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Enhancement of larval and cocoon traits of silkworm, *Bombyx mori* L. through the application of amino acids

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Abstract
Experiments were conducted by administering different amino acids *viz.*, Glycine, Alanine and Serine with mulberry leaves to enhance the larval and cocoon traits of *Bombyx mori* L. Supplementation of amino acids to silkworm larvae once daily in the morning from the first day of the fifth instar to spinning significantly enhanced the larval, protein, cocoon and silk reeling traits. Among the different amino acid combinations tested, it was found that the treatment with Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm registered significantly highest fifth instar larval weight (4.48 g), ERR (96.50%), silk productivity (6.38 cg/day), silk gland weight (1.12 g) and silk gland length (31.05 m), cocoon weight (2.63 g), shell weight (0.60 g), pupal weight (2.03 g), shell ratio (22..81%), filament length (1220 m) and filament weight (0.440 g). The proteins in the silk gland (64.70 mg/g) and haemolymph (48.50 mg/ml) were also found to be the highest in the above treatment apart from fibroin (420 mg/shell) and sericin (107.05 mg/shell) contents in cocoons. It is concluded that feeding the silkworm by amino acids *viz.*, glycine, alanine and serine fortified mulberry leaves during the fifth instar significantly improves the larval, cocoon and silk reeling traits.

Keywords: amino acids, glycine, alanine, serine, silkworm

Introduction
The silkworm, *B. mori.* secretes the lustrous raw material for the production of the queen of the textiles which could not be substantiated even by the advanced scientific innovations. "As it spins it protects the millions" says a popular Chinese adage. True to this proverb, sericulture provides gainful employment for more than nine million people in India.

Silkworm nutrition plays an important role in improving the growth and development of *B. mori.* The silkworm is a monophagous insect derives its entire nutritional requirement from mulberry leaves. Silk fibroin is derived mainly from four amino acids *viz.*, alanine, serine, glycine and tyrosine which come from their dietary source of protein and amino acids [1]. The silkworm obtains 72-86 per cent of their amino acids from mulberry leaves and more than 60 per cent of the absorbed amino acids are used for silk production [2]. It is well proved that amino acids play an important role in glucose, tryptophan and organic acid metabolism.

The nutritive value of mulberry leaves plays a crucial role in producing good quality cocoons and raw silk. The better growth and development of silkworm larvae and economic traits of cocoons were observed when the larvae were fed with good quality leaves [3]. Though the nutrients are balanced in mulberry leaves, the quantity available is not sufficient for vigorous and healthy growth and development of silkworm larvae [4]. The nutritional status of mulberry leaves is altered by supplementing them with different ingredients and food additives. Among various supplementation experiments with amino acids, vitamins, antibiotics, minerals, *etc.*, the fortification of mulberry leaves with amino acids seems to be much promising since it improved the quality of leaf and silk yield by 40-60 per cent [5].

Various studies conducted by different workers on amino acid supplementation revealed considerable improvement in silk production. The supplementation of various amino acids *viz.*, phenylalanine [6], glycine, alanine and serine [7], tyrosine, phenylalanine and alanine [8], glycine [9], glycine, phenylalanine, serine and aspartic acid [10], asparginine [11] and arginine and histidine [12] have improved the larval traits, cocoon traits and silk productivity. But, the studies on the effects of the amino acid combination on silkworm are very limited. By keeping this in mind, the experiments were carried out to assess the influence of amino acid combinations on the growth & development of silkworm and their impact on cocoon and silk reeling related traits.

Materials and Methods

The experiments were undertaken by supplementing amino acids *viz.,* Glycine, Alanine and Serine with mulberry leaf individually and in combination to improve the economic traits of silkworm larvae and cocoon. The methodology followed and materials used for the study are detailed below.

i) Disinfection of rearing house

Before commencement of rearing, the rearing house and appliances were thoroughly washed and disinfected with 2.5 per cent chlorine dioxide in 0.5 per cent slaked lime solution at 2 lit/m^2 floor area. After disinfection, the rearing house was kept closed in air tight condition for 24 hours and then opened to ward off the smell of chlorine completely [13].

ii) Silkworm rearing

Rearing of Bivoltine Double Hybrid silkworm, (CSR 6 × CSR 26) × (CSR 2 × CSR 27) was carried out using leaves from V1 mulberry variety by adopting standard method [14]. The larvae were fed three times a day with nutritious mulberry leaves without starvation. The bed cleaning was done as recommended and the required spacing was provided as the age of the larvae advanced. The bed disinfectant was applied @ 5g/sq. ft bed area to prevent diseases after bed cleaning as the prophylactic measure. The leaves were provided 45 minutes after the application of bed disinfectant [15].

iii) Application of amino acid to silkworm

The stock solution of 500 ppm was prepared by dissolving 500 mg of amino acid in one litre of distilled water and from the stock solution, different concentrations *viz.,* 10 and 100 ppm were prepared by serial dilution technique.

Weighed quantities of fresh mulberry leaves were separately sprayed with an aqueous solution of the respective amino acid by using a hand automiser. Two larval batches *viz.*, one with water spray and another with untreated mulberry leaves (Control) were also maintained. The treated leaves were shade dried and fed to silkworm larvae once daily in the morning from the first day of the fifth instar to spinning.

iv) Observations recorded

a) Estimation of protein

For the analysis of protein content, the haemolymph of silkworm was collected in microtubes, centrifuged at 14000 rpm and after removing the supernatant kept at –20 ^{0}C for analysis [16]. The silk gland was macerated with pestle and mortar in phosphate buffer (pH 7.0) and the supernatant was collected after centrifuging the content at 5000 rpm at 4 ^{0}C. The estimation of protein content was carried out by adopting astandard procedure [17].

Sericin and fibroin contents in cocoon were analyzed by taking three cocoons in a weighed crucible, to which 20 ml of 5 per cent NaOH was added and allowed to remain soaked for 12 hours. The sericin was removed by washing with boiling distilled water twice, leaving behind the fibroin. Then the crucible containing fibroin was oven dried at 90̊ C for 24 hours. The percentage of fibroin and sericin was calculated according to [18].

b) Larval and cocoon traits

The different observations on fifth instar larval weight, fifth instar larval duration, effective rate of rearing (ERR), silk productivity, cocoon weight, pupal weight, shell weight and shell ratio were recorded following standard procedure [19].

c) Silk reeling traits

The cocoons were harvested seven days after spinning separately replication wise. Three cocoons were randomly selected from each replication. The selected cocoons were cooked in boiling water and reeled individually using *Epprouvette* (Single cocoon reeler) and the filament length was determined. Then, all three reeled silk filaments were dried by keeping in a hot air oven at 70 ^{0}C for one hour and the average filament weight was recorded by using electronic balance. Data on the weight and length of silk filament were used to arrive at the denier (thickness) of the silk by using the following formula.

$$\text{Denier} = \frac{\text{Weight of the silk filament (g)}}{\text{Length of the silk filament (m)}} \times 9000$$

v) Statistical analysis

The experiments were conducted in Completely Randomized Design (CRD) with three replications for each treatment. The number of larvae per replication was 50. Statistical analysis of pooled data was done using the method suggested by [20].

Results and Discussion

Oral application of amino acid combinations to silkworm larvae once daily in the morning from the first day of the fifth instar to spinning significantly enhanced the larval, cocoon and silk reeling related traits of *B. mori*.

a) Effects of amino acid combinations on larval traits

Among the different treatments studied, it was found that the treatment with Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm registered significantly highest fifth instar larval weight (4.48 g), ERR (96.50%) and silk productivity (6.38 cg/day). This treatment was found to be statistically superior over all other treatments and was followed by Glycine @ 10 ppm + Alanine @ 100 ppm (4.41g, 94.50% and 6.27 cg/day, respectively). Here, the amino acids enhanced the fifth larval weight, ERR and silk productivity by 14.29, 14.07 and 10 per cent, respectively. The least fifth instar larval weight (3.92 g), ERR (84.60%) and silk productivity (5.80 cg/day) was recorded in control (Table 1).

Table 1: Effect of amino acid combinations on larval traits of *B. mori*

Treatment	V instar larval duration (h)	V instar larval weight (g)	ERR (%)	Silk productivity (cg/day)
Glycine @ 10 ppm	173.00	4.25	90.60	6.05
Alanine @ 100 ppm	176.00	4.16	89.00	5.96
Serine @ 100 ppm	177.00	4.10	87.25	5.92
Glycine @ 10 ppm + Alanine @ 100 ppm	166.50	4.41	94.50	6.27
Alanine @ 100 ppm + Serine @ 100 ppm	170.50	4.32	92.20	6.16
Glycine @ 10 ppm + Serine @ 100 ppm	169.00	4.35	92.80	6.18
Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm	164.00	4.48	96.50	6.38

Water spray	182.00	3.95	85.00	5.83
Control	182.50	3.92	84.60	5.80
SEd	1.00	0.03	0.75	0.04
CD (0.05)	2.00	0.05	1.50	0.08

Values are mean of three replications and pooled mean of two rearing.

The studies on supplementation of amino acids *viz.*, glycine at 10 ppm, phenylalanine at 100 ppm, serine at 100 ppm and aspartic acid at 100 ppm had resulted in improved larval weight, ERR and silk productivity [10]. Significantly increased larval weight, ERR and silk productivity due to feeding of the mulberry leaves fortified with glycine, alanine and serine have already been reported [19]. These findings are in line with the present observations. Corroborative evidence for having increased the larval parameters of silkworm due to the application of amino acids were also reported by earlier workers [7, 9].

The fifth instar larval duration was significantly reduced to 164.00 hrs due to the application of Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm from 182.50 hrs in control. The treatments *viz.*, Glycine @ 10 ppm + Serine @ 100 ppm and Alanine @ 100 ppm + Serine @ 100 ppm were found to be on par with each other in the case of all the above three larval traits (Table 1). This result falls in line with findings of previous researchers [7, 10], who reported a decrease in fifth instar larval duration by supplementing amino acids, glycine, alanine and serine.

b) Effect of amino acid combinations on silk gland traits and CFU

The size and weight of the silk gland have direct impact on cocoon shell size and weight. *Per os* application of amino acids to silkworm larvae significantly increased the silk gland weight, silk gland length and CFU. Among all the treatments, the amino acid combination with Glycine @ 10 ppm, Alanine @ 100 ppm and Serine @ 100 ppm was found to record significantly maximum silk gland weight, silk gland length and CFU of 1.12 g, 31.05 m and 90.75, respectively which was found to be superior over all other treatments. This was followed by Glycine @ 10 ppm + Alanine @ 100 ppm (1.03 g, 30.52 m and 88.50, respectively). The treatments *viz.*, Glycine @ 10 ppm + Serine @ 100 ppm and Alanine @ 100 ppm + Serine @ 100 ppm were found to be on par with each other. However, all the treatments with amino acid showed a significant difference from the control. The silk gland weight (0.78 g), silk gland length (28.24 m) and CFU (76.48) were found to be least in the control (Table 2).

Table 2: Effect of amino acid combinations on silk gland traits and CFU of B. mori

Treatment	Silk gland weight (g)	Silk gland length (m)	CFU
Glycine @ 10 ppm	0.86	29.50	83.50
Alanine @ 100 ppm	0.87	28.75	81.00
Serine @ 100 ppm	0.86	29.20	78.75
Glycine @ 10 ppm + Alanine @ 100 ppm	1.03	30.52	88.50
Alanine @ 100 ppm + Serine @ 100 ppm	0.93	29.93	85.60
Glycine @ 10 ppm + Serine @ 100 ppm	0.95	30.10	86.25
Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm	1.12	31.05	90.75
Water spray	0.78	28.30	76.60
Control	0.78	28.24	76.48
SEd	0.03	0.20	0.98
CD (0.05)	0.05	0.38	2.00

Values are mean of three replications and pooled mean of two rearing.

These observations synchronize with the previous findings of significant increase in silk gland weight, silk gland length and CFU of 1.03 g, 29.70 cm and 85.78, respectively in the silkworm larvae fed by leaves treated with glycine, alanine and serine [19]. Corroborative evidence were also reported by earlier workers [9, 10, 21], who reported that glycine application at minimum concentration either individually or in combination increased the silk gland related traits.

c) Effects of amino acid combinations on cocoon traits

Application of amino acid combinations elucidated the positive response on various cocoon related parameters of silkworm. The investigations with three amino acids showed that the treatment with Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm recorded highest cocoon weight (2.63 g), shell weight (0.60 g), pupal weight (2.03 g) and shell ratio (22.81%) which was found to be statistically superior over all other treatments. The improvement in cocoon weight, pupal weight and shell ratio was 36.98, 29.30 and 25.12%, respectively over the control. This was followed by Glycine @ 10 ppm + Alanine @ 100 ppm (2.48 g, 0.55 g, 1.93 g and 22.18%, respectively). The lowest value of 1.92 g, 0.35 g, 1.57 g and 18.23%, respectively were registered in control (Table 3).

Table 3: Effect of amino acid combinations on cocoon traits of *B. mori*

Treatment	Cocoon weight (g)	Shell weight (g)	Pupal weight (g)	Shell ratio (%)
Glycine @ 10 ppm	2.21	0.43	1.78	19.46
Alanine @ 100 ppm	2.16	0.41	1.75	18.98
Serine @ 100 ppm	2.05	0.40	1.65	19.51
Glycine @ 10 ppm + Alanine @ 100 ppm	2.48	0.55	1.93	22.18
Alanine @ 100 ppm + Serine @ 100 ppm	2.34	0.48	1.86	20.51
Glycine @ 10 ppm + Serine @ 100 ppm	2.37	0.50	1.87	21.10
Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm	2.63	0.60	2.03	22.81

Water spray	1.92	0.35	1.57	18.23
Control	1.92	0.35	1.57	18.23
SEd	0.04	0.02	0.03	0.30
CD (0.05)	0.10	0.04	0.06	0.58

Values are mean of three replications and pooled mean of two rearing.

Supplementation of amino acids *viz.*, glycine, alanine and serine through mulberry leaves during the fifth instar significantly increased the cocoon weight, pupal weight, shell weight and shell ratio [19]. *Per os* application of amino acids *viz.*, glycine at 10 ppm, phenylalanine at 100 ppm, serine at 100 ppm and aspartic acid at 100 ppm had resulted in enhanced cocoon weight, pupal weight, shell weight and shell ratio [10]. These findings are in line with the present observations. The present observations are also under the findings of earlier researchers [8, 22], who reported higher cocoon economic parameters when the silkworm larvae were fed by mulberry leaves supplemented with amino acids.

d) Effects of amino acid combinations on silk gland and haemolymph proteins

Fortification studies conducted to know the protein content in silk gland and haemolymph revealed that larvae treated with Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm recorded highest silk gland and haemolymph proteins of 64.70 mg/g and 48.50 mg/ml, respectively which were found to be statistically superior over all other treatments studied.

Table 4: Effect of amino acid combination on protein content in silk gland and haemolymph of *B. mori*

Treatment	Protein content	
	Silk gland (mg/g)	Haemolymph (mg/ml)
Glycine @ 10 ppm	56.90	41.05
Alanine @ 100 ppm	54.36	39.00
Serine @ 100 ppm	53.24	38.25
Glycine @ 10 ppm + Alanine @ 100 ppm	62.15	46.27
Alanine @ 100 ppm + Serine @ 100 ppm	59.47	43.20
Glycine @ 10 ppm + Serine @ 100 ppm	59.55	44.18
Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm	64.70	48.50
Water spray	50.68	36.00
Control	50.05	35.90
SEd	1.25	1.00
CD (0.05)	2.50	2.00

Values are mean of three replications and pooled mean of two rearing.

The next better treatment was Glycine @ 10 ppm + Alanine @ 100 ppm with the protein content of 62.15 mg/g and 48.50 mg/ml, respectively for silk gland and haemolymph proteins. The treatments *viz.*, Glycine @ 10 ppm + Serine @ 100 ppm (59.55 mg/g and 48.18 mg/ml, respectively) and Alanine @ 100 ppm + Serine @ 100 ppm (59.47 mg/g and 43.20 mg/ml, respectively) were found to be on par with each other and statistically differed from the control (Table 4). A high level of proteins in the present study might be due to incorporation of the amino acids into the haemolymph and silk gland.

The present result on increase in protein content of silkworm falls in parallel with the earlier findings that oral supplementation with 50, 100 and 150 µg/ml of arginine and histidine to the silkworm larvae resulted in a significant increase in silk gland and haemolymph proteins [12]. The other earlier findings that showed a significant increase in the protein content in larvae and silk gland due to supplementation of amino acids [10, 23, 24] were also fall in line with the present observations.

e) Effects of amino acid combinations on fibroin and sericin content

Application of amino acids to larvae of silkworm significantly enhanced the fibroin content in cocoon. Among all the treatments, the amino acid combination with Glycine @ 10 ppm, Alanine @ 100 ppm and Serine @ 100 ppm recorded highest fibroin content of 440 mg/shell which was found to be superior over all other treatments. This was followed by Glycine @ 10 ppm + Alanine @ 100 ppm (420 mg/shell). The treatments *viz.*, Glycine @ 10 ppm + Serine @ 100 ppm (398 mg/shell) and Alanine @ 100 ppm + Serine @ 100 ppm (387 mg/shell) did not differ statistically with each other and showed significant superiority over the control (310 mg/shell) (Table 5).

Table 5: Effect of amino acid combination on sericin and fibroin content in cocoon of *B. mori*

Treatment	Fibroin (mg/shell)	Sericin (mg/shell)
Glycine @ 10 ppm	368	85.00
Alanine @ 100 ppm	346	79.74
Serine @ 100 ppm	340	94.25
Glycine @ 10 ppm + Alanine @ 100 ppm	420	92.14
Alanine @ 100 ppm + Serine @ 100 ppm	387	99.08
Glycine @ 10 ppm + Serine @ 100 ppm	398	102.50
Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm	440	107.05
Water spray	317	72.10
Control	310	71.60
SEd	9.00	2.00
CD (0.05)	18.00	4.00

Values are mean of three replications and pooled mean of two rearing.

The present study is in parallel with the findings that, among the different amino acid tested, highest fibroin content of 370 mg/shell was recorded in the treatment with glycine 10 ppm, alanine 100 ppm and serine 100 ppm [19].

The amino acids also had a positive influence on the sericin level of cocoon. Among the different amino acid combinations studied, Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm recorded significantly highest sericin content (107.05 mg/shell) and was found to be superior over all other treatments. This was followed by Glycine @ 10 ppm + Serine @ 100 ppm (102.50 mg/shell) and Alanine @ 10 ppm + Serine @ 100 ppm (99.08 mg/shell) which were found to be on par with each other. The least sericin content of 71.60 mg/shell was registered in the control (Table 5). The present observations are supported by earlier workers [19] who registered the highest sericin content of 151 mg/shell was registered in when the fifth instar larvae were fed by mulberry leaves sprayed with alanine 100 ppm and serine 100 ppm. This also can be corroborated with the findings that the acidic amino acids would seem to be important as amino group donor, from which more glycine and serine residues will be formed and incorporated into fibroin or sericin [10, 25].

f) Effects of amino acid combinations on silk reeling related traits

The result of the present study indicated that *per os* administration of amino acid combinations to silkworm significantly enhanced reeling related traits. Among the various treatments, Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm registered maximum filament length (1220 m) and filament weight (0.440 g) which was found to be superior over all the treatments studied. Here, the improvement over control is 16.19 and 42.86%, respectively. The treatment with three amino acid combinations was followed by Glycine @ 10 ppm + Alanine @ 100 ppm (1169 m and 0.417 g, respectively). The minimum silk filament length (1050 m) and filament weight (0.308 g) was recorded in control (Table 6).

This result falls in line with previous workers [10, 26], who reported that supplementation of glycine and serine increased the filament weight by increasing fibroin and sericin respectively. Fibroin content has a direct effect on filament length which was largely influenced by lower concentration of glycine and a higher concentration of alanine. Reports of earlier researchers [5, 9, 21, 22] are in agreement with the present findings.

Table 6: Effect of amino acid combination on silk reeling related traits of *B. mori*

Treatment	Filament length (m)	Filament weight (g)	Denier
Glycine @ 10 ppm	1105	0.365	2.85
Alanine @ 100 ppm	1084	0.343	2.90
Serine @ 100 ppm	1080	0.336	2.90
Glycine @ 10 ppm + Alanine @ 100 ppm	1169	0.417	2.72
Alanine @ 100 ppm + Serine @ 100 ppm	1128	0.388	2.78
Glycine @ 10 ppm + Serine @ 100 ppm	1140	0.395	2.80
Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm	1220	0.440	2.66
Water spray	1058	0.312	2.91
Control	1050	0.308	2.93
SEd	10.00	0.01	0.02
CD (0.05)	20.00	0.02	0.04

Values are mean of three replications and pooled mean of two rearing.

Thin denier is a desirable character for quality silk filament. Silk industry should fulfill the choice of the people in silk fabrics. The necessity varies from coarse to fine textured cloths. This can be achieved only with fine denier filament. The supplementation of amino acid combination also altered the thickness of filament. The minimum denier of 2.66 was registered in Glycine @ 10 ppm + Alanine @ 100 ppm + Serine @ 100 ppm which was found to be statistically superior over all other treatment. On the contrary, the maximum denier was registered in control (2.93). Here, the improvement in denier was 9.22 per cent over the control (Table 6). The present result got strengthened with the findings that addition of alanine resulted in fine denier [10]. Further, the findings of [19], who recorded fine denier of 2.53 when the larvae were fed by mulberry leaves sprayed with glycine, alanine and serine is also synchronizes with the present observations.

Conclusion

It is concluded form the present study that fortification of mulberry leaves with amino acid combination having Glycine, Alanine and Serine, respectively at 10, 100 and 100 ppm and feeding to silkworm larvae once daily in the morning from the first day of the fifth instar to spinning significantly enhanced larval, cocoon and silk reeling related traits, the protein content of haemolymph, silk gland and cocoon.

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