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Impact of Bioactive Phytojuvenoid Compounds on the Silk Producing Potential of *Bombyx mori* (Lepidoptera: Bombycidae)

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Abstract: The objective of this investigation was to evaluate the effect of bioactive phytojuvenoid compounds on the silk producing potential (weight of silk gland, cocoon, cocoon shell and shell ratio per cent) of multivoltine mulberry silkworm (*Bombyx mori*). Bioactive phytojuvenoid compounds were derived after Soxhlet extraction from the leaves of *Azadirachta indica* and needles of *Pinus longifolia* and were used in 5%, 15%, 25% and 35% concentrations for single, double and triple treatment of third, fourth and fifth instars larvae of *B. mori* and further rearing was performed at normal rearing conditions with control set of study. The weight of silk gland, cocoon, cocoon shell and shell ratio per cent were increased gradually with increase in the concentrations of phytojuvenoid from 5 to 25% concentration in case of single, double and triple treated larvae whereas, in 35% concentration these parameters were increased slightly in the single treated larvae and further gradually decreased for the double and triple treated larvae. Maximum weight of silk gland (0.298±0.003 and 0.295±0.004 g), cocoon (1.298±0.020 and 1.289±0.024 g), cocoon shell (0.215±0.003 and 0.201±0.004 g) and shell ratio per cent cocoon (16.56±0.08 and 15.59±0.09) were recorded in the larvae triple treated with 25% concentration of phytojuvenoid derived from *P. longifolia* and *A. indica*, respectively, while control revealed minimum value for these parameters in *B. mori*. Thus, triple treatment of larvae with 25% concentration of phytojuvenoid of *P. longifolia* is most suitable to enhance the parameters of silk producing potential of *B. mori.* This study is important academically as well as economically and may be of importance to the sericulture industries. The study will further help to explore a new concept in this field.

Keywords: Bioactive, Phytojuvenoid, Silk gland, Cocoon, Cocoon shell, *Bombyx mori*, *Azadirachta indica*, *Pinus longifolia*

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Introduction

Silk industry is well known for its low investment and quick and high return round the year which makes it as an ideal industry fitting well in socio-economic frame of India. An analysis of international trends of the silk production suggests that sericulture has better prospects for growth in developing country than developed country. In multivoltine mulberry silkworm (*Bombyx mori*), the race *nistary* is a resistant variety which contributes to a great extent in the

commercial production of silk. The main aim of sericulture is to enhance the production of quality raw silk as per demand of the market. The economy of sericulture is definitely based upon the performance of the different stages of life cycle of *B. mori*. Silk producing potential of *B. mori* is an important parameter for commercial traits and better quality of raw silk in the sericulture industry.

To increase in the production of quality raw silk, efforts have been made to investigate the effect of temperature (Verma and Atwal, 1967), relative humidity (Upadhyay and Mishra, 2002), photoperiod (Jolly *et al*., 1971), X-rays (Kanarev and Chan, 1985), magnetic field (Tripathi, 2010) etc. on the performance of *B. mori*. The effect of bioactive phytojuvenoid compounds on *B. mori* has been of wide interest. Prothoracicotropic hormone (PTTH), juvenile hormone (JH) and ecdysone regulate growth and development in insects (Wigglesworth, 1985). Phytojuvenoids influence silk production (Pandey and Upadhyay, 2012) and reproductive parameters (Srivastava and Upadhyay, 2012) of *B. mori.* Effect of juvenile hormone and analogue on the commercial potential (Trivedy *et al*., 1997; Nair *et al*., 2003), delay in moulting (Sakurai *et al*., 1986), inhibitory action in early stage of protein synthesis and later bigger silk gland and improve in cocoon shell weight (Garel, 1983) in *B. mori* have been studied.

Keeping this in view, an attempt has been made to investigate the effect of bioactive phytojuvenoid compounds derived from leaves of *Azadirachta indica* and needles of *Pinus longifolia* on the weight of silk gland, cocoon, cocoon shell and per cent shell ratio of *B. mori*. The *A. indica* and *P. longifolia* are used for experiment due to their easily availability and juvenile properties. The study is important from academic as well as economic point of view and will explore a new concept for further investigation.

Materials and Methods

The seed cocoons of multivoltine mulberry silkworm (*B. mori* race *nistari*), were obtained from the silkworm grainage of Directorate of Sericulture, Uttar Pradesh at Behraich centre and were maintained in plywood trays (23 x 20 x 5 cm) under the ideal rearing conditions (26±1^{0}C, 75±5% RH and 12±1 h/day dim light) in the Silkworm Laboratory, Department of Zoology, Mahatma Gandhi P.G. College, Gorakhpur, India till the emergence of moths. The grainage operation was performed according to the Krishnaswami *et al.* (1973). Male and female newly emerged moths were allowed to mate and after 4 h of mating, the paired coupled moths were decoupled manually. The male moths were discarded while the female moths were allowed for egg laying on the sheet of paper in dark condition. After 24 h of egg laying, the female moths were individually examined for their disease freeness and crushed individually in mortar with pestles and blood smears which were examined by microscope under magnification of 15X45 for the detection of bacterial and protozoan pathogens.

The disease free laying (DFLs) thus prepared, were treated with 2% formalin for 15 min to increase the adhesiveness of eggs on the paper sheet and surface disinfection. Thereafter, the egg sheets with eggs laid, was thoroughly washed with running water to remove formalin and eggs were dried in shade. The dried eggs thus obtained, were allowed for hatching and larvae reared normally. The second, third and fourth instars larvae were taken for treatment with phytojuvenoid compounds derived from *Azadirachta indica* and *Pinus longifolia* under various experimental conditions.

Experimental design:

For the extraction of phytojuvenoid compounds the leaves from *Azadirachta indica* and needles of *Pinus longifolia* were collected, washed thoroughly with distilled water and dried in incubator at 37°C. The dried materials were grinded separately with the help of mechanical device. Further, 50 g grinded material of *A. indica* was subjected for extraction through Soxhlet apparatus with 250 ml of petroleum ether (B.P. 40-60°C) for 40 h. Extracted material was filtered and evaporated

and residue was further dissolved in acetone to separate polar compounds from extracted material (Srivastava *et al*., 1985).Acetone extract was concentrated at room temperature by evaporation. Thus, a little amount of concentrated solution of plant extract was obtained. 5 g dried acetone extracted residue was dissolved in 25 ml water and considered as a 100% concentration of phytojuvenoid. For further experiment the suitable narrow ranges of *A. indica* phytojuvenoid concentrations *viz.* 5%, 15%, 25% and 35% were formed. Thus, four concentrations of phyto-juvenoid compounds were applied by spraying as 1 ml on to 100 larvae of *B. mori* separately. Three sets of experiments were designed *viz.,* single, double and triple treatment of larvae. For single treatment, 100 larvae of fifth instar at the initial stage were taken out from the BOD incubator and treated with 1.0 ml of 5% concentrated solution of *A. indica* extract by sprayer. For double treatment, 100 larvae of fourth instar at initial stage were treated by 1.0 ml of 5% concentrated solution of *A. indica* extract by sprayer. The treated larvae were then transferred in BOD incubator for further rearing and development at normal rearing conditions. Further, similar second treatment for the same larvae was given at the initial stage of fifth instar larvae. Thus, in double treatment, the larvae at initial stage of fourth and fifth instar were treated. For triple treatment, 100 third instar larvae at initial stage were selected from BOD incubator and were treated with 1 ml of 5% concentrated solution of *A. indica* extract by sprayer and further kept in the BOD at normal rearing conditions. The second and third treatments were given at the initial stage of fourth and fifth instar and then larvae were kept in the BOD incubator. Similar experiments were performed by 15%, 25% and 35% concentrations of phytojuvenoid obtained from *A. indica* extract. A control set of experiment was also maintained with each experimental set. Similar experiments were performed with phytojuvenoid compounds extracted from *P. longifolia*. Weight of silk gland, cocoon, cocoon shell and per cent cocoon shell of *B. mori* were determined from the treated larvae.

Weight of silk gland: To determine the weight of silk gland, the fifth instar larvae were dissected in distilled water on 8th day and complete silk gland was taken out. The removed silk gland was cleaned and gently soaked with filter paper to remove the adhered water on it. For the weight of silk gland, 9 silk gland (three batches and each batch has three silk gland) were weighted for each replicate. Three replicates of each experiment were made.

Weight of cocoon: For determination of weight of single cocoon, 30 normal cocoons (three batches of 10 cocoons in each batch) were weighted for each replicate. Three replicates of each experiment were made. The weight of cocoon was taken on 5th day from the beginning of spinning.

Weight of cocoon shell: For determination of weight of single cocoon shell, 30 normal shells (three batches of 10 cocoon shell in each batch) were weighted for each replicate. Three replicate of each experiment were made. The weight of cocoon shell was also taken on the 5th day of the beginning of spinning.

Shell ratio per cent: To determine the shell ratio per cent, the weight of 20 good 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 (Ghosse, 1987) was calculated based on at least 20 cocoons and 20 cocoon shells, taken at random from the good lot.

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

Statistical analysis: Three replicates of each experiment were made and all the data obtained were analysed statistically by Two-way ANOVA.

Results

Weight of silk gland:

Data presented in Table 1 indicates that variation in the concentrations of phytojuvenoid extracted from *Azadirachta indica* and *Pinus longifolia* and

Table 1: Effect of phytojuvenoid compounds on the weight of silk gland (g) of *Bombyx mori*

Source of Phytojuvenoid compounds	Number of treatments	Phytojuvenoid Concentrations (%)				F_1-ratio n_1=3
		5%	15%	25%	35%	
	Control	0.221±0.002	0.221±0.002	0.221±0.002	0.221±0.002	16.98*
Azadirachta indica	Single	0.225±0.001	0.237±0.003	0.254±0.004	0.258±0.002	
	Double	0.234±0.003	0.248±0.002	0.276±0.004	0.245±0.004	
	Triple	0.255±0.003	0.282±0.004	0.295±0.004	0.229±0.003	
Pinus longifolia	Single	0.231±0.002	0.245±0.003	0.266±0.003	0.268±0.004	
	Double	0.243±0.004	0.262±0.003	0.281±0.004	0.256±0.003	
	Triple	0.261±0.002	0.280±0.004	0.298±0.003	0.230±0.002	

F_2-ratio= 19.25**; n_2=6; *P_1<0.01; **P_2<0.01; Each value represents mean±S.E. of three replicates

Table 2: Effect of phytojuvenoid compounds on the weight of cocoon (g) of *Bombyx mori*

Source of Phytojuvenoid compounds	Number of treatments	Phytojuvenoid Concentrations (%)				F_1-ratio n_1=3
		5%	15%	25%	35%	
	Control	1.001±0.011	1.001±0.011	1.001±0.011	1.001±0.011	14.75*
Azadirachta indica	Single	1.101±0.022	1.145±0.012	1.164±0.030	1.178±0.022	
	Double	1.110±0.032	1.155±0.025	1.195±0.032	1.182±0.030	
	Triple	1.200±0.031	1.231±0.025	1.289±0.024	1.163±0.026	
Pinus longifolia	Single	1.102±0.023	1.135±0.022	1.175±0.021	1.195±0.020	
	Double	1.131±0.020	1.163±0.028	1.198±0.032	1.175±0.031	
	Triple	1.221±0.023	1.256±0.030	1.298±0.020	1.156±0.020	

F_2-ratio= 13.18**; n_2=5; *P_1<0.01; **P_2<0.01; Each value represents mean±S.E. of three replicates

Table 3: Effect of phytojuvenoid compounds on the weight of cocoon shell (g) of *Bombyx mori*

Source of Phytojuvenoid compounds	Number of treatments	Phytojuvenoid Concentrations (%)				F_1-ratio n_1=3
		5%	15%	25%	35%	
	Control	0.142±0.002	0.142±0.002	0.142±0.002	0.142±0.002	12.62*
Azadirachta indica	Single	0.157±0.001	0.164±0.002	0.171±0.003	0.176±0.002	
	Double	0.160±0.003	0.170±0.001	0.178±0.001	0.169±0.001	
	Triple	0.175±0.002	0.183±0.003	0.201±0.004	0.161±0.002	
Pinus longifolia	Single	0.162±0.003	0.172±0.002	0.183±0.003	0.190±0.002	
	Double	0.170±0.001	0.181±0.002	0.191±0.004	0.185±0.003	
	Triple	0.185±0.003	0.198±0.002	0.215±0.003	0.173±0.002	

F_2-ratio= 11.42**; n_2=6; * P_1<0.01; **P_2<0.01; Each value represents mean±S.E. of three replicates

Table 4: Effect of phytojuvenoid compounds on the shell ratio per cent (%) of *Bombyx mori*

Source of Phytojuvenoid compounds	Number of treatments	Phytojuvenoid Concentrations (%)				F_1-ratio n_1=3
		5%	15%	25%	35%	
	Control	14.18±0.08	14.18±0.08	14.18±0.08	14.18±0.08	80.05*
Azadirachta indica	Single	14.25±0.11	14.32±0.13	14.69±0.10	14.94±0.13	
	Double	14.41±0.12	14.71±0.09	14.89±0.09	14.29±.08	
	Triple	14.58±0.11	14.86±0.11	15.59±0.09	13.84±0.05	
Pinus longifolia	Single	14.70±0.07	15.15±0.12	15.57±0.09	15.89±0.06	
	Double	15.03±0.09	15.56±0.11	15.94±0.08	15.74±0.11	
	Triple	15.15±0.07	15.76±0.09	16.56±0.08	14.96±0.10	

F_2-ratio=85.28**; n_2=6; *P_1<0.01; **P_2<0.01; Each value represents mean±S.E. of three replicates

their number of treatment of larvae influenced the weight of silk gland of *Bombyx mori***.** The weight of silk gland increased with increase in the concentration of phytojuvenoid compounds (*A. indica* and *P. longifolia* both) from 5 to 25% as well as number of treatment of larvae, while in 35% concentration it was slightly increased in case of single treated larvae and further gradually decreased in double and triple treated larvae. The trend of increase in the weight of silk gland with increase in concentration of phytojuvenoid was almost similar in single and double treated larvae while in triple treated larvae, the weight of silk gland was gradually increased from 5 to 35% concentration in case of single treated larvae whereas, it was increased from 5-25% and decreased in 35% concentration in double and triple treated larvae. Maximum weight of silk gland was recorded as 0.298±0.003 g in the larvae triple treated with 25% concentration of phyto-juvenoid extracted from *P. longifolia* and minimum weight of silk gland was recorded as 0.221±0.002 g for control. The statistical analysis by Two-way ANOVA showed that variation in the concentrations of phytojuvenoid compounds (P_1<0.01) and number of treatment of larvae (P_2<0.01) significantly influenced the weight of silk gland of the *B. mori*.

Weight of Cocoon:

Table 2 shows that variation in the concentration of phytojuvenoid and their number of treatments caused change in weight of cocoon of *B. mori***.** In single treated larvae, the weight of cocoon increased with increase in concentration of phytojuvenoid compounds from 5 to 35% concentration and it was maximum in case of 35% while in double and triple treated larvae, the weight of cocoon increased from 5 to 25% concentration and further decrease in 35% concentration treated larvae. In single and double treated larvae, the weight of cocoon increased slowly and it was maximum (1.198±0.032 g) in the larvae double treated with 25% concentration of phytojuvenoid extracted from *P. longifolia*. In triple treated larvae, the weight of cocoon was maximum (1.298±0.020 g) in 25% concentration of phytojuvenoid extracted from *P. longifolia*. The control study has minimum value of weight of cocoon (1.001±0.011 g). Two-way ANOVA indicated that variation in concentrations as well as number of treatment of phytojuvenoid compounds extracted from *A. indica* and *P. longifolia* significantly (P_1<0.01 and P_2<0.01, respectively) influenced the weight of cocoon of *B. mori*.

Weight of cocoon shell:

Table 3 shows that the change in concentration of phytojuvenoid and their number of treatments influenced the weight of cocoon shell of *B. mori***.** In case of single, double and triple treated larvae, the weight of cocoon shell increased with increase in concentration of phytojuvenoid compounds extracted from *A. indica* as well as *P. longifolia* from 5 to 25% concentration and was maximum (0.215±0.003 g) in 25% triple treated larvae with *P. longifolia*. In 35% concentration, the weight of cocoon shell slightly increased in single treated larvae and further decreased in double and triple treated larvae. The weight of cocoon shell was minimum (0.142±0.002 g) for control. Two-way ANOVA indicated that variation in the concentrations (P_1<0.01) and number of treatments (P_2<0.01) of phytojuvenoid compounds extracted from *A. indica* and *P. longifolia* significantly influenced the weight of cocoon shell of *B. mori*.

Shell ratio per cent (*%*):

Table 4 indicates that the shell ratio per cent varies due to change in the concentrations of phytojuvenoid extracted from *A .indica* and *P. longifolia* as well as their number of treatment to the larvae of *B. mori***.** In single treated larvae the shell ratio per centincreased with increase in the concentration of phytojuvenoid of *A. indica* and *P. longifolia* from 5 to 35% while in double and triple treated larvae, the shell ratio per cent increased up to 25% and further decreased in 35% concentration of phytojuvenoid. The trend of increase in the shell ratio per cent with increase in

the concentration of phytojuvenoid was almost similar and steady in single and double treated larvae up to 25% concentration. The maximum shell ratio per cent (16.56±0.08) was noticed in the larvae triple treated with 25% concentration of phytojuvenoid extracted from *P. longifolia* whereas it was minimum (13.84±0.05) in triple treated 35% concentration of phytojuvenoid extracted from *A. indica*. Two-way ANOVA indicated that variation in the concentrations of phytojuvenoid compounds (P_1<0.01) of *A. indica* and *P. longifolia* and their number of treatment for larvae (P_2<0.01) significantly influenced the shell ratio per cent of *B. mori*.

Discussion

Variation in the concentration of phytojuvenoid compounds extracted from *Azadirachta indica* as well as *Pinus longifolia* and their number of treatment of larvae influenced the weight of silk gland, cocoon, cocoon shell and shell ratio per cent of *B. mori*. Some plants like *Pinus longifolia*, *Abies bolsomea, Psorelea corylifolia* and *Azadirachta indica* act on *B. mori* larvae as bioactive juvenoid compounds (Nair *et al*., 1999). The larval development and their feeding status have impact on egg fertility in insects (Chaudhuri, 2003; Fischer *et al.*, 2004) and maternal ecdysteroid appear to be required at different tier for fertilization, embryogenesis and hatching of the silkworm larvae (Okuda *et al*., 1993). The thyroxin treated mulberry species as *Morus multicaulis* had significant effect on the denier of silk (Ahmad *et al*., 2009) whereas, administration of plant growth hormone Indole-3-acetic acid increased the denier of silk (Bharthi and Miao, 2003) and administration of phytojuvenoid at different age of V instar of *B. mori* increased reelability of silk filament and denier (Nair *et al*., 2005). Juvenile hormone considerably affect the incubation period of *B. mori* eggs (Reddy *et al.*, 1995) whereas, Juvenile hormone is claimed to inhibit protein synthesis in early treated larvae with later on regain protein synthesis resulting in bigger silk gland and the improvement of cocoon shell weight (Garel, 1983). In present study the weight of silk gland, cocoon and cocoon shell also increased with increase in concentration of phytojuvenoid extracted from *A. indica* and *P. longifolia* and their number of treatments of larvae.

Methoprene and fenoxycarb treated *B. mori* larvae showed significant enhance in the denier (Mamatha *et al*., 2006) and the process of growth and development in insects are regulated by prothoracicotropic hormone (PTTH), juvenile hormone (JH) and ecdysone, which either directly or indirectly manifest the moulting and metamorphosis (Wigglesworth, 1985). The response of silkworm to very small quantities of phytojuvenoid or its analogues may extend the larval maturation and spinning process, however, the response to such treatment varies and depending on the dosages of compounds showing duration and number of applications (Chowdhary, 2003). The juvenile hormone analogue also has been noticed to influence the commercial potential (Trivedy *et al*., 1997; Nair *et al*., 2003) whereas the delay in moulting is probably due to the inhibitory action of JH on ecdysone synthesis (Sakurai *et al*., 1986) in *B. mori*. Larval treatment with 20-hydroxyecdysone showed positive effect on the reproductive potential of *B. mori* (Prasad and Upadhyay, 2012) and the phytojuvenoid caused beneficial effect on the life pattern of silkworm (Srivastava and Upadhyay, 2013, 2016; Tripathi, 2022). In the present study, the shell ratio per cent of *B. mori* also increased with the increase in concentration of phytojuvenoid compounds extracted from *A. indica* and *P. longifolia* and their number of treatments.

Conclusion

The silk producing potential of *B. mori* i.e. weight of silk gland, cocoon, cocoon shell and shell ratio per cent increased with increase in number of treatment of larvae and concentration up to 25% of phytojuvenoid compounds extracted from *A. indica* and *P. longifolia*, whereas in 35% concentration, these increased in single treated larvae and further decreased gradually up to triple treated larvae. How exactly bioactive phytojuvenoid compounds influence the insects is

not clear but it may be due to their inhibitory effect on the ecdysone which delays moulting and increases larval duration, proper growth and development of silk gland of larvae which may cause better response for weight of silk gland, cocoon, cocoon shell and shell ratio per cent of *Bombyx mori*. The higher concentration of bioactive phytojuvenoid in triple treated larvae caused decrease in silk producing potential which may be due to their stress response. This study is important from academic and economic point of view and will explore a new concept for the further investigation.

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