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BIOACTIVE PHYTOJUVENOID COMPOUNDS INFLUENCE PROTEIN CONTENTS IN THE LARVAE AND PUPAE OF *BOMBYX MORI* (LEPIDOPTERA:BOMBYCIDAE)

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ABSTRACT

This study reports, the effect of bioactive phytojuvenoid compounds on the level of total protein contents in the silk gland, fat body and haemolymph of fifth instar larvae and fat body and haemolymph of pupae of multivoltine mulberry silkworm (*Bombyx mori*). The bioactive phytojuvenoid compounds were derived after soxhlet extraction from the leaves of the *Azadirachta indica* and needles of *Pinus longifolia* 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*. Further rearing was performed at normal rearing conditions with control set of study. The level of total protein contents increased in the silk gland, fat body and haemolymph of fifth instar larvae and fat body and haemolymph of pupae with increase in concentrations of phytojuvenoid from 5 to 25% in case of single, double and triple treated larvae whereas, in 35% concentration, the level of total protein contents was increased slightly in case of single treated larvae and further gradually decreased for the double and triple treated larvae. Maximum level of total protein contents in the silk gland of fifth instar larvae (60.248 ± 2.142 µg/mg tissue), haemolymph of fifth instar larvae (78.124 ± 3.002 µg/ml tissue) and pupae (71.146 ± 3.245 µg/ml tissue) and fat body of fifth instar larvae (68.124 ± 3.002 µg/mg tissue) and pupae (66.146 ± 2.245 µg/mg tissue) were recorded in case of larvae triple treated with 25% concentration of phytojuvenoid derived from *Pinus longifolia*, while control study revealed minimum values. Thus, triple treatment of larvae with 25% concentration of phytojuvenoid derived from *P. longifolia* is most suitable to enhance the level of total protein contents in the tissues of *B. mori.* This study is important from academic as well as economic point of view and will further help to explore a new concept in this field.

KEY WORDS: Bioactive, Phytojuvenoid, Silk gland, Haemolymph, Fat body, *Bombyx mori*

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 the developed country. In multivoltine mulberry silkworm (*Bombyx mori*), the race *nistary* is a resistance variety which contributes upto 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 synthesis of protein in the tissues of *B. mori*. Since, silk is chemically protein hence the protein synthesises and the level of total protein contents in the silk gland of *B. mori* are important parameters for its commercial traits.

To increase in the production of quality raw silk, efforts have been made to investigate the effect of temperature (Verma & Atwal, 1967), relative humidity (Upadhyay & Mishra, 2002), photoperiod (Jolly *et al.,* 1971), X-rays (Kanarev & Chan, 1985), magnetic field (Upadhyay & Tripathi, 2005; Tripathi, 2010) etc. on the performance of *B. mori*. The effect of bioactive phytojuvenoid compounds on *B. mori* has been wide interest. Prothoracicotropic hormone (PTTH), juvenile hormone (JH) and ecdysone regulate growth and development in insect (Wigglesworth, 1985). Phytojuvenoids influence silk production (Pandey & Upadhyay, 2012) and reproductive parameters (Srivastava & Upadhyay, 2012, Tripathi, 2022) of *B. mori.*

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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.

Objective of this study was to investigate the effect of bioactive phytojuvenoid compounds derived from *Azadirachta indica* and *Pinus longifolia* on the level of total protein contents in the fat body and haemolymph of fifth instar larvae and pupae and silk gland of fifth instar larvae of *Bombyx mori*. The *A. indica* and *P. longifolia* are used for experiment due to their easily availability and juvenile hormone 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 (23x20x5cm) 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 till the emergence of moths. The grainage operation was performed followed to the Krishnaswami *et al.* (1973). Male and female newly emerged moths were allowed to mate and after four hour 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 hours of egg laying, the female moths were individually examined for their disease freeness and crushed individually in the 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 minutes to increase the adhesiveness of eggs on the paper sheet and surface disinfection. Thereafter, the eggs 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.

Design of Experiment:

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^{0}C. The dried materials were grinded separately with the help of mechanical device. Further, 50 gm grinded material of *A. indica* was subjected for extraction through soxhlet apparatus with 250 ml of petroleum ether (B.P. 40-60^{0}C) for 40 h. Extracted materials 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. 5.0 gm 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 phytojuvenoid compounds were applied by spraying as 1.0 ml on to 100 larvae of *B. mori* separately. Three sets of experiments were designed

Table 1: Effect of phytojuvenoid compounds on the total protein content in the silk gland of fifth instar larvae of silkworm, *B. mori*

Source of Phytojuvenoid compounds	Number of treatments	Protein Content (µg/mg tissues) Phytojuvenoid Concentrations (%)				F_1-ratio n_1=3
		5	15	25	35	
	Control	48.142±1.502	48.142±1.502	48.142±1.502	48.142±1.502	
Azadirachta indica	Single	50.124±1.123	51.102±1.132	53.124±2.405	54.245±2.176	25.13*
	Double	52.152±1.356	53.547±1.102	54.234±2.006	53.143±1.954	
	Triple	54.124±2.201	55.147±2.175	58.427±2.135	50.142±1.128	
Pinus longifolia	Single	51.427±1.129	53.243±2.146	55.943±2.175	58.243±2.124	
	Double	53.751±1.164	55.243±1.128	57.143±2.124	57.124±1.153	
	Triple	55.257±1.118	57.752±2.148	60.248±2.142	55.129±2.24	

Note: Each value represents mean±S.E. of six replicates. F_2-ratio= 17.25** n_2=6 *P_1<0.01 **P_2<0.01

Table 2: Effect of phytojuvenoid compounds on the total protein content in larval haemolymph and pupae of silkworm, *B. mori*

Stages of Development	Source of Phytojuvenoid Compounds	Number of treatments	Protein Content (µg/mg tissues) Phytojuvenoid Concentrations (%) 5	15	25	35	F_1-ratio n_1=3
		Control	62.125±0.705	62.125±0.705	62.125±0.705	62.125±0.705	
V instar Larvae	*Azadirachta indica*	Single	64.541±2.002	64.985±1.002	66.582±2.101	68.521±2.001	19.18*
		Double	66.592±2.001	69.598±2.451	71.645±2.432	67.508±3.002	
		Triple	68.624±3.001	71.625±3.042	73.056±1.003	66.501±2.001	
	Pinus longifolia	Single	64.552±2.001	66.595±2.212	69.645±3.001	71.534±3.002	
		Double	66.899±2.452	69.635±3.241	73.689±3.242	70.518±3.001	
		Triple	69.626±3.103	74.656±3.453	78.124±3.002	68.508±2.003	
		Control	55.184±1.253	55.184±1.253	55.184±1.253	55.184±1.253	
Pupae	*Azadirachta indica*	Single	57.246±2.435	59.243±1.985	62.457±2.104	64.150±2.176	
		Double	60.124±2.156	63.247±2.473	65.658±2.104	63.154±2.164	
		Triple	62.165±2.451	65.143±2.145	68.952±2.895	61.245±2.654	
	Pinus longifolia	Single	59.245±2.154	61.265±2.143	64.329±2.654	66.427±2.457	
		Double	63.124±2.421	65.417±2.534	68.451±3.145	64.157±2.438	
		Triple	65.457±2.347	68.154±2.147	71.146±3.245	62.457±2.457	

Note: Each value represents mean±S.E. of six replicates. F_2-ratio= 12.85** n_2=13 *P_1<0.01 **P_2<0.01
Each value represents mean±S.E. of six replicates.

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 and then transferred in to BOD incubator for rearing and development. 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 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 separated from BOD incubator and were treated with 1.0 ml of 5% concentrated solution of *A. indica* extract by sprayer and further kept in the BOD at normal rearing. The second and third treatments were given at the initial stage of fourth and fifth instar of same larvae and then kept in the BOD incubator for rearing. 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*. Total protein contents from haemolymph and fat body of fifth instar larvae and pupae and silk gland of fifth instar larvae of *B. mori* was estimated from the treated larvae.

For estimation of total protein contents from the silk gland, haemolymph and fat body of fifth instar larvae and fat body and haemolymph of pupae, the fifth instar larvae and pupae were dissected in distilled water and 1.0gm, 1.0ml and1.0gm silk gland, haemolymph and fat body were taken respectively. Total protein content was estimated according to Lowry *et al*; (1951) and modified by Singh & Agarwal (1989).

Statistical analysis:

Six replicates of each experiment were made and all the data obtained were analysed statistically by Two-way ANOVA.

RESULTS

Total protein in the silk gland of fifth instar larvae:

Variations in the concentrations of phytojuvenoid extracted from *Azadirachta indica* and *Pinus longifolia* and their number of treatments for larvae influence to the level of total protein contents in the silk gland of *Bombyx mori* (Table 1). The level of total protein contents in the silk gland increased with increase in the concentration of phytojuvenoid compounds in both *A. indica* and *P. longifolia* 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 increasing trend in level of total protein contents in the silk gland with increase

in concentration of phytojuvenoid was almost similar in single and double treated larvae while in triple treated larvae, the level of total protein contents was gradually increase from 5 to 25% concentration and decreased in 35% concentration. Maximum amount of total protein in the silk gland was 60.248 ± 2.142 µg/mg tissue in case of larvae with 25% concentration of phytojuvenoid extracted from *P. longifolia* treated thrice and minimum level protein contents 48.142 ± 1.502 µg/mg tissue recorded in control. The statistical analysis by Two-way ANOVA shows that variation in the concentrations of phytojuvenoid compounds (P_1<0.01) and number of treatment of larvae (P_2<0.01) significantly influenced to the level of total protein contents in the silk gland of the fifth instar larvae of *B. mori*.

Total protein in the haemolymph of fifth instar larvae and pupae:

Results obtained shows that variation in the concentration of phytojuvenoid and their number of treatments caused change in the level total protein contents in the haemolymph of fifth instar larvae and pupae of *B. mori* (Table 2). Single treatment increased the total protein contents in haemolymph with the increase in concentration of phytojuvenoid compounds which ranged from 5 to 35% and it was highest with 35% concentration, while in double and triple treatment the level of total protein concentration increased from 5 to 25% and decreased with 35% concentration. The single and double treatment The level of total protein increased slowly and reached 73.689±3.242 µg/mg tissue with single treatment, whereas it increased to 68.451 ± 3.145 µg/mg tissue with double treated of 25% phytojuvenoid extracted from *P. longifolia*. In the larvae with triple treatment the level of total protein reached its highest 78.124 ± 3.002 µg/mg tissue for larvae and 71.146 ± 3.245 µg/mg tissue for pupae in the larvae treated with 25% concentration of phytojuvenoid extracted from *P. Longifolia,* whereas it was lowest 62.125 ± 0.705 µg/mg tissue in control. Two-way ANOVA indicates that variation in the concentrations as well as number of treatments with phytojuvenoid compounds extracted from *A. indica* and *P. longifolia* significantly (P_1<0.01 and P_2<0.01 respectively) influence to the level of total protein contents in the haemolymph of fifth instar larvae and pupae of *B. mori*.

Total protein in the fat body of fifth instar larvae and pupae:

The results of the present study cclearly that the change in the phytojuvenoid concentration and their number of treatments influence the level of total protein content in the fat body of fifth instar larvae and pupae of *B. mori*. In case of single, double and triple treated larvae, the level of total protein contents increased with the increase in concentration of phytojuvenoid compounds extracted from *A. indica* as well as *P. longifolia* from

Table 3: Effect of phytojuvenoid compounds on the protein content in larval fat body and pupae of silkworm, *B. mori*

Stag of Development	Source of Phytojuvenoid Compounds	Number of treatments	Protein Content (µg/mg tissues) Phytojuvenoid Concentrations (%) 5	15	25	35	F_1-ratio n_1=3
		Control	54.125±1.705	54.125±1.705	54.125±1.705	54.125±1.705	
V instar Larvae	*Azadirachta indica*	Single	55.124±1.002	55.785±2.001	56.296±1.001	57.521±1.004	17.26*
		Double	56.592±2.001	58.898±1.061	60.645±2.122	57.008±1.002	
		Triple	58.624±2.001	61.625±2.682	64.056±2.003	56.501±2.032	
	Pinus longifolia	Single	56.552±2.001	58.595±3.002	62.645±1.691	63.534±2.002	
		Double	57.599±2.002	59.635±2.031	63.689±2.002	62.518±2.301	
		Triple	59.626±2.003	63.656±3.003	68.124±3.002	61.508±2.003	
		Control	52.184±1.253	52.184±1.253	52.184±1.253	52.184±1.253	
Pupae	*Azadirachta indica*	Single	54.167±1.025	55.137±2.123	57.128±2.151	59.427±1.245	
		Double	56.129±2.321	58.143±2.145	60.246±2.538	58.854±1.457	
		Triple	57.652±1.345	61.245±2.143	63.552±2.895	57.146±2.134	
	Pinus longifolia	Single	54.578±1.0234	56.124±2.165	59.012±1.912	61.0425±1.854	
		Double	58.147±2.018	60.124±2.324	63.175±2.157	60.541±2.153	
		Triple	69.274±2.457	63.245±2.354	66.146±2.245	59.245±2.356	

Note: Each value represents mean ± S.E. of six replicates. F_2-ratio= 11.65** n_2=13 *P_1<0.01 **P_2<0.01

5- 25% and was maximum 68.124±3.002 µg/mg tissue in the larvae and 66.146±2.245 µg/mg tissue in pupae) in case of triple treated larvae with 25% concentration of phytojuvenoid extracted from *P. longifolia*. The level of total protein increased in with single treatment which decreased with double and triple treatment of 35%. The lowest total protein content was 52.184±1.253 µg/mg tissue recorded for the control larvae. Two-way ANOVA indicates that variation in the concentrations (P_1<0.01) and number of treatments (P_2<0.01) of larvae with phytojuvenoid compounds extracted from *A. indica* and *P. longifolia* significantly influence to the level of total protein in the fat body of fifth instar larvae and pupae of *B. mori*.

DISCUSSION

Variation in the concentration of phytojuvenoid compounds extracted from the *Azadirachta indica* as well as *Pinus longifolia* and their number of treatments given to the fifth instar larvae influenced the level of total protein contents in the silk gland, haemolymph and fat body larvae and pupae of *B. mori*. 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 impacted egg fertility in insects (Chaudhuri, 2003; Fischer *et al.*, 2004) and maternal ecdysteroid appear to be required at different tier for fertilization, embryogenesis and egg hatching in the silkworm (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 silk denier (Bharthi & Miao, 2003). Administration of phytojuvenoid at different age of fifth instar of *B. mori* increased both reelability of silk filament and denier (Nair *et al*., 2005) whereas, treatment of larvae with phytojuvenoid increases reproductive potential of *Bombyx mori* (Tripathi, 2022). 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 region protein synthesis resulting in bigger silk gland and the improvement of cocoon shell weight (Garel, 1983). In present study it was observed that the increase in level of total protein in silk gland, haemolymph and fat body of fifth instar larvae is dependent on the increase the concentration of the phytojuvenoid extracted from *A. indica* and *P. longifolia* .

Methopren and fenoxycarb treated *B. mori* larvae showed significant enhancement in the silk denier (Mamatha *et al*., 2006) and the process of growth and development in insect are regulated by prothoracicotropic hormone (PTTH), juvenile hormone (JH) and ecdysone, which either directly or indirectly manifest to 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 shown positive effect on the reproductive potential of *B. mori* (Prasad & Upadhyay, 2012) and the phytojuvenoid caused beneficial effect on the life pattern of silkworm (Srivastava & Upadhyay, 2013; Srivastava & Upadhyay, 2016). In present study, the total protein contents in the haemolymph and fat body of pupae of *B. mori* also increased with the increase in concentration and number of treatment with phytojuvenoid compounds extracted from *A. indica* and *P. longifolia*.

CONCLUSION

Thus, the level of total protein content in the fat body, haemolymph and silk gland of fifth instar larvae, pupae of *B. mori* increased with increase concentration 5-25% and dose of treatments using phytojuvenoid compounds extracted from *A. indica* and *P. Longifolia,* whereas, with 35% concentration, protein content increased with single treatment but further decreased gradually with third treatment to the larvae. The influence of bioactive phytojuvenoid compounds might have the inhibitory effect on the ecdysone, which leads to delay in moulting with increased larval duration, resulted more feeding and increased level of total protein contents in the tissues of *B. mori*. The higher concentration of bioactive phytojuvenoid in triple treated larvae caused decrease in level of total protein contents in the tissues of larvae and pupae which may be due to their stress response.

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