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Alteration in the primary metabolites in three different tissues of silkworm, *Bombyx mori* L. under the influence of a Juvenoid, R394

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ABSTRACT

A bivoltine silkworm hybrid, KA x NB_4D_2 was treated with the juvenoid R394 (Ethyl-9 cyclohexyl-3,7-dimethyl-2,4-nonadienoate) at a dose of 0.039 nl/larva at 24, 48, 72 and 96 h of 5th instar for silk yield improvement. Treatment specific significant increase was observed in the cocoon and shell weight with no such marked variation in the shell percentage. Notable changes were also seen in traits such as larval duration, larval weight and silk gland weight in the treated silkworm. Total protein, total carbohydrate and total lipid were analyzed in the posterior silk gland (PSG), haemolymph and fat body of fully grown larvae. The result indicated that the content of these primary metabolites varied significantly in the selected tissues depending on the time of juvenoid application. The highest content of protein in the haemolymph and silk gland was in the larvae treated at 72 h whereas the fat body protein content was lowest in the same group. The total carbohydrate was recorded lowest in the 72 h treated larvae as against the highest in the control both in haemolymph and fat body with no significant change in PSG. The total lipid content did not show any notable variation in the concentration on juvenoid administration except in the silk gland treated up to 72 h which showed a decline. The result indicates that the juvenoid induces tissue specific responses in terms of turnover in primary metabolites.

Key words: *Bombyx mori,* Fat body, Haemolymph, Juvenoid, R394, Silkworm

INTRODUCTION

Using juvenoid in sericulture for yield improvement has not been a concept confined to laboratories. Many countries led by Japan used various juvenoids to achieve 5 – 30 % increase in the cocoon production (Akai *et al.*, 1985). But the increase in production was largely dependent on the dose of the compound administered and time of administration (Chowdhary *et al.,* 1990). Some of the treatment resulted in prolonged larval period (Akai *et al.,* 1985) with complementary increase in the silk production, but some did not (Muroga *et al.*, 1975). Our previous studies indicate that some plant based juvenoids do not induce a very prominent prolongation but in such cases, the increase hovers between 8 and 10 % (Nair *et al.*, 2002). The juvenoid, R394 is bioactive at extremely low concentration and can be a good candidate for field use. This compound also is a good example for inducing varied response according to the time of application. It induced moderate response in terms of increased yield when treated at 24 h with no prolonged larval period. But when treated at 48 and 72 h, the increase in production was more which was accompanied by a prolongation in larval period by 24 h (Nair *et al.,* 2001). On this backdrop, this study was taken up to examine the biochemical constituents of three major tissues of juvenoid treated silkworm to understand the effect of the compound on these tissues and to understand whether juvenoid induced increase in silk yield is commensurate with the changes seen in the biochemical alterations.

MATERIALS AND METHODS

The juvenoid, R394 (Ethyl-9 cyclohexyl-3,7-dimethyl-2,4-nonadienoate) was procured from Dr. L. Streinz, Institute of Organic Chemistry and Biochemistry, Czech Republic. Bivoltine silkworm hybrid (KA x NB_4D_2) was reared on fresh mulberry leaves (S_{36} variety) in the laboratory at 25 ± 1 °C temperature, 75 ± 5 % RH under 12:12 (L:D) photoperiod following standard procedure (Krishnaswami, 1978).

On resumption to 5th instar, 100 larvae were counted and transferred to ventilated plastic trays measuring 56 x 36 cm for treating them with R394 followed by biochemical estimations. The juvenoid was prepared at a concentration of 0.31 nl/ml in the form of an emulsion using 2-3 ml of acetone and 2-3 drops of surfactant, tween-20 in water.

The emulsion was administered once at the rate of 12.5 ml/100 larvae. This dose of R394 was determined after a series of dose response studies conducted on silkworm using wide range of concentrations (Nair *et al*., 1999). After leaving for 30 minutes, the larvae were fed with fresh mulberry leaves *ad libitum*. Different such batches were treated at 24, 48, 72 and 96 h of 5th instar.

Untreated control was maintained in parallel for comparison of the results. Five male and five female larvae were collected from each batch including the control on the day when spinning was about to commence and dissected in insect ringer. Three tissues *viz*., PSG, haemolymph and fat body were isolated from the larvae and pooled treatment-wise.

The tissues were stored at –20°C until the estimations were carried out. The larval weight on maximum growth and survival was recorded. On maturation, the larvae were mounted for cocoon spinning. Last instar larval period *i.e* the duration from the time of resumption to 5th instar until the initiation of cocoon spinning was also calculated. Cocoons were harvested on the 6th day and cocoon and cocoon shell weights were recorded from which shell percentage was calculated.

Total protein was estimated following the method of Lowry *et a*l. (1951) using crystalline bovine serum albumin as standard. The total carbohydrate content was estimated following the method of Carroll *et al*. (1956) using glucose standard and the total lipid content by the method of Folch *et al*. (1957). The observations were computed and the data were statistically analyzed using ANOVA to ascertain the statistical significance between the control and the treated silkworm and also among the treated batches.

RESULTE

Influence of juvenoid on the economic traits

The results on the influence of R394 on larva and silk gland weight, 5th instar duration, survival and cocoon characters are presented in Table 1. The response of almost all economic traits to the treatment of juvenoid was dependent on the time of application. In the larva and silk gland weight, maximum improvement was noti-ced in the treatment at 48 and 72 h. The improvement was to the tune of about 7.5 % in larva weight and above 20 % in the silk gland weight when compared to the control. Both the positive changes are statistically significant ($P < 0.05$). The treatment at 24 h showed significant improvement of 12 % in the silk gland weight (Table 1). The difference in larval weight when treated at 24 and 96 h and that in silk gland weight when treated at 96 h from the control was not significant ($P > 0.05$).

A comparison of larval weight among the treatment shows significant difference between the larvae treated at 24 and 48 h ($P < 0.05$) while among the other treatments the difference was not significant ($P > 0.05$). In the silk gland weight, the larvae treated at 24 h had significant difference from that treated at 72 and 96 h. 48 h treatment had significant difference from 24 and 96 h treatment.

72 h treated larvae had significant increase in the silk gland weight when compared to 24 and 96 h treated larvae whereas 96 h treated larvae has significantly low silk gland weight in comparison to 24, 48 and 72 h treated larvae ($P < 0.05$).Fifth instar larval feeding period and survival are important economic characters of consideration. Compared to control, the larvae treated at 48 and 72 h took 24 hours more.

This was a significant difference not only from the control but from the larvae treated at 24 and 96 h which had no difference in larval duration from the control (Table 1). The difference in survival among the treated larvae and that from the control was negligible and was not significant ($P > 0.05$).

R394 administration had a substan-tial positive influence on the cocoon weight and shell weight. The pattern of increase was significant with a maximum change of 14.27% in the 72 h treatment followed by 9.5 % increase in the 24 and 48 h treated batches ($P < 0.05$). However, the difference in cocoon weight between the larvae treated at 96 h and control was low and was not significant ($P > 0.05$). The difference in cocoon weight between 24 and 72 h, 24 and 96 h, 48 and 72h, 48 and 96 h, and 72 and 96 h were found significant ($P < 0.05$). Similar was the trend in cocoon shell weight as well. Inter- treatment significance followed the same pattern. Compared to the control, maximum improvement was noticed in the 72 h treated batches.

Table 1: Influence of the juvenoid, R394 on the economic traits of silkworm, *Bombyx mori* L (Hybrid: KA x NB_4D_2). Each value is the mean of 10 male and 10 female larvae.

Treatment hour in 5th instar	Larva weight (g)	Silk gland weight (g)	5th instar period (h)	Survival (%)	Cocoon weight (g)	Shell weight (g)	Shell percentage
24 (**a**)	4.425 ^{b} (3.02)	1.679 ^{bcd} (12.53)*	168 (0.00)	87.11 (0.78)	2.076 ^{cd} (9.56)*	0.417 ^{cd} (8.37)*	20.09 (-1.09)
48 (**b**)	4.627 ^{a} (7.72)*	1.798 ^{ad} (20.51)*	192^{ad} (14.29)*	86.67 (0.27)	2.074 ^{cd} (9.44)*	0.423 ^{cd} (10.01)*	20.42 (0.52)
72 (**c**)	4.620 (7.55)*	1.810 ^{cd} (21.31)*	192^{ad} (14.29)*	86.59 (0.17)	2.165 ^{abd} (14.27)*	0.445 ^{abd} (15.73)*	20.57 (1.28)
96 (**d**)	4.457 (3.76)	1.536 ^{abc} (2.96)	168 (0.00)	86.00 (-0.51)	1.938 ^{abc} (2.26)	0.395 ^{abc} (2.52)	20.36 (0.25)
Control	4.296	1.492	168	86.44	1.895	0.385	20.31
SE ±	0.045	0.036	3.650	0.98	0.023	0.004	NS
CD at 5 %	0.178	0.118	10.94	2.75	0.065	0.011	

Values in the parentheses are percentage difference from the control. * Shows statistical significance (P < 0.05). a, b, c and d in parentheses are used to designate different treatment hours for subsequent expression of statistical significance. Values when superscribed with a,b,c or d denotes statistical significance from the corresponding values.

Here as well, this was followed by the 48 h and 24 h treated batches. The maximum improvement was 15.73 %. In the shell percentage though, there was no significant difference either among the treatments or between treatment and control.

Influence on biochemical constituents

The influence of the juvenoid treatment on the biochemical constituents of the three selected tissues was prominent. The total protein content of the haemolymph of the treated silkworm varied significantly (P < 0.05) among themselves and also when compared to the control. The highest haemolymph protein content was observed in the silkworms treated at 72 h which was 33 % more than that of the control followed by 48 h (19.60 %) and 24 h (17 %). Interestingly, there was no much difference (P > 0.05) between total protein content of 96 h treated silkworm and that of the control (Table 2). Except between the treatment at 24 and 48 h and between 48 and 72 h, all other treatments had significant difference in total protein content in the haemolymph on juvenoid administration.

The sequence was reversed in the fat body almost in the same manner. In this case, though the protein content ranged from about 67 to 83 mg/g tissue among the treated silkworms, the lowest level of protein was observed in the silkworm treated at 72 h. This was 22.29 % less than that of the control. The difference between the control and treated was significant except between 96 h and the control. In the PSG, there was a marked positive and significant difference in total protein content in the silkworm treated at 24, 48 and 72 h compared to the control (P < 0.05). The maximum protein content was noticed in the silkworm treated at 72 h with a percentage change of 37.88 followed by that treated at 48 h with 25.25 % change (Table 2). The difference among the treatment was also significant except that between 24 and 96 h treated larvae.

The concentration of total carbohydrate content also followed varied pattern in the different tissues studied. In haemolymph, the total carbohydrate was maximum in the control (Table 3).The lowest content was available in the 72 h treated silkworms, which was 22.69 % less than that of the control. The 48 h treated silkworms followed this with 20.09 % reduction. The difference in the carbohydrate content in the case of treatment at 24 and 96 h also was prominent and all these values were statistically significant.

Data also show that the difference between 24 and 72 h and 72 and 96 h are significant where as difference between 48 and 72 h and 24 and 96 h are statistically not significant (P < 0.05). The total carbohydrate content in the fat body followed a different pattern. The maximum content was in the control silkworm but unlike in the haemolymph, the minimum was in the 24 h treated silkworm.

The prominently low content of fat body total carbohydrate in all the treatments when compared to the control are significant. Further perusal of the data reveals that the difference from 24 h treatment to 48, 72 and 96 h were significant. Differences in the carbohydrate content between 48 and 72 h treatment also found to be statistically significant (P < 0.05).

Table 2: Changes in the total protein content in the tissues of silkworm, *Bombyx mori* L. on administration of the juvenoid, R394. Each value is the mean ± SD of 5 separate observations. Tissues of 10 larvae (5 males and 5 females) were pooled for each sample.

Treatment hour in 5th instar	*Tissues*		
	Haemolymph mg/ml	*Fat body mg/g wet tissue*	*Silk gland mg/g wet tissue*
24 (a)	45.360 ± 2.353^{cd} (17.01)*	74.275 ± 3.434^{cd} (-13.45)*	112.112 ± 2.916^{bc} (11.79)*
48 (b)	46.366 ± 2.055^{d} (19.60)*	72.447 ± 4.094^{d} (-15.58)*	125.605 ± 6.320^{acd} (25.25)*
72 (c)	51.617 ± 1.752^{ad} (33.15)*	66.695 ± 2.807^{ad} (-22.29)*	138.280 ± 5.543^{abd} (37.88)*
96 (d)	39.114 ± 1.432^{abc} (0.89)	82.702 ± 2.586^{abc} (-3.63)	105.496 ± 2.581^{bc} (5.19)
Control	38.767 ± 1.815	85.380 ± 2.282	100.287 ± 3.155
SE ±	1.200	1.491	1.999
CD at 1 %	5.697	7.073	9.485

Values in parentheses are percentage difference from the control. * Shows statistical significance (P < 0.05). a, b, c and d in parentheses are used to designate different treatment hours for subsequent expression of statistical significance. Values when superscribed with a,b,c or d denotes statistical significance from the corresponding values.

At the same time, the difference in the content between 48 and 96 h treatment and 72 and 96 h treatment did not show significance.

In silk gland on the other hand, the differences in carbohydrate content were statistically insignificant (P > 0.05). The highest carbohydrate concentration was found in the larvae treated at 24 h and the lowest in the 96 h treated batch. The juvenoid administration did not exert any significant effect on the total lipid content in haemolymph and fat body.

The difference in concentration of total lipid between the treated larvae at different hours and control was meager and statistically not significant (P > 0.05). The difference among various treatment also was insignificant. The lowest lipid content in the haemolymph was noticed in the silkworm treated at 72 h, which was 8.71 % less than that in the control. The other treated batches were almost near to the control.

In the fat body, the total lipid content varied from 80 to 85 mg/g tissue, the highest being 85.67 mg/g in the control silkworm. The lowest content was found in the larvae treated at 72 h which was 5.88 % less than that of the control.

In the silk gland, the treatment at 24, 48 and 72 h induced a significant decline (P < 0.05). The highest lipid content was noticed in the control and the lowest in the 48 h treated silkworms. The silk gland of the 96 h treated silkworm had total lipid content almost at par with the control (Table 4). The difference among the treatments was not significant.

DISCUSSION

In the silkworm, *B. mori*, the final larval stage is the most active feeding period during which the larvae accumulate large quantity of bimolecular reserves in various tissues and are endowed with unique biochemical adaptation to conserve nutritional resources for cocoon spinning, metamorphosis and reproduction (Hugar & Kaliwal, 1998).

The three tissues viz., haemolymph, fat body and silk gland selected to study the effect of juvenoid on the silk production assume much significance when the role played by these tissues in silk synthesis is considered. At the stage of cocoon spinning, the cells in the silk glands, especially posterior silk gland synthesize large amount of fibroin, the main protein in the silk filament.

This protein synthetic activity implies coordinated functioning of all elements of the cell machinery devoted to fibroin assembling and maturation which would primarily depend on the availability of resources.

The changes in the haemolymph pool of nutrients certainly affect silk gland development and link it to food digestion and reserve mobilization (Sehnal & Akai, 1990). Since haemolymph is the immediate environment of the organs in the silkworm, the metabolic activity and the development are affected by the haemolymph (Nakayama *et al*., 1990). Fat body plays a very vital role in the storage of biomolecules and responds to the fluctuation of the metabolites in the haemolymph fairly quickly (Tojo *et al*., 1981).

Proteins are the chief organic constituents of the cell. These macromolecules are concerned with the regulation of all biochemical events in the organism (Harper *et al*., 1993).

Table 3: Changes in the total carbohydrate content in the tissues of silkworm, *Bombyx mori* L. on administration of the juvenoid, R394. Each value is the mean ± SD of 5 separate observations. Tissues of 10 larvae (5 males and 5 females) were pooled for each sample.

Treatment hour in 5th instar	*Tissues*		
	Haemolymph mg/ml	*Fat body mg/g wet tissue*	*Silk gland mg/g wet tissue*
24 (**a**)	15.229 ± 1.012^{c} (-12.89)*	46.970 ± 1.275^{bcd} (-43.83)*	12.167 ± 1.576 (7.35)
48 (**b**)	14.035 ± 0.807 (-20.09)*	60.916 ± 2.044^{ac} (-27.15)*	10.467 ± 0.613 (-7.67)
72 (**c**)	13.578 ± 0.818^{ad} (-22.69)*	55.192 ± 1.708^{ab} (-33.99)*	10.937 ± 0.358 (-3.53)
96 (**d**)	15.898 ± 0.287^{c} (-9.48)*	57.933 ± 2.179^{a} (-29.92)*	10.062 ± 0.396 (-8.60)
Control	17.563 ± 0.746	83.620 ± 2.358	11.337 ± 0.636
SE ±	0.466	1.178	NS
CD 5 %	1.519	3.845	

Values in parentheses are percentage difference from the control. * Shows statistical significance ($P < 0.05$). a, b, c and d in parentheses are used to designate different treatment hours for subsequent expression of statistical significance. Values when superscribed with a,b,c or d denotes statistical significance from the corresponding values. NS: Non-significant

Table 4: Changes in the total lipids content in the tissues of silkworm, *Bombyx mori* L. on administration of the juvenoid, R394. Each value is the mean ± SD of 5 separate observations. Tissues of 10 larvae (5 males and 5 females) were pooled for each sample.

Treatment our in 5th instar	*Tissues*		
	Haemolymph mg/ml	*Fat body mg/g wet tissue*	*Silk gland mg/g wet tissue*
24	32.369 ± 1.751 (-5.59)	82.437 ± 3.607 (-3.77)	42.421 ± 1.868 (-11.06)*
48	32.697 ± 2.768 (-4.63)	81.578 ± 3.769 (-4.77)	43.278 ± 2.588 (-9.23)*
72	32.287 ± 2.612 (-8.74)	80.635 ± 3.963 (-5.88)	42.826 ± 2.001 (-10.21)*
96	33.678 ± 2.822 (-1.77)	84.637 ± 3.068 (-1.20)	46.232 ± 1.456 (-3.07)
Control	34.285 ± 2.368	85.668 ± 3.581	47.695 ± 1.902
SE ±	NS	NS	1.252
CD 5 %			4.084

Values in parentheses are percentage difference from the control.
* Significant ($P < 0.05$), NS: Non-significant.

Accumulation of proteins in haemolymph and fat body during final instar insect development has been established by Chen (1985). Apart from this, in silk gland, accumulation of protein is a well-established fact. The data in this study show that the juvenoid treated mature larvae had more haemolymph protein than the control had. But in the fat body, the total protein content was comparatively low in the treated larvae compared to control. In silk gland on the other hand, there was a significant positive difference in the total protein content especially in silkworms treated at 72 h. The decreased protein content in the fat body on JH administration might be because some of the proteins were channelised to haemolymph through which it was transported to silk gland. Such high protein content seen in the haemolymph of the treated larvae suggests that the protein synthesis and accumulation is mediated by the juvenoid to an extent. The report of Thomas and Nation (1966) that withdrawal of JH inhibits protein synthesis may be correlated to this. The role of haemolymph and fat body in synthesis and storage of proteins in *B. mori* towards silk spinning have been documented earlier (Tojo *et al.,* 1981). It was also indicated that such synthesis and release is hormonally mediated and JH and ecdysterone play important roles in this (Riddiford & Truman, 1978). The elevated amount of protein content in the haemolymph and silk gland of treated larvae towards spinning indicates a hormonally mediated

preparation of these tissues mainly silk gland for attaining competence for cocoon spinning. It is vivid from the present study that the total carbohydrate has declined in the treated silkworm in all the three tissues investigated. But the extent of reduction is less in silk gland compared to fat body and haemolymph.

This indicates an increased mobilization of carbohydrate reserves both from fat body and haemolymph mainly towards the cocoon spinning process. Interestingly, such prominent changes were not visible in the silk gland, which implies that silk gland is independent of the exogenous juvenoid in respect of its carbohydrate content.

Lipids constitute not only an essential and integral component of cell membranes but also act as an important source of energy for various metabolic activities of which reproduction and flight are important (Gilbert, 1967). In domesticated silkworm, since flight is not an important function and is limited to a short precopulatory phase, its physiological role for reproduction may assume much more importance.

Nonetheless, the role of lipids as an energy source for cocoon spinning and metamorphic activity cannot be overemphasized. In the present study, however, the haemolymph lipid did not respond to the exogenous juvenoid application and a very little change was observed between treated and control. In the fat body lipid content, the larvae treated at 72 h showed the lowest concentration and compared to the control the difference was highest. Similarly, in the silk glands of the larvae treated at 24, 48 and 72 h, it declined to a significant level. The literature reveals that removal of active CA resulted in an accumulation of fat body triacylglycerol and such hypertrophy can be reversed through implantation of CA to the allatectomized insects. As per Gilbert (1967), inclusion of CA with incubated fat body tissue suppresses the incorporation of fatty acid into triacylglycerol. These are indications that JH plays a crucial role in lipid metabolism. But in this study such prominent changes was not noticed in the fat body lipids.

These observations on the changes in the concentration of primary metabolites especially total protein and carbohydrate correspond to the changes noticed in the cocoon and cocoon shell weight and the weight put on by the silk gland as well as the larval body. Survival of the larvae was not much affected by the treatment mainly because the dose of the compound was tolerable level and intended to induce positive changes. Similarly, there was no significant change in shell percentage because, the change in cocoon weight and shell weight was almost in the same level.

The increment in the larval body weight, silk gland weight, cocoon weight and cocoon shell weight is directly correlated to the prolongation in the larval duration. The treatment at 48 and 72 h had a significant effect on the larval duration whereas treatment at 24 and 96 h were unaffected with regard to larval duration. All other economic traits almost followed the same trend. The juvenoid effect in 5th instar silkworm at the threshold concentration is usually prominent when treated at the critical period between 48 and 72 h. Treatment before completion of 24 h turns out to be less effective as target tissue programming and preparation is not probably on for juvenoid action or the dose at that level for relatively smaller body mass suitable.

Quite interestingly, the treatments at 96 h of 5th instar have not shown any notable effect on any of the parameters studied. This observation can be examined in the light of the report that JH application to early last instar larvae lowers the threshold of PG sensitivity to PTTH while treatment later in the instar stimulates it (Cymborowski and Stolarz, 1979). It is emerging that to induce JH effect in 96 h treated larvae, a much higher concentration may be required than what was used, based on the physiological status of the silkworm owing to the body size and the ratio of the titre of JH and ecdysone. It may also be a probability that by 96 h of age in the 5th instar, the target tissues have almost taken over by the higher level of ecdysone, for the reversal of which a still higher dose of juvenoids is required. But such a reversal may end up in inordinate delay of spinning or may end up in non-spinning.

This study makes it clear that the major tissues such as silk gland, fat body and haemolymph of silkworm respond to juvenoid treatment in ways specific to these tissues which culminates in increased silk production. This underlies the fact that juvenoids are bioactive compounds on silkworm and the enhanced yield on its exogenous administration is substantiated by the biochemical changes.

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