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STUDIES ON THE COMPARATIVE FEED EFFICACY OF *BOMBYX MORI* (L.) (LEPIDOPTERA: BOMBYCIDAE) FED WITH SILVER NANOPARTICLES (AGNPS) AND *SPIRULINA* TREATED MR_2 MULBERRY LEAVES IN RELATION TO GROWTH AND DEVELOPMENT

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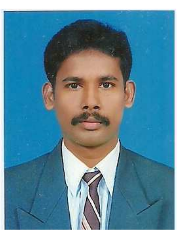

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ABSTRACT

Silkworm *Bombyx mori* is an important economic insect and also a tool to convert leaf protein into silk. The industrial and commercial use of silk, the historical and economic importance of production and its application in all over the world finely contributed to the silkworm promotion as a powerful laboratory model for the basic research in biology. The mulberry leaf is the only important source of the silkworms. The present study has been undertaken to the effectiveness of foliar applications of silver nanoparticles (AgNps) (25%) and single cell protein (SCP) containing microalgae *Spirulina* (0.03%) treated MR_2 mulberry leaves fed with the popular Indian bivoltine hybrid (CSR2×CSR4) silkworm *B. mori* larvae (3^{rd}, 4^{th} and 5^{th} instar) to study the larval growth and development (length, width and weight) and economic parameters like cocoon (length, width and weight) shell and pupal weight were significantly increased than control group. This study was indicated that the AgNps and *Spirulina* exhibit the presence of certain growth stimulant and can be used to increase the silk yield in commercial silkworm rearing.

KEYWORDS

Bombyx mori, Silver Nanoparticles (AgNps), *Spirulina*, Mulberry leaves.

INTRODUCTION

The silkworm rearing is a traditional industry in Asia and the life of many people is depended on it. Increase of larval growth and cocoon quality and quantity would result better economics for this industry and meet the production needs. Consequently, the enrichment of mulberry leaves by supplementary compounds with the aim of increasing the production of cocoon is a very important aspect. Many investigations have been done on this topic and various reports have been published [1-3]. Fortification of mulberry leaves with complementary compounds was found to increase the larval growth and post cocoon characters [1, 4].

Morus alba L. (mulberry), a woody plant belonging to the genus *Morus*, family Moraceae, is a widely distributed plant in China, whose leaves, root bark and branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, facilitate discharge of urine and lower blood pressure [5-6]. The mulberry leaves are rich in flavonoids, alkaloids and polysaccharides components which are known as the most potently major active compounds by chemical constituent investigations [7]. Among those, the flavonoids like rutin, quercetin, isoquercitrin and quercetin 3-(6-malonylglucoside) [8] are found in mulberry leaves. The health benefits of flavonoids are well known and are displayed as a remarkable range of biochemical and pharmacological properties that may significantly affect the function of various mammalian cells [9]. The anti-inflammatory, antioxidant, antithrombotic and anticarcinogenic effects of flavonoids are some of the properties that have been under consideration in view of therapeutical purposes for several human diseases [10]. Flavonoids are thought to be one of the most critical constituents in mulberry that have therapeutic functions. But the anti-fatigue effects of flavonoids from mulberry leaves (FML) have also been reported [11].

Silkworm mortality is mainly caused by bacteria and other pathogenic microorganisms. AgNps have antimicrobial activity against several pathogenic microorganisms. Antibacterial properties of silver are documented since 1000 B.C., when silver vessels were used to preserve water. The first scientific papers describing the medical use of silver report the prevention of eye infection in neonates in 1881 and internal antisepsis in 1901. After this, silver nitrate and silver sulfadiazine have been widely used for the treatment of superficial and deep dermal burns of wounds and for the removal of warts [12]. The term ‘‘silver nanomaterials’’ refers to any silver-containing materials with enhanced activity due to their nanoscale features. In some cases, commercial products containing metallic AgNps in the range of 5–50 nm or ionic silver are given the name ‘nanosilver’ [13]. AgNps mode of action is presumed to be dependent on Ag^{+} ions, which strongly inhibit bacterial growth through suppression of respiratory enzymes and electron transport components and through interference with DNA functions [14]. AgNps in a nanometric scale (less than 100 nm) has different catalytical properties compared with those attributed to the bulk form of the noble metal, like surface plasmon resonance, large effective scattering cross section of individual AgNps, and strong toxicity to a wide range of microorganisms [15].

Mulberry leaf supplemented with *Spirulina* as a feed to *B. mori* L. (Lepidoptera: Bombycidae) oraly found to be effective in enhancing the larval and cocoon characters. *Spirulina*, blue-green algae contains 18 amino acids viz., glutamine, glycine, histidine, lysine, methionine, creatine, cysteine, phenylalanine, serine, proline, tryptophan, asparagine, pyruvic acid and vital vitamins like biotin, tocopherol, thiamine, riboflavin, niacin, folic acid, pyrodozoic acid, beta-carotene and vitamin B_{12} [16]. Various researches have been carried out on the diet supplementation of mulberry leaves fed to silkworms. These supplementations include vitamins such as ascorbic acid, thiamine, niacin, folic acid and multivitamins. Its nutrients are very easy to digest protein (biliprotein), carbohydrates (mucopolysaccharides, rhamnose and glycogen), 50 different minerals and trace minerals, beta-carotene, chlorophyll, GLA omega3 fatty acid, and many other nutrients [2]. The dietary supplements like protein, vitamins, lipids etc. evinced their specificity at specific dose for various metabolic activities of silkworm [17]. Amino acids such as aspartic acid and glutamic acid are considered to be essential for silkworm growth [18]. Nutritional study on silkworm is an essential prerequisite for its proper commercial exploitation. Nutrition of silkworm is sole factor which almost individually augment quality and quantity of silk [19].

MATERIALS METHODS

Silkworm rearing method

The eggs of popular Indian bivoltine hybrid (CSR2×CSR4) silkworm *B. mori* were collected from farmers training centre at Jayankondapattinam, Tamilnadu, India. The eggs were placed at ambient temperature of 25±2º C and relative humidity of 70 to 80% in an incubator for hatching. After hatching, larvae were isolated from stock culture. The larvae were divided into 3 experimental groups including control (distilled water treatment), each group consisting of 6 larvae. The larvae were reared in card board boxes measuring 22×15×5 cms covered with nylon net and placed in an iron stand with ant wells. The control and treated MR_2 mulberry leaves were fed to silkworms, five feedings/day. They were maintained up to cocoon stage.

Preparation of Silver Nanoparticles and *Spirulina* solution

Silver Nitrate $AgNO_3$ (Sigma Aldrich, UK) and Trisodium Citrate $C_6H_5O_7Na_3$ (Sigma Aldrich, UK) of analytical grade purity, were used as starting materials without further purification. The AgNps were prepared by using chemical reduction method according to the description of Lee and Meisel [20]. All solutions of reacting materials were prepared in distilled water. In typical experiment, 50 ml of $1{\cdot}10^{-3}$ M $AgNO_3$ was heated to boiling. To this solution, 5 ml of 1% Trisodium Citrate was added drop by drop. During the process, solution was mixed vigorously. Solution was heated until colour change is evident (pale yellow). Then it was removed from the heating element and stirred until cooled to room temperature.

Mechanism of reaction could be expressed as follows:

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^{0} + C_6H_5O_7H_3 + 3Na^{++} + H^{+} + O_2\uparrow$$

Spirulina powder were purchased from Pharmaceutical company at Chidambaram and to prepare the experimental dose for 0.03% concentration.

Mulberry (*Morus alba L.*) MR_2 leaves treated with Silver Nanoparticles (AgNps) and *Spirulina* (SCP)

AgNps and *Spirulina* were diluted to 25% and 0.03% concentrations respectively; fresh MR_2 mulberry leaves were soaked in this two concentrations for 15 minutes and then were dried in air for 10 minutes. The treated leaves were used for feeding the 3rd, 4th and 5th instars larvae of silkworm *B. mori*. They were maintained up to cocoon stage.

Experimental groups

There are three experimental groups. 3rd, 4th and 5th instars of *B. mori* larvae fed with the following treated MR_2 mulberry leaves. Group T_1 larvae fed with distilled water treated mulberry leaves, it serve as a control, group T_2 larvae fed with 25% AgNps treated mulberry leaves, group T_3, larvae fed with 0.03% *Spirulina* solution treated mulberry leaves. They were maintained up to cocoon stage.

Statistical analysis

Data were analyzed by One-Way Analysis Of Variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available statistics software package (SPSS® for Windows, V. 16.0, Chicago, USA). Results were presented as means ± SD. P values < 0.05 were regarded as statistically significant.

RESULTS

Larval Parameters

Morphometric analysis of 3rd, 4th and 5th instar larvae

Table 1 displays that the morphometric data of control MR_2 leaves, AgNps and *Spirulina* treated MR_2 leaves fed 3rd instar *B. mori* larvae length, width and weight. The mean value of group T_1 were (1.6833±0.1472cm, 0.3500±0.0548cm and 0.1117±0.0075gm), respectively. The mean value of group T_2 were (1.9333±0.1633cm, 0.3833±0.0408cm and 0.1233±0.0103gm), respectively. The mean value of group T_3 were (1.7500±0.1049cm, 0.3500±0.0548cm and 0.1167±0.0121gm), respectively. In these three observations, 25% AgNps treated 3rd instar larvae length were significantly increased than the other two groups (T_1 and T_3).

Table 1

Morphometric data of AgNps and Spirulina treated 3rd instar of B. mori larvae.

3rd instar of *B. mori* larvae			
Groups	Length (cm) (Mean ± S.D)	Width (cm) (Mean ± S.D)	Weight (gm) (Mean ± S.D)
Control (T_1)	1.6833±0.1472[a]	0.3500±0.0548[a]	0.1117±0.0075[a]
MR_2 Leaves + 25% AgNps (T_2)	1.9333±0.1633[b]	0.3833±0.0408[a]	0.1233±0.0103[a]
MR_2 Leaves + 0.03% *Spirulina* (T_3)	1.7500±0.1049[a]	0.3500±0.0548[a]	0.1167±0.0121[a]

Values are Mean ± Standard Deviation of six observations. Values in the same column with different superscript letters (a & b) differs significantly at P<0.05 (DMRT).

Table 2 shows that the morphometric data of control MR_2 leaves, AgNps and *Spirulina* treated MR_2 leaves fed 4th instar *B. mori* larvae length, width and weight. The mean value of group T_1 were (5.7333±0.2422cm, 0.5333±0.0816cm and 0.4233±0.0344gm), respectively. The mean value of group T_2 were (6.0167±0.1472cm, 0.6167±0.0753cm and 0.5450±0.0404gm), respectively. The mean value of group T_3 were (5.8833±0.1941cm, 0.5667±0.0816cm and 0.4883±0.0183gm), respectively. In these three observations, 25% AgNps treated 4th instar larvae length, width and weight were significantly increased than the other two groups (T_1 and T_3).

Table 2
Morphometric data of AgNps and Spirulina treated 4th instar of B. mori larvae.

4th instar of *B. mori* larvae			
Groups	Length (cm) (Mean ± S.D)	Width (cm) (Mean ± S.D)	Weight (gm) (Mean ± S.D)
Control (T_1)	5.7333±0.2422^{a}	0.5333±0.0816^{ab}	0.4233±0.0344^{a}
MR_2 mulberry + 25% AgNps (T_2)	6.0167±0.1472^{b}	0.6167±0.0752^{b}	0.5450±0.0404^{c}
MR_2 Leaves + 0.03% *Spirulina* (T_3)	5.8833±0.1941^{ab}	0.5667±0.0816^{a}	0.4883±0.0183^{b}

Values are Mean ± S.D of six observations. Values in the same column with different superscript letters (a, b & c) differs significantly at P<0.05 (DMRT).

Table 3 shows that the morphometric data of control MR_2 leaves, AgNps and *Spirulina* treated MR_2 leaves fed 5th instar of *B. mori* larvae length, width and weight. The mean value of group T_1 were (6.7167±0.2483cm, 1.0333±0.1211cm and 2.8350±0.0855gm), respectively. The mean value of group T_2 were (7.2500±0.1871cm, 1.1333±0.0816cm and 3.5583±0.2369gm), respectively. The mean value of group T_3 were (7.0500±0.1871cm, 1.0667±0.0816cm and 3.4467±0.2965gm), respectively. In these three observations, 25% AgNps and 0.03% *Spirulina* treated 5th instar larvae length and weight were significantly increased than control group (T_1).

Table 3
Morphometric data of AgNps and Spirulina treated 5th instar of B. mori larvae.

5th instar of *B. mori* larvae			
Groups	Length (cm) (Mean ± S.D)	Width (cm) (Mean ± S.D)	Weight (gm) (Mean ± S.D)
Control (T_1)	6.7167±0.2483^{a}	1.0333±0.1211^{a}	2.8350±0.0855^{a}
MR_2 mulberry + 25% AgNps (T_2)	7.2500±0.1871^{b}	1.1333±0.0816^{a}	3.5583±0.2369^{b}
MR_2 Leaves + 0.03% *Spirulina* (T_3)	7.0500±0.1871^{b}	1.0667±0.0816^{a}	3.4467±0.2965^{b}

Values are Mean ± S.D of six observations. Values in the same column with different superscript letters (a & b) differs significantly at P<0.05 (DMRT).

Cocoon and pupal parameters

Table 4 shows that the morphometric data of the control MR_2 leaves, AgNps and *Spirulina* treated MR_2 leaves fed *B. mori* larvae was found to be produced cocoon length, width and weight. The mean value of group T_1 larvae produced cocoon were (3.4000±0.3847cm, 2.1333±0.1211cm and 1.5117±0.1007gm), respectively. The mean and of group T_2 larvae produced cocoon were (3.6667±0.1633cm, 2.4000±0.0894cm and 2.2117±0.3632gm), respectively. The mean value of group T_3 larvae produced cocoon were (3.5333±0.1862cm, 2.3000±0.0894cm and 1.9933±0.0975gm), respectively. In these three observations, 25% AgNps and 0.03% *Spirulina* treated larvae produced cocoon width and weight was significantly increased than control group (T_1).

Table 4

Morphometric data of AgNps and Spirulina treated B. mori larvae produced cocoon.

	Cocoon of *B. mori*		
Groups	Length (cm) (Mean ± S.D)	Width (cm) (Mean ± S.D)	Weight (gm) (Mean ± S.D)
Control (T_1)	$3.4000±0.3847^a$	$2.1333±0.1211^a$	$1.5117±0.1007^a$
MR_2 mulberry + 25% AgNps (T_2)	$3.6667±0.1633^a$	$2.4000±0.0894^b$	$2.2117±0.3632^b$
MR_2 Leaves + 0.03% *Spirulina* (T_3)	$3.5333±0.1862^a$	$2.3000±0.0894^b$	$1.9933±0.0975^b$

Values are Mean ± S.D of six observations. Values in the same column with different superscript letters (a & b) differs significantly at P<0.05 (DMRT).

Table 5 shows that the morphometric data of the control MR_2 leaves, AgNps and *Spirulina* treated MR_2 leaves fed *B. mori* larvae produced cocoon shell and pupal weight. The mean value of group T_1 larvae produced cocoon shell and pupal weight were (0.3883±0.0376gm and 1.1233±0.1256gm), respectively. The mean value of group T_2 larvae produced cocoon shell and pupal weight were (0.4767±0.0308gm and 1.5683±0.0673gm), respectively. The mean value of group T_3 larvae produced cocoon shell and pupal weight were (0.4250±0.0187gm and 1.5542±0.0964gm), respectively. In these three observations, 25% AgNps treated larvae produced cocoon shell weight was significantly increased than other two groups (T_1 and T_2). But 25% AgNps and 0.03% *Spirulina* treated larvae produced pupa weight was significantly increased than control group (T_1).

Table 5
Morphometric data of AgNps and Spirulina treated B. mori larvae produced cocoon shell and pupal weight.

Groups	Cocoon shell Weight (gm) (Mean ± S.D)	Pupal Weight (gm) (Mean ± S.D)
Control (T_1)	0.3883±0.0376[a]	1.1233±0.1256[a]
MR_2 mulberry + 25% AgNps (T_2)	0.4767±0.0308[b]	1.5683±0.0673[b]
MR_2 Leaves + 0.03% *Spirulina* (T_3)	0.4250±0.0187[a]	1.5542±0.0964[b]

Values are Mean ± S.D of six observations. Values in the same column with different superscript letters (a & b) differs significantly at P<0.05 (DMRT).

DISCUSSION

In the present study, the larval and cocoon length, width and weight were significantly increased in some groups. Many researchers showed that the larval characters improve by different concentrations of complementary compounds such as ascorbic acid, folic acid, thiamin, vitamin B complex etc., [2, 21, 22]. Muniandy *et al.* [23] have been reported that multi-vitamins and mineral compounds could increase the food intake, growth and conversion efficiency of silkworm. In the present study, it has been observed that silkworms fed by the particular dose of AgNps (25%) and *Spirulina* (0.03%) have enhanced the larval, cocoon and pupal characters were concomitantly increased from 3rd to 5th instars, suggested that AgNps and *Spirulina* which were stimulate silkworm to feed more amount of nutrients intake than the control. This work is corroborated with Nirwani and Kaliwal [22], suggested that this enhancement in larval, cocoon and pupal parameters related to phagostimulation of folic acid. Several authors also reported these effects about ascorbic acid [24-27].

AgNps attach the surface of pathogenic bacterial cell membrane and penetrate to release the silver ions and disturb the functions of bacteria [28-29]. The AgNps target the bacterial cell membrane, leading to a dissipation of the proton motive force, Consequently AgNps need to reach the cell membrane to achieve an antibacterial effect [28, 30]. Considering that the bacterial cell membrane is the site of active transport, respiratory chain components, energy transducing systems, membrane stages in the biosynthesis of phospholipids, peptidoglycan, LPS and capsular polysaccharides, and the anchoring for DNA [31], an alteration of the membrane's integrity would have a great impact in bacterial growth. The AgNps were effective in inhibiting bacterial growth in a dose and time dependent manner [32]. The Pathogenic bacterial cells exposed to milli-molar Ag^+ doses suffer morphological changes such as cytoplasm shrinkage and detachment of cell wall membrane, DNA condensation and localization in an electron-light region in the center of the cell, and cell membrane degradation allowing leakage of intracellular contents [33, 34]. Physiological changes occur together with the morphological changes. Bacterial cells enter an active, but non-culturable state in which physiological levels can be measured but bacteria are not able to growth and replicate. The AgNps enter the gut of silkworm *B. mori* from AgNps (25%) treated MR_2 mulberry leaves it inhibit the pathogenic bacterial growth and multiplication to prevent the bacterial diseases and enhance the feed efficacy, larval growth rate, silk protein synthesis and silk production.

Since most of this multi-vitamin compounds is composed of ascorbic acid, it could be thought that the increase of larval weight is due to an enhancement of feeding activity. Although, in treated larvae the vitamin as cofactors can facilitate the metabolic pathway. Similar findings have also been observed in the present study that AgNps and *Spirulina* act as vitamins to stimulate the feeding activity in the silkworms. Therefore, AgNps and *Spirulina* can improve the food digestibility and increase the larval, cocoon and pupal parameters. *Spirulina* is a rich source of protein and the most powerful and well balanced source of nutrition. One kg of *Spirulina* is said to be equivalent to 1000kg of assorted vegetables [35]. The growth promoting effect of water soluble proteins and vitamins *viz.,* B_2, B_6 and C are found in *Spirulina* and it is treated on silkworm with vitamins and amino acids enhance the larval and cocoon length, width and weight, cocoon shell and pupal weight. Therefore, in the end, the study recommends that 0.03% concentration of aqueous solution of *Spirulina* as feed to silkworm found to effectively increases single larval and cocoon weight, shell and pupal weight. The specific dose of *Spirulina* 0.03% concentration contain maximum amount of essential amino acids and vitamins which determines the specificity for various metabolic activities in silkworm *B. mori*.

In this study, cocoon parameters were changed in different treatments. Previously, it was reported that enrichment of mulberry leaves by some vitamins could increase the cocoon yield. Nirwani and Kaliwal [22] have determined that folic acid causes a significant increase in economical parameters such as female and male cocoon weight. Evanglista *et al.* [36] and Balasundaram *et al.* [37] have also reported that the larval and cocoon length, width and weight were increase under multi-vitamin and vitamin C treatments. The enrichment of mulberry leaves with AgNps and *Spirulina* increase the larval, cocoon and pupal parameters increase in these insects was related to metabolisms other than proteins. It is assumed that fortification of diet supports the metabolism of carbohydrates and lipids.

CONCLUSION

In conclusion, the AgNps and *Spirulina* could increase some biological characters of silkworm *B. mori*, but this enhancement could economically improve the Sericultural goals. In the present study, the treatment of AgNps and *Spirulina* at the concentration of 25% and 0.03% respectively may have beneficial effects on the growth of the silkworm larval, cocoon and pupal parameters and also increase the quantity of silk by enhancing the feed efficacy. So, this supplementation could be prescribed to the farmers to get more quantity of silk.

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