RESEARCH

Spermidine Enhances the Silk Production by Mulberry Silkworm

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ABSTRACT. Polyamines are ubiquitous low molecular weight polycationic aliphatic amines involved in diverse cellular processes. Spermidine (Spd), a polyamine, has been proved to be crucial for cell survival in various organisms. Our study reports the effect of Spd on the growth of *Bombyx mori*. Silkworms showed improved silk gland weight and economic parameters in the fifth instar larval stage when treated with different concentrations of Spd, in the range of 25–75 μM. The worms treated with Spd produced 31% more silk when compared with the control worms. Altogether, this study establishes that Spd-treated leaves can be fed into the larvae for better silk production.

Key Words: polyamine, spermidine, larval weight, economic trait

Polyamines have been known for a long time and are found in all organisms (Bachrach 2010). As these are polycations, one of their main features is to interact with negatively charged molecules, such as DNA and RNA. Intensive research on the functions of polyamine in mammals has revealed that they are essential regulators of growth, gene transcription, and ribosome-mediated translation (Childs et al. 2003, Thomas and Thomas 2003, Umekage and Ueda 2006). These are important players in plant growth, stress, and disease resistance (Hussain et al. 2011). Earlier studies in our laboratory showed 1,3,4-diamino-substituted thiadiazole as possible growth enhancer of *Bombyx mori* larvae in fifth instar (Umadevi et al. 2012).

Spermidine (Spd) is synthesized from putrescine and is involved in an array of crucial molecular processes. It is essentially present in all body fluids like semen, blood, saliva, tears, and milk. Feeding *B. mori* in the fifth instar larval stage with bovine milk-treated mulberry leaves improved the growth of the worms (Niharika et al. 2013). In addition, Spd is a natural component of our diet, and several foods including soybeans, tea leaf, and mushrooms (Binh et al. 2010) are known to be rich in Spd. Evidence suggests that eating a Spd-rich diet results in increased blood Spd levels (Soda 2009). Spd at 0.1 and 2 μm concentration caused the growth of root and shoot and increased the total biomass of lime seedling (Elias et al. 2011). In blow fly, *Calliphora erythrocephala* rapid growth during development coincided with high levels of Spd concentrations (Andersson and Heby 1972). Treatment of Spd in micromolar concentrations to Tasar silkworm showed increased silk production (Renuka et al. 2013). Spd can also interact with RNA to modulate mRNA translation, and, in lymphocytes, it is estimated that approximately 60% of Spd binds to RNA (Igarashi and Kashiwagi 2009). Spd at high concentrations (120 mM) did not show any positive effect on transcription and growth (Byus and Herbst 1976, Ramot et al. 2011). Further, the addition of other polyamines like spermine, putrescine, cadaverine, and ethylene diamine did not show any increase in the RNA transcription (Byus and Herbst 1976). Spd in micromolar concentrations (0.1–1 μM) has been found to promote human hair shaft elongation and prolonged hair growth (Ramot et al. 2011). It had a beneficial anti-aging effect on nematodes and fruit flies when added to their food, in millimolar concentrations (Madeo et al. 2010). Spd prolonged the mean and the maximum lifespan of the nematode *Caenorhabditis elegans* by 15% and in the fly *Drosophila melanogaster* by up to 30% when supplemented in lower concentrations (0.2 mM) (Eisenberg et al. 2009). Recently, Spd was shown to be beneficial against two age-related diseases: cataract formation and multiple sclerosis (Lentini et al. 2011), thus suggesting that it has a potential antiaging effect.

The mulberry silkworm *B. mori* (Lepidoptera: Bombycidae) is an economically important insect and the silk it produces is called mulberry silk. It is domesticated and has been exploited for over 4000 yr. The larvae mostly feed on the leaves of mulberry. They require an optimum temperature of about 23–25°C and the larval duration in the life cycle ranges from 25 to 30 d. The larval stage is divided into five instars, separated by four molts. On an average, one generation of *B. mori* spans 40–45 d. During the last larval stage (fifth instar), the silk gland produces the silk for the cocoon. The weight of silk gland accounts for about 25% of the weight of larvae in the late fifth instar (spinning stage). One of the most important factors in the rearing of silkworm is the cultivation of mulberry. The nutritive value of mulberry plays a very effective role in producing good quality cocoons of silkworms (Legay 1958). It was observed that silkworms obtain 72–86% of their amino acids from mulberry leaves and more than 60% of the absorbed amino acids is used for silk production (Lu and Jiang 1988).

Polyamines were found to play a crucial role in the development of *B. mori*. Spd was observed to be abundant, especially in the silk glands, gonads, mucous gland, and sucking stomach. The concentrations of most of the polyamines in the silk glands remained constant during the larval stage and decreased markedly at the pupal stage (Hamana et al. 1984).

The present study demonstrates the effects of Spd on the growth of mulberry silkworm during the fifth instar stage in which the development of the silk glands takes place. Treatment with Spd showed positive results on the silk gland weight. The economic parameters such as cocoon weight, pupal weight, shell weight, and the amount of silk reeled also increased in the Spd-treated groups. Therefore, the present study suggests that mulberry silkworms can be fed with mulberry treated with Spd in micromolar concentrations for better growth and silk production.

Materials and Methods

Standard Polyamine. Spd-free base was purchased from HiMedia Chemicals.

Collection of Silkworms. The disease-free layings and bivoltine race of mulberry silk worm *B. mori* were collected and the fifth instar larvae

were picked up at random for treatment with Spd. The worms were collected in the months of October/November/December from Kathipudi area in East Godavari district of Andhra Pradesh.

Design 1. The fifth instar (day 1) mulberry silkworms were initially divided into four groups of 10 each, out of which three groups were treated with different concentrations of Spd (25, 50, and 75 μM) and the fourth group worms were kept as control without any drug treatment. For each concentration group (25, 50, and 75 μM), five numbers of mulberry leaves were taken for 1 ml of foliar application of Spd. The sample solutions were prepared by dissolving 15 mM Spd in 10 ml of distilled water (stock solution) from which 0.033, 0.066, and 0.1 ml were dissolved in 20 ml of distilled water in order to obtain the desired working concentrations, i.e., 25, 50, and 75 μM, respectively. Spd was applied uniformly on both sides using paintbrush method. The leaves were allowed to air dry before feeding. The worms were grown in separate trays and fed three times a day in the above manner for the first 2 d. The number of the leaves and the volume of Spd was increased in each treatment (10 numbers and 2 ml Spd) from day 3 to day 7 of the fifth instar. The control worms were fed with the same number of untreated leaves till the onset of spinning. As it was difficult to maintain the number and the mass of the leaf constant for every treatment, we recorded the total mass of the leaf given and also the mass of the leaf left over on each day from all the groups. These data were used to calculate the total mass of the leaf consumed from day 1 to day 7 of the fifth instar stage in the control and the treated groups.

Treatment was given to a small group of worms (10–30 in each group) in all experimental groups initially and was repeated three times. The similar treatment was also given once to a larger group of worms (83 worms in each).

Larval Weights. Pre-treatment weights of all the worms in different groups were taken. Body weights of the larvae in all the groups were taken on alternate days, i.e., 1, 3, 5, and 7 of the fifth instar during the treatment period using a digital balance. The mass of all the worms from each group was recorded and then the average larval weight was calculated. The gland weights of the larvae were taken on the seventh day of fifth instar (spinning stage).

Leaf Consumed. The total amount of leaf consumed by the larvae during the fifth instar stage was calculated by subtracting the unconsumed leaf weight from the total weight of leaves provided initially.

$$\text{The amount of leaf consumed} = \text{Total weight of leaves provided (g)} - \text{weight of leaves left over (g)}$$

Tissue Sample Preparations. The silk glands were dissected out from the larvae on the first and seventh day of fifth instar stage in all the treated and untreated groups in insect ringer solution and homogenized in pre-chilled phosphate buffer in cold conditions. The homogenate was centrifuged at 3000 rpm for 15 min in cold condition (4°C) and the supernatant was collected and stored at −80°C till use.

Protein, Carbohydrate, and RNA Estimations. Protein assay of various gland samples was done by Lowry method (Lowry et al. 1951), using bovine serum albumin as standard. The quantitative estimation of total sugars in the samples was done by Anthrone method (Dubois et al. 1956), using glucose as standard. RNA estimation was done by Orcinol method. These assays were performed on the first (pre-treatment assays) and seventh day of the fifth instar larval stage.

Economic Parameters. All the parameters were measured based on the standard procedures (FAO Manual 1972).

1. Cocoon weight/Pupal Weight
   By the end of fifth instar stage, cocoons were formed in all the experimental groups. The cocoons were harvested, weighed, and the values were noted down. The cocoons were cut open, and the pupal weights were also recorded.

2. Shell Weight

$$\text{Shell weight} = \text{Cocoon Weight} - \text{Pupal weight.}$$

3. Shell Ratio
   The formula for shell ratio is

$$\text{Shell ratio (\%)} = \frac{\text{Shell Weight}}{\text{Cocoon Weight}} \times 100$$

4. Reelability: The reelability of cocoons is the fitness of cocoons for economically feasible reeling. This is calculated by the following formula:

$$\text{Reelability (\%)} = \frac{\text{Weight of the silk reeled}}{\text{Weight of the cocoons}} \times 100$$

5. Length/weight of the filament (FAO Manual 1972)
   Cocoon epprouvette was used to measure the filament length. It has an axis around which four wooden sticks were arranged at equal distance with the circumference of 9/8 m. The number of rotations multiplied by the circumference gives the filament length (Length of filament = One revolution on epprouvette = 9/8 m or 1.125 m).

$$\text{Filament length} = 9/8 \text{ m} \times \text{No. rotations}$$

   The reeled silk weight was measured with an electronic digital balance in grams.
6. Denier: Denier is obtained by the following formula:
   Weight of the silk reeled

$$\text{Denier} = \frac{\text{Weight of the silk reeled}}{\text{Length of the silk reeled}} \times 9000$$

Statistical Analysis of Data. The differences in the larval weights, silk gland weights, total amount of leaf consumed during treatment, post cocoon parameters like cocoon weights, pupal weights, shell weights, etc., and the protein, carbohydrate, and RNA estimation values

Table 1. Effect of Spd (25, 50, and 75 μM) on the body weights and silk gland weights of fifth instar larvae

Treatment	Weight of worms before treatment (g)	Silkworm weight (before spinning) (g)	Silk gland weight (before spinning) (g)	Total leaf consumed during fifth instar (g)
Control	0.72 ± 0.01	2.76 ± 0.11a	0.68 ± 0.02	143.98 ± 11.67a
25 μM	0.77 ± 0.02	3.40 ± 0.23a	0.90 ± 0.02*	125.85 ± 5.27a
50 μM	0.81 ± 0.03	3.29 ± 0.29a	1.11 ± 0.02*	125.47 ± 8.78a
75 μM	0.86 ± 0.02	3.31 ± 0.25a	0.69 ± 0.04	130.66 ± 7.09a

The body weights were taken prior to Spd treatment and also at the end of fifth instar (before spinning). The total leaf consumed by the control group and the Spd-treated groups (25, 50, and 75 μM) from day 1 to day 7 of the fifth instar larval stage was tabulated. Means (±S.E.) followed by same letters within the same column are not significantly different (a). Asterisk indicates that the values are statistically significant (*t*-test, $\alpha = 0.05$) from the control group.

between the control and the Spd-treated groups were compared using Student's *t*-test and the probability $\alpha = 0.05$ was taken as the critical value for all the tests.

Results

Effect of Spd on Body and Gland Weight. In order to test the effect of Spd on the growth of silk worm, body and silk gland weights were checked after treatment with different concentrations. Treatments involving 25, 50, and 75 μM Spd concentrations showed varied results. The larval weights of the control and the treated groups increased from day 1 to day 7 (Table 1). Though the results of the Spd-treated groups showed that the average body weights enhanced to a greater extent than that of the control group, the increase was not found to be significant as variations were observed among replicates, both in the control and in the Spd-treated groups. This could possibly be due to various other factors involved during rearing (Fig. 1).

We also examined the effect of Spd on the silk gland weights. The 25 and 50 μM Spd-treated groups showed appreciably higher silk gland weights than that of the control group (Table 1). The 50 μM Spd-treated group showed more pronounced increase in the gland weight than the 25 μM treated group. With further increase in concentration to 75 μM, the silk glands did not show any substantial weight gain over the control. In addition, the silk glands at 75 μM weighed yet lesser than that of the 50 and 25 μM Spd-treated groups. In conclusion, 50 μM Spd-treatment was more effective for silk gland growth.

We further checked if the increase in the silk gland weights in the 25 and 50 μM Spd-treated groups was due to consuming more leaf than the untreated worms in the control group. The amount of leaf consumed was weighed for the treated and control groups from day 1 to day 7 of the fifth instar larval period. Twenty-eight worms were taken in each group. We did not find any measurable difference in the leaf consumption between the control and the different treated groups (Table 1).

Effect of Spd on the Economic Traits of B. mori. Our overall aim was to assess the effect of Spd on the economic traits of mulberry silkworm. Economic traits of the treated and the control cocoons were determined in accordance with the standard procedures (FAO Manual 1972). The post-cocoon parameters of mulberry silkworm *B. mori*, including cocoon weight (g), pupal weight (g), shell weight (g), shell ratio (%), reelability (%), weight of the silk reeled (g), and length of the silk reeled (m), showed better results in all the treated groups than in the control group (Table 2) (Fig. 2). Of all the treated groups, 75 μM concentration showed higher values when compared to other groups in all the economic parameters. The increase in the pupal and shell weights of all the treated groups was found to be statistically significant. The increase in the shell weight indicates an increase in the amount of silk produced. All the treated groups showed relatively considerable increase in length of the silk reeled. Further, the amount of silk reeled was found to be highest in 75 μM Spd treatment. The three concentrations of Spd-treated groups and control group showed similar denier values. Spd showed positive effect on all the economic parameters of mulberry silk worm.

Effect of Spd on the Protein, Carbohydrate, and RNA Levels of Silk Glands of B. mori. Finally, the effect of Spd on the protein, carbohydrate, and RNA levels of silk glands of *B. mori* were checked on the seventh day of fifth instar stage (Table 3). Whole silk gland was used for these estimations. Standards for the three estimations were maintained as per the protocols. The results obtained were as follows: no appreciable differences were noted in the protein and carbohydrate levels of the control and the Spd-treated groups. There were no important changes in the total silk gland RNA levels as estimated on seventh

Fig. 1. The control and Spd-treated larvae (25, 50, and 75 μM) on day 7 of the fifth instar stage of *B. mori*.

Fig. 2. Cocoons of the control and Spd-treated larvae (25, 50, and 75 μM).

Table 2. Post cocoon parameters of the control and the Spd-treated larvae (25, 50, and 75μM) of *B. mori*

S. No	Concentration of Spd	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell ratio (%)	Reelability (%)	Weight of the silk reeled (g)	Length of the silk reeled (m)	Denier
1	25 μM	1.87 ± 0.004[a]	1.50 ± 0.01*	0.37 ± 0.005*	19.78	14.81	0.277	692	3.6
2	50 μM	1.90 ± 0.009[a]	1.51 ± 0.005*	0.38 ± 0.004*	20.26	15.15	0.288	720	3.6
3	75 μM	1.94 ± 0.005[a]	1.52 ± 0.005*	0.42 ± 0.008*	21.53	16.15	0.315	787	3.6
4	Control	1.70 ± 0.012	1.39 ± 0.023	0.31 ± 0.021	18.23	13.64	0.232	600	3.47

All the parameters like cocoon weights, shell weights, shell ratio, reelability, weight of the silk reeled, and the length of the silk reeled of control and all the treated groups were tabulated. Means (±SE) followed by same letters within the same column are not significantly different (a). Asterisk indicates that the values are statistically significant (*t*-test, $\alpha = 0.05$) from the control group.

Table 3. Effect of Spd on the protein, carbohydrate, and RNA levels of silk gland of *B. mori*.

Treatment	Total gland protein (mg/ml)	Total gland carbohydrate (mg/ml)	Total gland RNA (mg/ml)
Control seventh day	42.35 ± 1.95a	7.08 ± 0.84a	2.87 ± 0.42a
25 μM	39.70 ± 2.40a	4.61 ± 0.13a	3.32 ± 0.20a
50 μM	42.35 ± 5.25a	5.48 ± 0.62a	2.35 ± 0.23a
75 μM	48.00 ± 3.00a	6.82 ± 0.80a	2.91 ± 0.56a

The estimations were performed on seventh day of fifth instar for the control and the Spd-treated groups and the results were tabulated. Means (±SE) followed by same letters within the same column are not significantly different.

day of the fifth instar larvae between the control and treated groups. These observations suggest that Spd treatment did not influence the overall protein, carbohydrate, and the RNA levels during the fifth instar stage.

Discussion

The rearing of mulberry silkworm *B. mori*, which produces mulberry silk, is of great economic importance. Numerous efforts have been made to enhance its growth by several biotechnological methods. Although Spd is known to have a positive effect on the growth of various organisms (Eisenberg et al. 2009), its effect on the growth of the economically important mulberry silkworm *B. mori* was not ascertained. The present report shows a positive effect of Spd treatment on the growth and economic parameters of mulberry silkworm. The exact reason for the improved silk gland weights with lower Spd concentrations, i.e., 25 and 50 μM and decreased gland weights with further increase in concentration to 75 μM was not clear. Earlier reports have also shown increase in transcription levels and growth at lower concentrations and inhibition of growth at higher concentrations of Spd treatment (Byus and Herbst 1976, Ramot et al. 2011). We did not observe any change in the total RNA levels though Spd was known to increase transcription levels (Igarashi and Kashiwagi 2009). We observed a slight increase in protein level in one of the treated groups. As Spd is known to interact with promoter sequences specifically (Søren et al. 2005), we hypothecate that interaction of Spd with some specific promoters like fibroin resulted in increased silk production.

The results correlate with the earlier findings of the role of polyamines in the larval development of silkworm (Hamana et al. 1984, Renuka et al. 2013). From the present study it can be interpreted that polyamines, namely, Spd can be given as an extra supplement for the better growth of silkworms. More elaborate studies with various concentrations of Spd must be tried at farm level to determine the minimum Spd concentration required to obtain best economic traits.

The present study is the first report showing the effect of Spd on the growth parameters of mulberry silkworm *B. mori*. It can be concluded from the present study that the *B. mori* silkworms can be fed with Spd-treated leaves for better growth and for increased silk production.

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References Cited

Andersson, G., and O. Heby. 1972. Polyamine and nucleic acid concentrations in Ehrlich ascites carcinoma cells and liver of tumor-bearing mice at various stages of tumor growth. J. Natl Cancer Inst. 48, 165–172.

Bachrach, U. 2010. The early history of polyamine research. Plant Physiol. Biochem. 48: 490–495.

Binh, P. N. T., K. Soda, C. Maruyama, and M. Kawakami. 2010. Relationship between food polyamines and gross domestic product in association with longevity in Asian countries. Health 2: 1390–1396.

Byus, C. V., and E. J. Herbst. 1976. The effect of polyamines on the synthesis of ribonucleic acid by *Drosophila melanogaster* larvae. Biochem. J. 154: 23–29.

Childs, A. C., D. J. Metha, and E. W. Gerner. 2003. Polyamine-dependent gene expression. Cell. Mol. Life Sci. 60: 1394–1406.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method of determination of sugars and related substances. Anal. Chem. 28: 350–356.

Eisenberg, T., H. Knauer, A. Schauer, H. Fussi, S. Buttner, and D. Carmona-Gutierrez. et al. 2009. Induction of autophagy by Spd promotes longevity. Nat. Cell Biol. 11: 1305–1314.

Elias, A., M. Mohammad, and M. Majid. et al. 2011. The effects of Spd and putrescine polyamines on growth of pomegranate (*Punica granatum* L. cv 'Rabbab') in salinity circumstance. Int. J. Plant Physiol. Biochem. 3: 43–49.

FAO. 1972. Manual on sericulture, Vol. 3, Silk reeling, Agricultural Services Bulletin No. 72/3.

Hamana, K., S. Matsuzaki, and K. Inoue. 1984. Changes in polyamine levels in various organs of *Bombyx mori* during its life cycle. J. Biochem. 95: 1803–1809.

Hussain, S. S., M. Ali, M. Ahmad, and K. H. M. Siddique. 2011. Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. Biotechnol. Adv. (doi: 10.1016/j.biotechadv.2011.01.003).

Igarashi, K., and K. Kashiwagi. 2009. Modulation of cellular function by polyamines. Int. J. Biochem. Cell Biol. (doi: 10.1016/j.biocel.2009.07.009).

Lu, S. L., and Z. D. Jiang. 1988. Absorption and utilization of amino acids in mulberry leaves by *Bombyx mori* L. Acta Sericol. Sancta 14: 198–204.

Legay, J. M. 1958. Recent advances in silkworm nutrition. Annu. Rev. Entomol. 3: 75–86.

Lentini, A., C. Tabolacci, P. Mattioli, B. Provenzano, and S. Beninati. 2011. Spd delays eye lens opacification in vitro by suppressing transglutaminase-catalyzed crystalline cross-linking. Protein J. 30: 109–114.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with folin-phenol reagent. J. Biol. Chem. 193: 265–275.

Madeo, F., T. Eisenberg, S. Büttner, C. Ruckenstuhl, and G. Kroemer. 2010. Spd: a novel autophagy inducer and longevity elixir. Autophagy 6: 160–162.

Niharika, K., A. Praveena, B. Venugopal Reddy, and M. Anitha. 2013. Effect of bovine milk on the growth of *Bombyx mori.* J. Insect Sci. 13: 98.

Ramot, Y., S. Tiede, T. Biro, M. H. Abu Bakar, K. Sugawara, M. P. Philpott, W. Harrison, M. Pietila, and R. Paus. 2011. Spd promotes human hair growth and is a novel modulator of human epithelial stem cell functions. PLoS One 6: 1–12.

Renuka, G., M. Anitha, and G. Shamitha. et al. 2013. Effect of Spd on the economic traits of Tasar silk worm *Antheraea mylitta* (Daba TV). Int. J. Innov. Biol. Res. 2: 15–20.

Soda, K. et al. 2009. Long-term oral polyamine intake increases blood polyamine concentrations. J. Nutr. Sci. Vitaminol. (Tokyo). 55: 361–366.

Søren, L., E. Peter Nielsen, and E. M. Niels. 2005. Polyamines preferentially interact with bent adenine tracts in double-stranded DNA. Nucleic Acids Res. 33: 1790–1803.

Thomas, T., and T. J. Thomas. 2003. Polyamine metabolism and cancer. J. Cell Mol. Med. 7: 113–126.

Umadevi, P., B. Venugopal Reddy, and M. Anitha. 2012. Synthesis and evaluation of diamino substituted 1,3,4-thiadiazole as possible *Bombyx mori* growth enhancer. Int. J. Pharma Biosci. 3: 604–611.

Umekage, S., and T. Ueda. 2006. Spd inhibits transient and stable ribosome subunit dissociation. FEBS Lett. 580: 1222–1226.

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