation period and per cent fertilized eggs, obtained from the larvae treated with low phytoecdysteroid concentration may be due to ultra structural changes in the cell contents and enzyme activity during larval and pupal stages caused positive effect on the incubation period and per cent fertilized eggs resulting increase in the per cent fertilized eggs but decrease the incubation period of Bombyx mori eggs. The higher concentration of phytoecdysteroid caused adverse effect on the cellular level, therefore, incubation period of Bombyx mori eggs increased and the per cent fertilized eggs declined.

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REFERENCES