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Research Article

# GREEN SYNTHESIZED SILVER NANOPARTICLES AND GROWTH CHANGES IN THE LARVAE OF THE MULBERRY SILK WORM *BOMBYX MORI*

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**A B S T R A C T**

Silkworm being a monophagous insect derives almost all the nutrients required for its growth from the mulberry leaf itself. The silk worm nutrition is considered as a major area of research in sericulture. The traditional method of feeding the larvae with mulberry leaves is suitably replaced by mulberry leaf mixed with supplementation and artificial diets. The pathogenic microbes in the mulberry leaf may affect the gut micro biome of silkworm. This issue can be solved by supplementing the mulberry leaf with natural antibiotics like silver Nanoparticles. In the present study silver Nanoparticles were synthesized using the extract of the plant *Acalypha indica* as a reducing agent (GAgNPs). Two doses of GAgNPs (10% and 20%) were prepared from the stock solution. The multivoltine race L x CSR2 was reared by feeding with MR2 variety of mulberry leaves. Prior to feeding the larvae, healthy leaves of mulberry were coated with GAgNPs. GAgNPs treated 3rd, 4th and 5th instars larvae of *B.mori* showed an enhancement in the growth significantly when compared with the control. Our study emphasized that GAgNPs treatment has influenced the metabolic process in the larvae leading to a progressive weight gain.



## INTRODUCTION

Sericulture provides gainful employment, economic development and improvement in the quality of life to the people in rural area and therefore it plays an important role in antipoverty programme and prevents migration of rural people to urban area in search of employment. The main outcome from sericulture is the silk for fashionable clothings. The silk is the conversion of the nutrients present in the mulberry leaves which are the role source of food for silk worm, *Bombyx mori* L, Nutritional quantity of leaves play a vital role in determining the health and growth of the larvae. The feeding of nutritionally enriched leaves showed better growth and development of silk worm larvae, as well as directly influences the quality and quantity of silk production (Krishnaswami *et al*., 1971). Nearly 70% of the silk proteins produced by silk worm are directly derived from the protein of mulberry leaves (Narayanan *et al*., 1967).

Although the nutritional levels of $MR_2$ mulberry influence the larval growth of silk worm, supplements given along with mulberry leave has been reported to enhance a higher growth rate when compared to mulberry leaves alone given groups (Amalarani *et al*., 2011a,b; Ganesh prabu *et al*., 2012; Jeyaraj *et al*., 2016).

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Mulberry leaves treated with silver nano particles, is reported to influence the feed efficacy of silk worm larvae (Ganesh Prabu *et al*., 2012). In recent decade green synthesis of metallic nanoparticles has become a promising field of research. Bio- nanoparticles were feasible, scalable, non - corrosive and economical when compared to synthetic nano particles, which can be achieved easily through the plant mediated approach (Ganesh Prabu *et al*., 2012). Silver is a renowned resource metal exploited for the common usage of mankind, which is well known for it intake antimicrobial activity towards the microbe organisms (Tiang *et al*., 2004). Silver precursors were reduced by several reducing agents like chemicals, plant leaf extract, plant seed extract, micro algae extracts, microorganisms like bacteria and fungi. It has been shown that feeding silk worms with $TiO_2$ NPS can increase feed efficiency and promote silk protein synthesis (Ni *et al*., 2015). Anyhow work on the effect of green synthesized silver nano particles on larval growth and protein profile of *B.mori* is very meager. So the present study was planned to find out the impact of green synthesized silver nano particles on the larval parameters of *B.mori.*

The metamorphosis of the larvae 3rd instar to 5th instar was closely followed using nano particle treatment. The changes in the larval weight and protein content were recorded. Protein was estimated using the method of Lowry *et al*., (1951).

## MATERIALS AND METHODS

### *Collection of plant materials*

Fresh and healthy *Acalypha indica* plants were collected from M.S.University campus.

Kingdom: Plantae
Clade : Angiosperms
Clade : Eudicots
Clade : Core eudicots
Clade : Rosids
Order : Malpighiales
Family : Euphorbiaceae
Subtribe : Acalyphinae
Genus : Acalypha
Species : A.indica

### *Preparation of Acalypha indica aqueous extract for the synthesis of NPs*

The collected plant parts were thoroughly washed under running tap water, dried in shade and triturated into fine powders by using an electric grinder. The powder was stored in air sealed bottles separately and kept under laboratory condition (Temperature 30 ± 2 $^{o}$C; RH 70 ± 5 %). Twenty grams of dried powder (leaf, stem and root) were mixed with 100 ml of Millipore water with continuous shaking on orbital shaker at 37$^{o}$C in 24 hrs. The suspension was left for 3 hrs, filtered through Whatman No.1 filter paper and the filter was stored in amber-colored air-tight bottles at room temperature

### *Synthesis of Silver Nanoparticle*

The broth solution of *A.indica* leaves, stem and root was prepared, separately, by taking 20 gm of dried powder in a 500 ml Erlenmeyer flask along with 100 ml of Millipore water.

The extract was filtered with whatman no.1 filter paper, stored at refrigerator and used within a week. The filtrate was treated separately with aqueous 1mM $AgNo_3$ solution in Erlenmeyer flask and incubated at room temperature. The reaction mixture was checked for the development of color change and absorbance spectra read by UV-visible spectroscopy with the ranges of 300-900 nm. Ninety five ml of aqueous solution of 1mM $AgN0_3$ was reduced using 5 ml of *A.indica* plant parts *viz*., leaves, stem and root extract separately at room temperature for 10 min resulting in a brown colour of the solution indicating the formation of AgNPs. It was found that aqueous silver can be reduced by aqueous extract of *A.indica* to generate extremely stable AgNPs in water (Prashar *et al.*, 2009; Sathiskumar *et al.*, 2014).

### *Selection of larvae (B.mori L×CSR2)*

For the present study 3$^{rd}$ instar stage of *B. mori* reared in a private farm at Pavoorchathiram was chosen. The larvae were fed with Green synthesized AgNPs from 3$^{rd}$ instar stage to the 5$^{th}$ instar stage.

### *Experiment*

Healthy 3$^{rd}$ instar larvae were isolated and separated in to 1 control group and 2 test groups. Each group contained 30 larvae. The control larvae were fed with normal mulberry leaves alone. The experimental groups (T1 & T2) were fed with mulberry leaves treated with green synthesized SNP at a concentration of 10% &20% respectively.

### *Application of Green synthesised nano materials on mulberry leaves*

Fresh MR2 mulberry leaves (*Morus alba)* were collected and separately treated with 10% & 20% SNP. The leaves were separately soaked with each concentration for 15 min and then dried in air for 10 min. The green synthesized silver nano materials treated leaves were used for feeding the larvae.

**Table 1** Weight changes in the 3$^{rd}$ instar larvae of *Bombyx mori* treated with green synthesized silver nano particles coated mulberry leaves (Value represent mean of 3 groups-each group with 30 larvae)

| Group | Mean weight changes in larvae in different days | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Control | 13.22 ± 0.14 | 15.42 ± 0.36 (16.64) | 18.02 ± 0.16 (36.30) | 21.46 ± 0.16 (62.32) | 24.08 ± 0.41 (82.14) | 26.62 ± 0.36 (101.36) |
| T1 10 % treatment | 13.54 ± 0.21 | 16.08 ± 0.14 (18.75) | 19.42 ± 0.31 (43.42) | 25.81 ± 0.46 (90.32) | 29.02± 0.42 (114.32) | 32.50 ± 0.26 (140.02) |
| T2 10% treatment | 12.03 ± 0.4 | 14.84 ± 0.16 (23.35) | 21.42 ± 0.26 (78.05) | 26.68 ± 0.17 (127.77) | 30.017±0.31 (149.95) | 33.64 ± 0.27 (179.63) |

Percentage of weight changes over the 1$^{st}$ day of larval weight is given in parenthesis.

**Anova: Two-Factor Without Replication**

| *SUMMARY* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Row 1 | 6 | 118.82 | 19.80333 | 26.64711 |
| Row 2 | 6 | 136.37 | 22.72833 | 56.82762 |
| Row 3 | 6 | 138.627 | 23.1045 | 73.06953 |
| Column 1 | 3 | 38.79 | 12.93 | 0.6331 |
| Column 2 | 3 | 46.34 | 15.44667 | 0.384933 |
| Column 3 | 3 | 58.86 | 19.62 | 2.92 |
| Column 4 | 3 | 73.95 | 24.65 | 7.8213 |
| Column 5 | 3 | 83.117 | 27.70567 | 10.1076 |
| Column 6 | 3 | 92.76 | 30.92 | 14.1924 |

ANOVA

| *Source of Variation* | *SS* | *Df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Rows | 39.18966 | 2 | 19.59483 | 5.950629 | 0.019845 | 4.102821 |
| Columns | 749.7923 | 5 | 149.9585 | 45.53993 | 1.47E-06 | 3.325835 |
| Error | 32.929 | 10 | 3.2929 | | | |
| Total | 821.9109 | 17 | | | | |

**Table 2** Weight changes in the 4th instar larvae of *Bombyx mori* treated with green synthesized silver nanoparticles coated mulberry leaves. (Value represent mean of 3 groups-each group with 30 larvae)

| Group | Mean weight changes in larvae in different days | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Control | 27.50 ± 0.14 (110.28) | 30.12 ± 0.21 (127.83) | 34.23 ± 0.57 (142.7) | 40.41 ± 0.62 (205.67) | 48.21 ± 0.82 (264.97) |
| T1 10 % treatment | 33.06 ± 0.04 (144.16) | 34.84 ± 0.16 (157.31) | 39.54 ± 0.16 (178.64) | 43.61 ± 0.16 (214.69) | 51.36± 1.1 (279.32) |
| T2 20% treatment | 36.06 ± 0.23 (199.91) | 39.12 ± 0.51 (225.18) | 39.86 ± 0.48 (238.07) | 44.55 ± 0.68 (249.62) | 58.4±0.92 (384.29) |

Percentage of weight changes over the 1st day of larval weight is given in parenthesis.

| Anova: Two-Factor Without Replication | | | | |
|---|---|---|---|---|
| *SUMMARY* | *Count* | *Sum* | *Average* | *Variance* |
| Row 1 | 5 | 180.47 | 36.094 | 69.61133 |
| Row 2 | 5 | 202.41 | 40.482 | 53.98022 |
| Row 3 | 5 | 217.99 | 43.598 | 77.71302 |
| Column 1 | 3 | 96.62 | 32.20667 | 18.86453 |
| Column 2 | 3 | 104.08 | 34.69333 | 20.26613 |
| Column 3 | 3 | 113.63 | 37.87667 | 9.999233 |
| Column 4 | 3 | 128.57 | 42.85667 | 4.710533 |
| Column 5 | 3 | 157.97 | 52.65667 | 27.22003 |

**ANOVA**

| *Source of Variation* | *SS* | *Df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Rows | 142.1234 | 2 | 71.06168 | 28.42812 | 0.000232 | 4.45897 |
| Columns | 785.2207 | 4 | 196.3052 | 78.5316 | 1.86E-06 | 3.837853 |
| Error | 19.99757 | 8 | 2.499697 | | | |
| Total | 947.3416 | 14 | | | | |

The larvae were fed 3 times a day and the larval weight was recorded for control and experimental groups.

***Protein Estimation***

The total amount of protein in the entire body of control and green synthesized silver nanoparticles treated worms (4th day of 3rd, 4th and 5th instar) were estimated using the method of Lowry *et al*., (1951).

## RESULTS

The growth of the control group, 10% and 20% GAgNPs treated larvae were monitored from 3rd instar stage to 5th instar stage. The difference in weight gain/less between control and AgNPs treated groups are presented in (Table 1)The mean weight of the larvae of control and experimental groups T1 and T2 were 13.22± 0.42 g (30 worms) , 13. 54 ±0.21 g (for 30 worms) and 12.03 ± 0.42 g (30 worms) respectively on the first day of experiment (Table 1 Fig 3). The larval weight increased progressively in the control and treated worms. On the 6th day of 3rd instar stage the weight of the larvae in the control group was 26.62 ±0.36 g. But in the 10 % and 20% AgNPs supplemented feed fed larvae the mean weight on the 6th day of 3rd instar larvae were 32.50 ±0.26 g and 33.64 ±0.27 g. When compared with control the GAgNPs fed larvae showed a higher growth than the mulberry leaf alone fed larvae. In the control group the growth difference between 1st and 6th day of 3rd instar larvae was 101.36 % high. But in the AgNPs treated larvae the weight gain was 140.12% in 10 % treated group and 179.63% in 20% GAgNPs treated group. This observation clearly indicates that the mulberry leaf soaked with AgNPs promoted growth higher than the mulberry leaf alone fed 3rd instar larvae of *B. mori* (P<0.01).

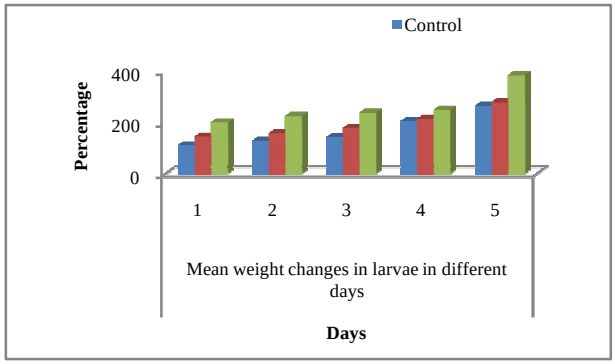


**Fig 1** Weight changes in the 3rd instar larvae of *Bombyx mori* treated with green synthesized silver nano particles coated mulberry leaves

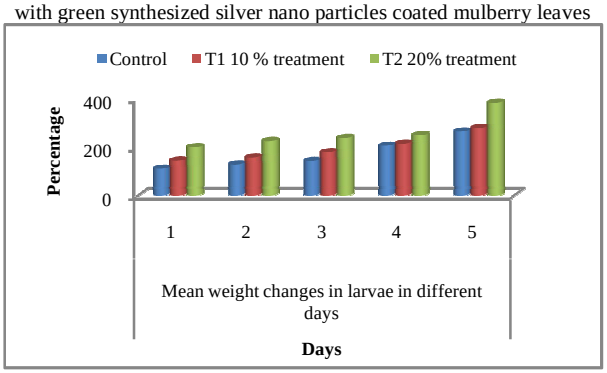


**Fig 2** Weight changes in the 4th instar larvae of *Bombyx mori* treated with green synthesized silver nanoparticles coated mulberry leaves

**Table 3** Weight changes in the 5th instar larvae of *Bombyx mori* treated with green synthesized silver nanoparticles coated mulberry leaves. (Value represent mean of 3 groups-each group with 30 larvae)

| Group | Mean weight changes in larvae in different days | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Control | 50.40 ± 0.47 (264.67) | 51.38 ± 0.21 (288.65) | 54.60 ± 0.32 (344.68) | 50.36 ± 0.78 (280.93) |
| T1 10 % treatment | 53.2 (315.14) | 54.41 (331.84) | 60.21 (178.64) | 55.14 (214.69) |
| T2 20% treatment | 60.73 (404.82) | 39.12 ± 0.51 (418.20) | 39.86 ± 0.48 (455.36) | 44.55 ± 0.68 (385.53) |

Percentage of weight changes over the 1st day of larval weight is given in parenthesis.

| Anova: Two-Factor Without Replication | | | | |
|---|---|---|---|---|
| *SUMMARY* | *Count* | *Sum* | *Average* | *Variance* |
| Row 1 | 4 | 206.74 | 51.685 | 3.999033 |
| Row 2 | 4 | 222.96 | 55.74 | 9.520467 |
| Row 3 | 4 | 184.26 | 46.065 | 101.3642 |
| Column 1 | 3 | 164.33 | 54.77667 | 28.54163 |
| Column 2 | 3 | 144.91 | 48.30333 | 65.54543 |
| Column 3 | 3 | 154.67 | 51.55667 | 110.477 |
| Column 4 | 3 | 150.05 | 50.01667 | 28.12543 |

ANOVA

| *Source of Variation* | *SS* | *Df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Rows | 188.8441 | 2 | 94.42203 | 2.048682 | 0.209812 | 5.143253 |
| Columns | 68.116 | 3 | 22.70533 | 0.492639 | 0.700332 | 4.757063 |
| Error | 276.535 | 6 | 46.08917 | | | |
| Total | 533.4951 | 11 | | | | |

Both the control and treated larvae consumed the mulberry leaves voraciously in the 4th instar stage. This has been well reflected in the %of weight gain during the different days of 4th instar stage. The weight gain in the larvae were different in the control and treated larvae. In the 5th day of the 4th instar stage the percentage of weight gain was high. On the 5th day 10% GAgNPs treated larvae had the 279.32% increase in

weight. In the 20% AgNPs treatment the % weight gain was 384.29 (i.e) tenfold increase from the first day.(Table 2). When it is compared to the control (264.97 on 5th day of 4th instar) the AgNPs treated larvae exhibited a higher % of weight change. This weight gain in 20% AgNPs treatment was 90% higher than the control and it is significant at P<0.2 level.

In the present study weight gain in the 5th instar larvae of *B.mori* treated with GAgNPS was also traced. Due to high environmental temperature the duration of the 5th instar larval stage was reduced. There is a progressive weight gain in the first 3 days. The weight gain on the first day of 5th instar and 3rd day was 264.67 and 313.01 on the control larvae. Whereas the weight gain on the first and third days of 10% GAgNPS treatment was 315.14 and 344.68 respectively (Fig 4). Likewise the weight gain of larvae treated with 20% GAgNPs on the 1st and 3rd days of 5th instar stage was 404.82 and 455.36. This observation indicated that GAgNPs has a great impact on the growth of the Larvae of *B.mori.*

**Table 4** Changes in the protein content of the entire body in the control and silver nanoparticle supplemented group (Mean ± SE)

| Stages | Protein content (mg/ml) | | |
|---|---|---|---|
| | Control | Nanoparticle treated | |
| | | 10 % | 20 % |
| 3rd instar (4th day) | 35.31 ± 0.28 | 39.42 ± 0.34 | 41.42 ± 0.21 |
| 4th instar (4th day) | 39.21 ± 0.36 | 43.61 ± 0.64 | 44.76 ± 0.74 |
| 5th instar (5th day) | 42.64 ± 0.47 | 45.73 ± 0.81 | 48.46 ± 0.36 |

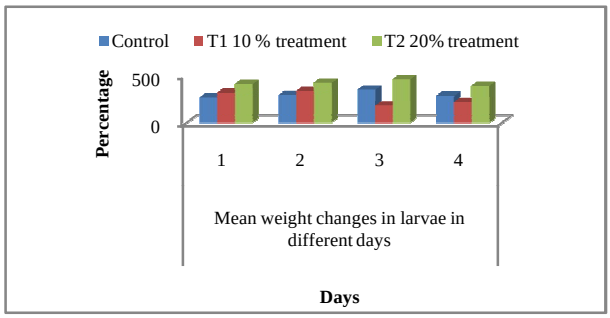


**Fig 3** Weight changes in the 5th instar larvae of *Bombyx mori* treated with green synthesized silver nanoparticles coated mulberry leaves

**Fig 4** Showing III,IV & V instars of *B.mori*

In the present experiment the mulberry leaf supplemented with silver nanoparticles was found to enhance protein content (Table 4). The amount of protein in the entire body of control and silver nanoparticles supplemented larvae was estimated on the 4th day of 3rd, 4th and 5th instar stages. The protein content in the silver nanoparticle treated worms showed a higher value when compared to the mulberry leaf alone treated larvae. In the mulberry leaf treated group the total protein in the body was 35.31 ± 0.28, 39.21 ± 0.36 and 42.64 ± 0.47 in the 3rd, 4th and 5th instar larvae respectively. But in the silver nanoparticle supplemented larvae the protein content was41.42 ± 0.21, 44.76 ± 0.36 in the 4th day of 3rd, 4th and 5th instar larvae. The increase in the protein content in the 5th instar larvae shows, its preparatory phase for spinning cocoon. The study shows that the green synthesised silver nanoparticle elevated feed intake and that is reflected in the protein metabolism.

## DISCUSSION

Ganesh Prabu *et al*, (2012) reported that fortification of mulberry leaves with complementary AgNPs was found to increase the larval growth. This has been also observed by earlier workers who have tried with other complementary compounds (Etebari, and Fazilati, 2002, Sheeba, 2008, Amala Rani 2014). Silver Nanoparticles are reported to express a good antimicrobial activity (Jeyaraj Pandiarajan, *et al.*, 2016; Beckar, 1999). Jeyaraj Pandiarajan *et al*., (2016) have reported that the biologically synthesised nanoparticles are safer materials which do not have any danger in the living host. Further have stated that the AgNPs can be utilized as an ancillary complex which can boost up the growth and development of the larvae and also the quality and quantity of cocoon. Bhattacharya and Mukherjee, (2008) had reported the biological properties of “naked” metals and can stimulate more production of fibroin in silkworm.

Meng *et al.*, (2017) studied the effect of AgNPs on growth and body proteins in silkworms (*Bombyxmori*) and report that low concentrations (<400mg/L) of AgNPs promotes the growth and cocoon weights of *B.mori*. In the present study also the growth promoting effects of green synthesised AgNPs has been observed.

The 4th& 5th instar of *B. mori* fed with AgNPs also showed a progressive weight gain till the 4th of 5th instar stage. This clearly indicates the influence of AgNPs in the metabolism of *B. mori* larvae.

Wu *et al.*, (2008) reported that Arginine Kinase plays a critical role in the metabolism, storage and utilization of energy in invertebrates. Kang *et al.*, (2011) reported that Arginine Kinase (AK) play an important role in the insect immune response and environmental adaptation. Further studies showed that AK protects silkworm larvae against viral infection. Glutathione S-transferase and Arginine Kinase expressions were up regulated after silkworms being fed AgNPs (Meng *et al*, 2017). They have also reported that AgNPs exhibit the presence of certain growth stimulant activities. The increase in the protein content of the silver nanoparticle supplemented larvae was due to the higher –need intake due to the inclusion of silver nanoparticle in the mulberry leaf.

In the present study, GAgNPs was found to enhance the growth of the larvae of *Bombyx mori*, and GAgNPs usage can be recommended to farmers.

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