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Influence of the diterpenoids (Retinol and Phytol) (Race: PM x CSR2) on the cocoon and silk parameters in silkworm, *Bombyx mori* (L) (Race: PM x CSR2)

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ABSTRACT

Topical application of ten microliters of one milligram per liter strength acetone solution of retinol phytol to the individual fifth instar larvae of multivoltine cross breed race of silkworm, *Bombyx mori* (L) (PM x CSR2) at 48, 54, 60 and 66 hours after the fourth moult was found variously reflected into prolongation of larval age (14 – 39 % for retinol treated groups and 17 – 42 % for phytol treated groups); improvement in the tissue somatic index (TSI) of silk glands (3.053 – 3.343 for retinol treated groups and 3.070 – 3.092 % for phytol treated groups); cocoon shell ratio (2.790 – 3.177 for retinol treated groups and 2.693 – 3.629 for phytol treated groups) and denier scale of silk filament ($p < 0.01$). Retinol and phytol, thus chiefly lengthening the larval age in silkworm, *Bombyx mori* (L). The synergistic activity of diterpenoids in the present attempt is hypothesized to be due to changes in the membrane fluidity, interference with membrane bound signaling proteins and cell cycle arrest. Efficient utilization of diterpenoids like, retinol and phytol, through acetone solvent for topical application to the fifth in stared larvae of silkworm, *Bombyx mori* (L) may open a new biotechnological avenue in the sericulture industries.

***Keywords*:** Retinol; Phytol; TSI; Shell Ratio; Denier Scale of Silk Filament

1. INTRODUCTION

Vitamins are the organic compounds required by organism a vital nutrient in limited amounts. Supplementation of vitamins serve to orchestrate the metabolism. The larvae of silkworm, *Bombyx mori* (L) deserve appreciation for synthesis of silk for it's metamorphosis. Sericultural practices are serving a lot to provide the silk fibre. The silkworm, *Bombyx mori* (L) exert a significant influence on the concept of insect metamorphosis through it's simple life cycle and efficient utilization of the nutrients from the mulberry, *Morus alba* (L). Interplay of juvenile hormone and moulting hormone in the insect larval body serves to orchestrate the progression of metamorphosis from one instar to next, with moulting hormone regulating the onset and timing of moulting cycle and juvenile hormone regulating the quality of moult (Riddiford, 1985, 1994; Sehnal, 1985).

During the last larval stadium of holometabolous insects, such as silkworm, *Bombyx mori* (L), a reduction of JH in haemolymph is the necessary step in the initiation of metamorphosis. It has been demonstrated that, haemolymph ecdysteroid and JH level undergo the developmental changes during larval - larval and larval - pupal cycles in silkworm, *Bombyx mori* (L) (Calvez *et al*, 1976; Kiguchi and Agui, 1981). Juvenoids are well known in prolonging the larval life in the insect and have long been tried for qualitative improvement of silk (Ratnasen, 1988; Granier and Granier, 1983 and Mamatha *et al*., 1999).

There is considerable evidence that juvenile hormone mimics occur in plants, which occasionally leads to economically important consequences in the insect development (Slama, 1979). Juvenile hormone active compounds are found in many higher plants, exogenous application through suitable solvents of which exhibited potent activity in the insects (Prabhu and John, 1975). Efficient use of available system, the principle of quality improvement made man to use juvenoids for pest control as well as for the silk yield. Use of juvenoids (synthetic, plant derived and animal derived) in the rearing of silkworm larvae had positive influence, especially in the silk yield (Ching et al, 1972; Nihmura et al., 1972; Muroga et al, 1975; Kamada and Shimada, 1988; Rajashekhargouda, 1991; Vitthalrao *et al*, 2002, 2003 and Vitthalrao, 2004).

The terpenes and terpenoids mimics the action of natural insect juvenile hormone. They are widespread in nature and are involved in much more biological activities including morphogenesis, embryogenesis and cellular differentiation. Diterpenoids are structurally highly complex, but the biosynthetic routes are relatively simple: only two enzyme families are involved in the key steps. Geranylgeranyl-diphosphate (GGPP), the ubiquitous C-20 precursor for all diterpene biosynthesis is cyclised by diterpene synthases (diTPS). The resulting diterpenes are further functionalised by enzymes of the cytochrome P450 mono-oxygenase class (P450) (Hamberger and Bak, 2013). The retinol is a diterpenoid, a terpenoid derived from a diterpene, which include the compounds with C20 skeleton of the parent diterpene, which has been rearranged or modified by the removal of one or more skeletal atoms (generally, methyl groups).

The retinoids deserve important role in the process of morphogenesis and in immune response in the insects like R. prolixus, suggesting that the molecular mechanism recognize the terpenoid backbone as one of the important structural determinant in insects (Angelica Nakamura, *et al*., 2007). Insects, such as the sumac flea beetle, are reported to use phytol and its metabolites (e.g. phytanic acid) as chemical deterrents against predation (Vencl and Morton, 1998).

These compounds originate from host plants. Indirect evidence has been provided that, in contrast to humans, diverse non-human primates can derive significant amounts of phytol from the hindgut fermentation of plant materials (Watkins, *et al* , 2010 and Moser, *et al*., 2013). Terpenoid hormones seems to act as the morphogens throughout the metazoan. Phytol is an acyclic diterpene alcohol that can be used as a precursor for the manufacture of synthetic forms of vitamin E and vitamin K1. In ruminants, the gut fermentation of ingested plant materials liberates phytol, a constituent of chlorophyll, which is then converted to phytanic acid and stored in fats (Van Den Brink and Wanders, 2006). The regulatory activities of terpenoid hormones range from controlling metamorphosis in insects (Riddiford and Ashburner, 1990) and to determine the germ cell fate in the mammalians (Bowels, *et al*., 2006). In the metamorphosis, the interplay of the juvenile hormone and ecdysone serve to orchestrate the progression from one instar to the next, with ecdyosteroid regulating the onset and timing of the moult and JH determining whether the moult would be larval – larval or larval – pupal (Gilbert, *et al*, 1996; Mamatha, et al., 2005).

Phytophagous insects like silkworm, *Bombyx mori* (L) derive their juvenoid nutrients through the plant material available for them (Khyade, *et al*., 2007). Retinol and phytol like vitamin nutrients may either be synthesized by the insect tissue or derived from the plant material. Nutrition with vitamins is playing important role in the improvement of growth and development in silkworm, *Bombyx mori* (L).

Juvenoids are known for disruption of normal developmental pattern leading to the deformities in the insects. Interestingly, the silkworm, *Bombyx mori* (L) is known to have a stimulatory influence on the administration of exogenous Juvenoids (JHA) in an appropriate quantities. The specific titer of juvenoids, either topical or through the food, at the specific period of the larval instars of silkworm, *Bombyx mori* (L) are positively reflected into the retention of larval features long enough enabling the larvae to consume maximum quantity of mulberry leaves and to synthesize paramount silk to be used in spinning the qualitative cocoon (Akai, *et al*., 1990; Mamatha, *et al*., 2005, 2006, 2008; Chowdhary, *et al*., 1990; Miranda, *et al*., 2002; Mamatha, *et al* , 2006, 2008). Topical application of acetone solution of retinol has been reported for juvenoid activity and recommended for rearing the fifth instar larvae of silkworm, *Bombyx mori* (L) (Vitthalrao and Sarwade, 2013; Vitthalrao *et al*., 2015). Diterpene structure , insect juvenoid activity and vitamin nature of Retinol and Phytol made to plan for the efforts on it's topical application through the acetone to the fifth instar larvae of silkworm, *Bombyx mori* (L) (PM x CSR2).The objective of the study is to analyze the diterpenoid, especially, Retinol and Phytol for the cocoon and silk characters of silkworm, *Bombyx mori* (L) through the Race: PM x CSR2.

2. MATERIAL AND METHODS

All the facilities at the Sericulture Farm of Agriculture Development Trust, Baramati were utilized for the experimentations. The entire attempt was divided into the parts, which include: Rearing of the silkworm larvae; Topical application of retinol through the acetone solution to the fifth instar larvae; Analysis of parameters (Larval, cocoon and silk filament) and Statistical analysis of the data. The larvae of silkworm belongs to polyvoltine cross breed (PM x CSR_2) race were reared in the laboratory through standard methods (Krishnaswami *et* *al.*, 1978). The vitamin A, retinol and Phytol were procured through local dealer. One mg of retinol was dissolved in the acetone and the stock solution of one ppm was prepared. Likewise, one mg of phytol was dissolved in the acetone and the stock solution of one ppm was prepared.

Soon after fourth moult, the fifth instared larvae were grouped into one control group; one acetone treated group and eight (A1 and A2; B1 and B2; C1 and C2; D1 and D2) experimental groups, each with hundred individuals. Ten microliters of acetone solution of retinol was used for topical application to the individual larva in each group (A1; A2; A3 and A4) separately (Table – 1).

Ten microliters of acetone solution of phytol was used for topical application to the individual larva in each group (B1; B2; B3 and B4) separately (Table – 1). The experimental group: "A" received the topical application of retinol at 48 hours after the fourth moult. The experimental group: "B" received the topical application of retinol at 48 and 54 hours after the fourth moult. The experimental group: "C" received the topical application of retinol at 48; 54 and 60 hours after the fourth moult. And the experimental group: "D" received the topical application of retinol at 48; 54; 60 and 66 hours after the fourth moult. Untreated control (UTC) and Acetone treated control (ATC) groups of larvae were also maintained.

Table 1. Schedule of topical application of acetone solution (ten microlitere of one ppm) of retinol and phytol to the fifth instar larvae of silkworm, *Bombyx mori* (L).

Hours after IV moult→ group↓	**48**	**54**	**60**	**66**
UTC	-	-	-	-
ACT	-	-	-	-
A1	+	-	-	-
A2	+	-	-	-
B1	+	+	-	-
B2	+	+	-	-
C1	+	+	+	-
C3	+	+	+	-
D1	+	+	+	+
D3	+	+	+	+

\+ indicates topical application of ten microliters of one ppm solution of acetone to individual larvae of silkworm, *Bombyx mori* (L).

\- Indicates No Treatment. (ACT group were treated with Acetone).

Topical application was followed by feeding the larvae with tender mulberry leaves. The schedule of feeding was 100 gms of mulberry leaves for each time (48, 54, 60, 66 hours after the fourth moult) for each group of 100 larvae. Acetone treated control and untreated control groups of larvae were also maintained. Daily larval weight was recorded. For the purpose to calculate tissue somatic index (TSI) of silk glands, ten larvae from each group were selected at random on the fifth day, anesthetized, dissected and silk glands were separated. The silk glands were bottled and weighed on electronic balance. The weight of silk glands was divided by weight of larva. The quotient thus obtained was multiplied by 100. Weight of silk glands and larval body weight, thus, were accounted for the calculation of tissue somatic index (TSI) of silk glands.

The matured larvae (having transparent skin, feeding stopped and moving its head in specific manner for searching the surface for attachment of fluid silk) were transferred to the mountage for spinning the cocoon. The larval duration (right from zero hour of fifth instar to fifty percent spinning) was recorded. The cocoons were harvested on sixth day after mounting the mature larvae on the mountage. Cocoon weight, shell weight and pupal weight were recorded. Shell ratio was calculated. Ten cocoons per replication were reeled and length (m) of unbroken silk filament was obtained by using eprouvate. Weight of silk filament from individual cocoon was recorded. Length (m) and weight (gm) of silk filament were accounted for the calculation of Denier scale. The experimentation was repeated for thrice for the purpose of consistency in the results. The statistical methods were employed to calculate the mean, standard deviation, percent variation and student "t" – test (Norman and Bailey, 1955). The data collected belong to three successive trials.

3. RESULTS AND DISCUSSION

The larval parameters get reflects on the quality of the cocoon and silk fibre. Extension of fifth instar larval period was observed in groups of larvae topically applied with one ppm acetone solution of retinol and phytol. The extension of fifth instar larval period was 14 - 39 % in all the retinol treated groups of larvae. The extension of fifth instar larval period was 17 - 42 % in all the phytol treated groups of larvae. Maximum increase in the larval duration was recorded in larvae received four times (at 48, 54, 60 and 66 hours after the fourth moult) the topical application of retinol phytol.

Corresponding to the extension in the larval duration, an increase in the larval growth by the body weight (17-24% for retinol treated groups and 21-25% for phytol treated groups) was observed in all the treated groups. Tissue somatic index (TSI) designate the status of tissue and signify the percentage of tissue in entire body. Tissue somatic index (TSI) of the silk glands of untreated larvae in the percent study was found measured 23.928. Treating the larvae with one ppm retinol solution and phytol through acetone at 48, 54, 60 and 66 hours after fourth moult was found variously reflected into most significant improvement in the TSI of silk glands (Table 2).

The economic parameter in sericulture is the Cocoon spinned by the mature fifth instar larvae of silkworm, *Bombyx mori* (L). Cocoon is the most important aspect in sericulture as it is used for reeling the commercial silk fibre. Cocoon weight, shell weight and thereby the shell ratio were found influenced by the topical application of acetone solution of retinol to the fifth instar larvae of silkworm, *Bombyx mori* (L).

The range of percent increase in the cocoon weight and shell weight in the retinol treated groups was 42.911 to 50.268 and 66.565 to 77.243 respectively. And the range of percent increase in the cocoon weight and shell weight in the phytol treated groups was 44 to 69 and 67.477 to 103.95 respectively. Shell ratio of the cocoons was found improved in the corresponding groups of treatment.

Most significant ($p < 0.001$) shell ratio belonged to cocoons harvested from the group of larvae treated with one ppm acetone solution of retinol and phytol at 48 hours after the fourth moult. Silk filament is sole aim in sericulture. Length and weight of entire silk filament are the qualitative measurements to be accounted for the Tex and Denier scale. Both the parameters (Tex and Denier) of silk filament were found influenced through treating the larvae with retinol and phytol solution (one ppm). The retinol and phytol through acetone were found resulted into fortified silk filament, with reference to Tex and Denier scale. The silk reeled from the cocoons belong to the group C and D (Table 4) were exhibited most significant improvement.

Prolonged larval duration in the larvae treated with retinol and phytol fed in the present study is as good as tendency of larvae retaining their larval stage. Extension of larval duration is one of the distinguishing features of insect larvae recipient of exogenous juvenoid (Akai and Kobayashi, 1971). With this, retinol and phytol deserve juvenoid activity in fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2). The larvae of all the treated groups in the present study were found increased in their body weight. The retinol and phytol received by larvae through the acetone topical application, may influence the appetite, nutrition and absorption of digested food. This may be responsible for accelerated growth of silk glands.

Cocoon is the material used for reeling the commercial silk fibre. It is in fact, a protective shell made up of a continuous and long proteinaceous silk filament spun by mature silkworm prior to pupation for self protection from adverse climatic situations and natural enemies. Juvenile hormone (JH) controls insect metamorphosis. High JH titers maintain the larval state while a decrease in the JH titer initiates the pupation sequence as well as a change in tissue commitment away from synthesis of larval tissues to pupal tissues at the pupal stage.

The drop in JH titer at the beginning of the last larval instar in the Lepidoptera appears to be due to a combination of increased metabolism.

The juvenoid titre (endogenous and / or exogenous) in the body of larvae stimulate hypermetabolism (Slama, 1971). The terpenoids are known to arrest the cell cycle at G1, S and G2 M- phases Zore, *et al*, 2011). The synergistic activity of diterpenoids in the present attempt is hypothesized to be due to changes in the membrane fluidity, interference with membrane bound signaling proteins and cell cycle arrest. Use of retinol and phytol through the acetone for topical application, thus chiefly reflected into lengthening fifth instar larval duration.

The time required for eating and amount of mulberry leaves eaten both may have been increased and were practically reflected into the improvement of cocoon quality, shell ratio and silk filament quality. Retinol and phytol topically applied may be utilized by the silkworm larvae for the extra synthesis of silk. The retinol and phytol are the most popular vitamin suppliment used by man. Use of retinol and phytol through acetone for rearing of silkworm larvae is much more easy method. Use of diterpenoids (like retinol and phytol) may open a new avenue in sericulture for the qualitative cocoon and silk filament.

Table 2. Effect of Retinol and Phytol on the larval parameters of silkworm, *Bombyx mori* (L).

Parameter → Groups ↓	**Larval duration (hours)**	**Larval weight (g)**	**Weight of silk glands (g)**	**Tissue somatic index (TSI)**
UTC	156 (±23.786)	3.126 (±0.457)	0.748 (±0.019)	Tissue somatic index (TSI)
ATC	156 (±29.261)	3.132 (±0.478)	0.751 (±0.028)	23.928
A1	178** (±5.943)	3.658** (±0.539)	0.987** (±0.112)	26.981 3.061
A2	184** (±8.889)	3.786** (±0.557)	1.023*** (±0.239)	27.020 3.092
B1	193** (±4.614)	3.789*** (±0.438)	1.014*** (±0.236)	26.761 2.833
B2	199** (±4.769)	3.917*** (±0.453)	1.081*** (±0.339)	27.597 3.619
C1	198*** (±5.651)	3.881*** (±0.457)	1.058*** (±0.417)	27.261 3.333
C2	200*** (±5.213)	4.011*** (±0.613)	1.094*** (±0.786)	27.275 2.445
D1	217*** (±5.503)	3.781*** (±0.947)	1.035*** (±0.376)	26.373 2.445
D2	223*** (±5.728)	3.915*** (±0.743)	1.057*** (±0.453)	26.998 3.020

- Each figure is the mean of the three replications.
- Figure with ± sign in the bracket is SD.
- Figure below the standard deviation is the increase for calculated parameter and percent increase for the others over the control.

* - $P < 0.05$; ** - $P < 0.005$; *** - $P < 0.01$

Table 3. Effect of Retinol and Phytol on the cocoon parameters of silkworm, *Bombyx mori* (L).

Parameter → Groups ↓	**Cocoon weight (g)**	**Shell weight (mg)**	**Shell Ratio**
UTC	1.856 (±0.273)	0.329 (±0.078)	17.726
ATC	1.856 (±0.291)	0.331 (±0.091)	17.834
A1	2.671** (±0.445)	0.548** (±0.102)	20.516 2.790

A2	2.678*** (±0.384)	0.551** (±0.111)	20.575 2.693
B1	2.694*** (±0.786)	0.553** (±0.181)	20.527 2.801
B2	2.688*** (±0.739)	0.557** (±0.148)	20.721 2.887
C1	2.716*** (±0.643)	0.571*** (±0.187)	21.023 3.297
C2	2.789*** (±0.416)	0.594*** (±0.128)	21.297 3.571
D1	2.789** (±0.671)	0.583*** (±0.105)	20.903 3.177
D2	3.142*** (±0.786)	0.671*** (±0.116)	21.355 3.629

- Each figure is the mean of the three replications.
- Figure with ± sign in the bracket is SD.
- Figure below the standard deviation is the increase for calculated parameter and percent increase for the others over the control.

* - P < 0.05
** - P < 0.005
*** - P < 0.01

Table 4. Effect of Retinol and Phytol on the silk filament parameters in silkworm, *Bombyx mori* (L).

Parameter → Groups ↓	**S.F length (meter) (A)**	**S.F weight (mg) (B)**	**Tex = (B÷A) x 1000**	**Denier = (B÷A) x 9000**
UTC	794 (±23.245)	0.181 (0.039)	0.228	2.051
ATC	794 (±23.289)	0.181 (0.055)	0.228	2.051
A1	958 (±8.679)	0.254* (0.083)	0.265	2.386
A2	992 (±11.987)	0.278* (0.062)	0.280	2.522
B1	963 (±11.748)	0.277** (0.041)	0.287	2.588
B2	979 (±11.867)	0.301** (0.033)	0.308	2.776
C1	959 (±11.764)	0.321*** (0.052)	0.334	3.012
C2	981 (±13.781)	0.352*** (0.001)	0.358	3.229
D1	976 (±23.789)	0.324*** (0.086)	0.332	2.987

D2	1004 (±49.452)	0.363*** (0.089)	0.361	3.253

- Each figure is the mean of the three replications.
- Figure with ± sign in the bracket is SD.
- Figure below the standard deviation is the increase for calculated parameter and percent increase for the others over the control.

* - $P < 0.05$
** - $P < 0.005$
*** - $P < 0.01$

4. CONCLUSIONS

Retinol and Phytol, chiefly lengthening the larval age in silkworm, *Bombyx mori* (L). Prolonged larval duration in the larvae treated with retinol and phytol fed in the present study is as good as tendency of larvae retaining their larval stage. Extension of larval duration is one of the distinguishing features of insect larvae recipient of exogenous juvenoid. Retinol and Phytol deserve juvenoid activity in fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2). The larvae of all the treated groups in the present study were found increased in their body weight. The retinol and phytol received by larvae through the acetone topical application, may influence the appetite, nutrition and absorption of digested food. This may be responsible for accelerated growth of silk glands.

The synergistic activity of Retinol and phytol (diterpenoids) is hypothesized to be due to changes in the membrane fluidity, interference with membrane bound signaling proteins and cell cycle arrest. Efficient utilization of diterpenoids like, retinol and phytol, through acetone solvent for topical application to the fifth instared larvae of silkworm, *Bombyx mori* (L) may open a new biotechnological avenue in the sericulture industries.

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