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POLYAMINE SUPPLEMENTS TO THE DIET ENHANCE LARVAL AND SILK GLAND CHARACTERISTICS IN TASAR SILKWORM *Antheraea mylitta* D

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AUTHORS' CONTRIBUTIONS
This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Polyamines (PAs) are polycationic, biosynthetic intermediate metabolites of amino acids and regulate many metabolic processes inside cells i.e., organization of DNA, RNA, transcription and translation etc., which contribute to promoting growth and development in animals. The DABA bivoltine (2 crops/year) ecorace of Tasar silkworm, *Antheraea mylitta* is reared by tribal populations in Indian forest ecosystems mainly for livelihood. Due to its rearing in natural wild conditions, the abiotic and biotic environmental stress led to 60-70% crop loss in every rearing. Silk yarn is used in textile industries while raw and fabricated products possess export value. Recent investigations revealed that silk proteins viz., fibroin and Sericin tend to have high potential biomaterial for tissue engineering. Hence, there is a need to select high-yielding and disease-resistant varieties for sustainable crop improvement. As silk production relies on the fifth instar larval and silk gland development, which in turn is determined by quality food intake and molecular mechanism contributed by nutritive supplements, the present work is taken up which was not explored to date. The fifth instar larvae of *A. mylitta* D (Daba TV) were allowed to feed on the *Terminalia arjuna* leaves treated with *polyamines (Spermidine, Spermine and Putrescine)* in 50 µM, 100 µM and 150 µMconcentrations. The larval behaviour was studied; larval characteristics, silk gland development, silkworm Body Mass Index (BMI), mortality and Effective Rearing Rate (ERR) were estimated statistically and interpreted. The study revealed significant enhancement in larval, silk gland weight and disease resistance in certain specified concentrations of *PAs*.

Keywords: *A. mylitta*; *polyamine* supplements; V instar larvae; silk gland; development; effective rearing rate (ERR); mortality; behaviour.

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1. INTRODUCTION

Tasar culture in India is an age-old traditional and cultural heritage practised by backward tribal populations for livelihood in various geographical zones of Indian remote forests. Among 44 eco races, *Antheraea mylitta* with 10 diversified ecorace and their food plants, *Terminalia tementosa, Terminalia arjuna, Shorea robusta* etc., occupied in southern and south-east Indian states Maharashtra, Jharkhand, Chhattisgarh, Odisha, West Bengal, Andhra Pradesh and Telangana [1]. Tasar silkworms are a precious source of high-quality protein [2]. The main silk protein i.e., fibroin (70-80%) and the coated protein Sericin (20-30%) contribute to making sophisticated biomaterial for medical, surgical and pharmaceutical applications due to their biocompatibility, biodegradability and higher levels of protein content made up of essential amino acids [3-6]. Being a wild silkworm species A. *mylitta* provides an ample opportunity for its exploitation as well as the conservation and utilization of natural forest genetic material for sustainable development. Owing to its unique features and nature, the silk fibre is known as the "Queen of Textiles" to the world for centuries. Lightweight, more absorbance, soft-touch, innate affinity [7] for dyes and the highest level of durability extended its applications in the fabrication of textiles, cosmetics and automobiles etc.

Daba ecorace of *Antheraea mylitta* is a high crop yielding Tasar silkworm [8]. Due to adverse climatic factors such as rainfall, drought, temperature and humidity etc., and biotic invaders such as pests and predators, there is a diminished Tasar silk production.

The present investigation carried on the effect of various concentrations of PAs (Spermidine, Spermine and Putrescine) on growth, development, resistance to adverse Abiotic and biotic stress and crop yield in Silkworm Antheraea *mylitta* DABA- BV ecorace at culturing field of Kakatiya University, Warangal, Telangana.

2. POLYAMINES

Polyamines are polycationic in nature and biological derivatives of amino acid arginine catabolism are involved in many vital metabolic processes [9]. *Polyamines (PAs)* such as Putrescine *(Put), Spermidine (Spd),* and *Spermine (Spm)* are products of Ornithine [10]. *Spermine* and *Spermidine* protect the DNA and RNA from denaturation [11]; *PAs* regulate transcription, translation and deficiency led to congenital malformations [12,13,14] and [15] *PAs* achieve stress resistance [16-18]. *Spermidine* increases autophagy during stress and ageing [17] *PAs* act as scavengers of oxygen free radicals (ROS) protect nucleic acids and cellular components from oxidative damage [19], decreases age-related oxidative stress, increase cell viability and *Spm* develop anti-oxidant potential in *B.mori* [20], increased metabolic activity in silk glands [21]. PAs enhance life span in mice, *C. elegans and* in *D. melanogaster* [22], prevented death from necrosis [17]. *Spd* enhance immunity [17,14,23-25], facilitate embryonic development in *D. melanogaster* [26], PAs contribute to repairing of tissues [12,27], participate in several physiological functions [28], reduces larval mortality [29].

3. OBJECTIVES OF THE STUDY

As silk production mainly depends on fifth instar larval and silk gland development, which in turn is determined by quality food intake, the present work aimed to explore the influence of various concentrations of PAs i.e., spermidine, Spermine and Putrescine as dietary supplements on the growth and development of larvae and silk glands during V instar. Rate of mortality, Effective Rearing Rate (ERR) and behaviour aspects were also studied under the same conditions in Tasar silkworm *A. mylitta*, DABA BV ecorace, which has not been done to date.

4. MATERIALS AND METHODS

4.1 Experimental Design: Larval Culture and Polyamine Treatment

Tasar silkworm *Antheraea mylitta (*Daba BV ecorace) was reared at average monthly temperature (25-28 C) and Relative Humidity (75-80%) at the well-maintained *T. arjuna* garden rosed at Kakatiya University, during July-August 2019. *Polyamines (PAs)* as (1mg/1ml), diamine *Putrescine*, triamine *Spermidine*, and tetraamine *Spermine*, purchased from Hi-Media Laboratories Pvt. Ltd., Hyderabad, India. *PAs* were used as an additional food supplement to test their effect on larval, silk gland growth and development.

Unusual twigs, branches and un-matured leaves were trimmed off from the 10 selected *T. arjuna* trees and covered with nylon net to prevent the entry of pests and predators. Each *PA* with 50 µM, 100 µM and 150 µM were prepared according to manufacturer instructions freshly just before application on leaves of food plants. 2-3 ml drug was uniformly applied on both surfaces of healthy matured leaves with a sprinkler contained a narrow nozzle followed by air-drying. Three hundred (300) healthy V Instar Day 4 larvae from culturing field were selected and

segregated into 10 groups each contain 30 worms for experimental investigation (9 for PAs fed and 1 for Control). Larvae were placed on separately labelled and treated plants after two hours of starvation to feed for 2 days till completion of maximum leaves. All the branches were masked with plastic bags to protect the treated leaves from unwanted washing out from precipitation and snowfall. Larvae were transferred to normal trees for routine feeding separately till day 10 (Fig. 1). On Day 11 of V Instar, once again the drug application was repeated as usual and larvae were allowed to ingest for the same period as previously done, followed by normal graze till Day 16 (up to complete development of silk gland).

4.2 Estimation of Larval Characteristics

All the larval and silk gland characteristics were estimated (as per FAO sericulture manual, 1976).

Larval Weight: All the groups (9 treated & one Control) of post-treatment larvae weights on V instar Day 16 was measured using a digital balance and calculated mean in PAs treated and Control groups.

Silk Gland Weight: Silk glands of fully matured larvae of Day 16 were extracted by scarifying eight silkworms of randomly selected in each group, washed in 1%NaCl solution, wet weight was measured on digital balance and mean with standard deviation was calculated in all PAs treated and Control groups.

Body Mass Index (BMI): Silk gland Body mass Index= wt. of the silk glands/Larval wt. X100.

Larval Mortality and ERR: During the experimental period (day 4 to day 16) dead and live larvae were counted periodically and calculated percentage in PAs treated and Control groups.

Behavioural Aspects: Feeding and Locomotory behaviour observed throughout the experimental period daily in the morning 7.00 am to 11.00 am (active feeding time) and recorded in PAs treated and Control groups.

Fig. 1. Net Rearing of tasar silkworm, *A. mylitta* on *T. arjuna* plantation (KU). (Arrow marks showing the larvae on *PAs* treated plant)

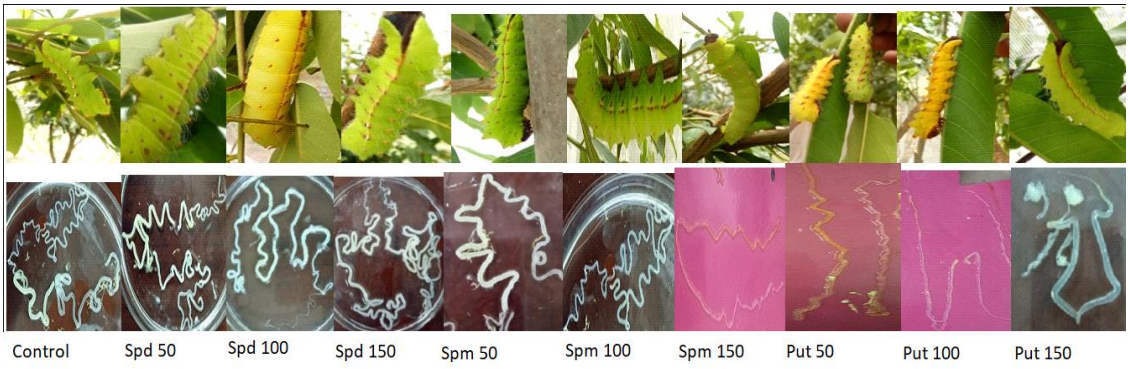


Fig. 2. Silkworms of 10 experimental groups collected (above) and Silk gland extracts (below)

4.3 Statistical Analysis

ANOVA single factor of excel used to analyze *F distribution (F ratio to F critical value)*, probability (P) for significance (at **α** = 0.05) in between larval weight and silk gland weight. A larger *F- value* that is bigger than the *F critical value* means something is significant, while the level of statistical significance is often expressed as a *p-value* between 0 and 1. The smaller the *p-value* indicates the stronger the evidence that rejects the null hypothesis (H_0). Rejected H_0 indicates the occurrence of variation between independent (larval weight) and dependent variable (silk gland). A *p-value* with i.e., $p \leq 0.05$ is statistically significant. The degree of association or relationship between two variables larvae and silk gland weight was measured by a *Pearson Correlation Coefficient (r)* from Coefficient determination (r^2). The correlation coefficient can be positive or negative falls between -1 and + 1. A positive correlation indicates that the two variables are varying in the same direction while a negative indicates an inverse direction. *Correlation coefficient value* larger than 0.70 (r) explain the perfect positive correlation between, yet.

5. RESULTS AND DISCUSSION

5.1 Larvae and Silk Gland: Regression and Correlation

Regression and coefficient evaluation of present study of *PAs* treated *A. mylitta* shown highly significant variation *(F ratio > Fcrit.) and p < 0.00001* in between larval weight to silk gland weight by all the treated and control groups. The perfect positive correlation is shown by *Spm 100 µM, Spd 100 µM, Spd 50 µM* and *Spm 50 µM* and *Spd 150 µM* treated larvae. But weak positivity in *Spm* 150 µM and perfect negative correlation (r = **< 0)** was observed in all *Put* treated groups (Table 1).

Increase in larval weight of *B.mori* correlated with other silkworm characteristics [30,31] *Spd* and *Spm* exhibited significant variation and positive correlation in larval and all silk yield characteristics in *B.mori,* CSR2 × CSR4 strain [32], *Spd 50 µM* increased larvae and post cocoon characteristics in *A. mylitta* [33]. In the present investigation, larvae nourished by polyamines of *Spm 50 µM and Spm 100 µM* showed a predominant rise in Silk gland development whereas, larvae fed with *Spd50 µM and Spd 100 µM* were more effective in increasing larval body weight. All *Put* treated larvae showed a negative correlation in silk gland development to larval growth, but *Spm 150 µM* showed a weak positive correlation whereas *Spd* 150 µM showed a perfect positive correlation which is in contrast to the previous reports in *B.mori* [34].

5.2 Mean Larval and Silk Gland Weight

Spd 50 µM increases larvae and silk gland weight in Daba TV ecorace of *A. mylitta* [33] PAs are essential to rapid cell division [12], increased larval weight [30,35], improved silk gland weight [36] in *B.mori*, increased silk secretion by silk glands of *A. mylitta* [37]. In this present study, mean larval weight by the end of day 16 of V instar experimental groups resulted (Graph 1) as *Spd 100 µM > Spm100 µM > Spd 50 µM > Spm 50 µM > Control*. Whereas, mean silk gland weight in the groups of *Spm 100 µM >Spd100 µM > Spd 50 µM > Spm 50 µM > Control* treated. Predominant increase in larval weight by *Spd 100 µM* and silk gland weight by *Spm 100 µM* fed silkworm. Whereas, *Spd 150 µM, Spm 150 µM* and all the *Put (50 µM,100 µM,150 µM*) fed larval and silk glands weights were lower even than Control.

5.3 Silk Gland Body Mass Index (BMI) (%)

Silk gland Body mass index calculations shown as *Spm 100 µM > Spd 100 µM > Spm 50 µM > Spd 50 µM > Control.* Contrarily, the rest of the groups were left over the lower BMI values even than Control (Graph 2).

In the present investigation, the V instar experimental groups of silkworm larvae fed on *Spd 50 µM, Spd 100 µM, Spm 50 µM* and *Spm 100 µM* treated leaves were shown development in larval and silk gland weight which is similar to the work carried out in *B.mori* [34] and with our previous work *A. mylitta* Daba TV ecorace [1]. Especially, *Spd 100 µM* treatment in larval weight and *Spm 100 µM* treatment in silk gland weight increased results revealing their predominant effect in silkworm growth and development which is novel and elucidated the first time. However, further extension is required to study the effect of *PAs* application in the remaining life cycle of Tasar silkworms such as cocoon, shell and silk fibre characteristics and metamorphosis of pupae.

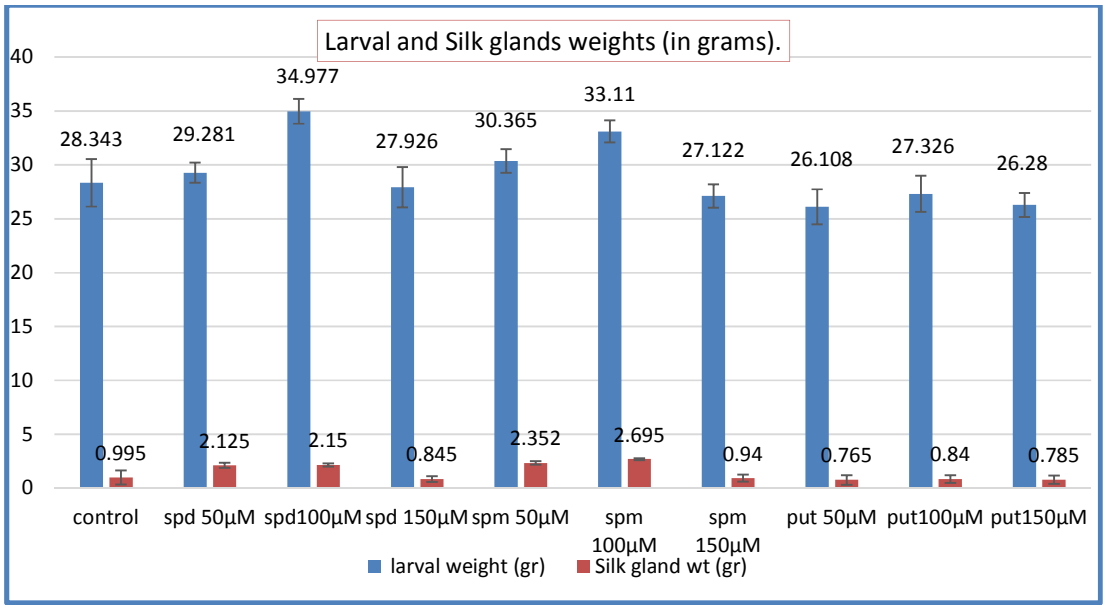
5.4 Effective Rearing Rate (ERR) and Mortality (%)

Effective Rearing Rate (ERR) exhibited as 100 %, 96.67% and 90% by the larvae fed by *Spd100 µM* and *Spm 100 µM* and *Spm 50 µM* batches, respectively. *Spd 50 µM* and *Spd 150 µM* also showed better results in ERR with 83.34% and 80%. Likewise, *Spm 150 µM, Put 50 µM, Put 100 µM& Put 150 µM* silkworms also showed a little bit of intensified results in ERR with 76.67%, 63.34%, 73.33% & 66.67%respectively, compared to diminished results in Control groups with 53.34% (Graph 3A & 3B). It is also observed that most of the worms died from the attack of pathogenic diseases.

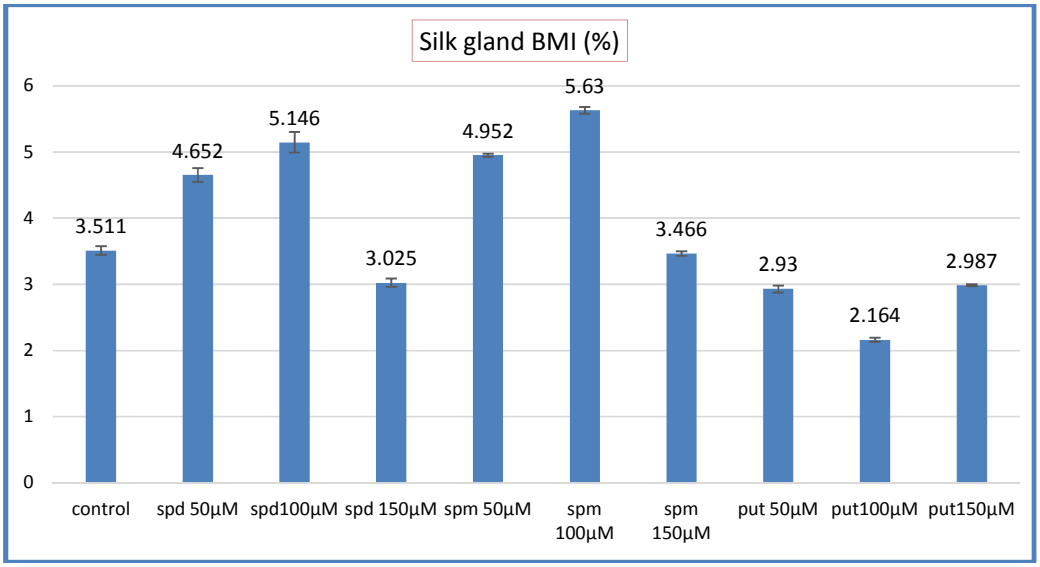
Table 1. Control and PAs treated Tasar silkworm *A. mylitta* larvae and silk glands of V instar as on day 16

Type and concentration of polyamine	coefficient determination (r^2)	Pearson correlation coefficient value (r)	correlation	p value (α = 0.05)	F value to F crit.	Null Hypothesis ($H_{0)}$	result of p & F values	Regression equation
control	0.61	0.78***	perfect +Ve	< 0.00001	3884.79 > 4.96	Rejected	significant	Y=0.0976x-1.772***
Spd 50 µM	0.91	0.97***	perfect +Ve	< 0.00001	1474.73 > 4.96	Rejected	significant	y= 1.1899x-32.25***
Spd 100 µM	0.98	0.99***	perfect +Ve	< 0.00001	4212.87 > 4.96	Rejected	significant	y= 0.3134x-7.79***
Spd 150 µM	0.83	0.91***	perfect +Ve	< 0.00001	8167.86 > 4.96	Rejected	significant	y= 0.0485x+2.08***
Spm 50 µM	0.58	0.76***	perfect +Ve	< 0.00001	1327.07 > 4.96	Rejected	significant	y= 0.3974x-10.71***
Spm 100 µM	0.95	0.97***	perfect +Ve	< 0.00001	4876.52 > 4.96	Rejected	significant	y= 0.5121x-14.14***
Spm 150 µM	0.1	0.32**	weak +Ve	< 0.00001	6057.12 > 4.96	Rejected	significant	y= 2.7111x+12.40**
Put 50 µM	0.16	-0.39*	perfect -Ve	< 0.00001	9962.20 > 4.96	Rejected	significant	y= -0.052x+2.1236*
Put 100 µM	0.013	-0.11*	perfect -Ve	< 0.00001	1201.34 > 4.96	Rejected	significant	y= -0.0102x+1.114*
Put 150 µM	0.04	-0.21*	perfect -Ve	< 0.00001	14.35.12> 4.96	Rejected	significant	y= -0.0255x+1.454*

**** Prefect Positive Correlation ** Weak Positive Correlation *Perfect Negative Correlation*

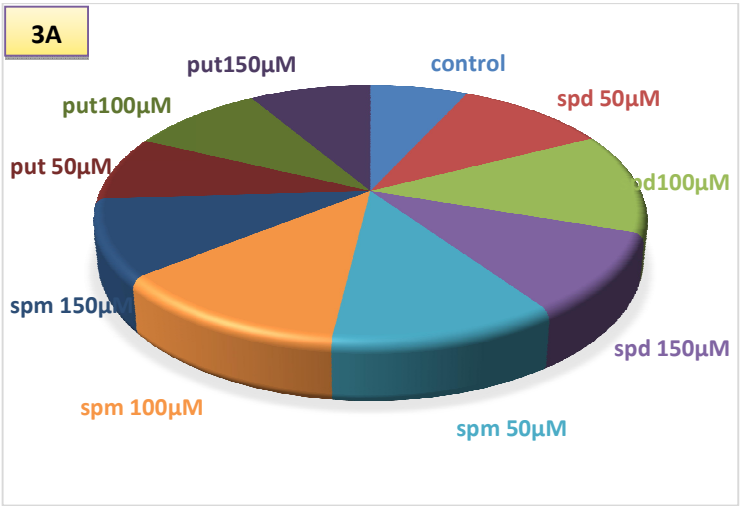


Graph 1. Larval and silk gland weights of V instar, day 16 larvae of Tasar silkworm, *A. mylitta*

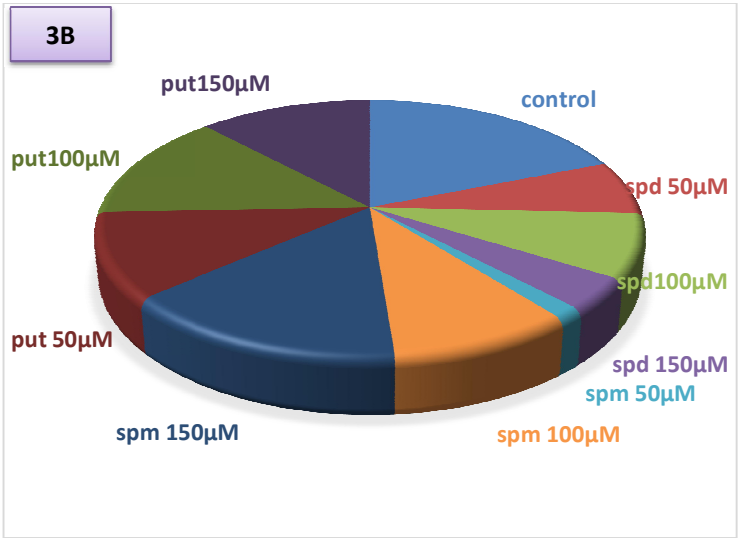


Graph 2. Silk gland body mass index (BMI) of V instar, day 16 larvae of Tasar silkworm, *A. mylitta*

Almost all the PAs showed resistance to pathogenic conditions up to some extent. Additional nutrients in food decrease mortality [28], *PAs* enhance immunity and prevent ageing-associated diseases [12], and function against viral infection [13]. In this present investigation, specifically, *PAs* by *Spd 50 µM*, *Spd 100 µM*, *Spm 50 µM* and *Spm 100 µM* additives in feed might be involved in larval tissue development and also exhibited immunological resistance to pathogenic invasion. However, it is observed that *Spd, Spm* with *150 µM* and *Put* of all concentrations might be adversely affected silkworm growth and development. It was revealed that a high concentration of *PAs* reduced larval weight in *A. mylitta* [33], *weight* loss and lethal [24], increased larval mortality [24]. Hence, it can be deduced that an application of optimal concentration of *PAs* are found to be effective for Tasar silkworm growth and development. However, the study needs further probing in *PAs* treatment at other stages of development and explore the molecular mechanism of disease resistance in Tasar silkworm.



Graph 3A. Effective Rearing Rate of Larvae during the experimental period



Graph 3B. Larval mortality during experimental period

5.5 Behavioral Aspects

The locomotion and feeding behaviour of the *PAs* treated Tasar silkworms was examined. It was observed that they were found to be active in all *Spd* and *Spm* treated larval batches throughout the experimental period, except *Spd 150 µM* batch. In *Put* treated larvae, the majority have exhibited sluggish movement and are passive in all behavioural aspects like feeding and foraging. *PAs* with *Spd* and *Spm* by *50 µM* and *100 µM* might be used for growth and development in larval tissues, whereas, increased concentration of *Spd 150 µM* and *Put (50 µM, 100 µM,150 µM)* might be detrimental to the larval growth and development.

6. CONCLUSION

*Polyamines, Spd 50 µM, Spd 100 µM, Spm 50 µM*and *Spm100 µM* treated larvae shown enhanced growth in larval weight and silk gland development. A significant enhancement was also observed in by *Spd 50 µM* and *100 µM* on larval weight and *Spm 50 µM* and *100 µM* on silk gland weight. Almost all the applied *polyamines* have shown considerable impact on resistance to diseases.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research

and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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