

High levels of endogenous polyamines indicate superiority of *Morus alba* L. varieties for improved silk production in *Bombyx mori* L.

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ABSTRACT: Mulberry (*Morus alba* L.) is an essential plant for sericulture. The composition of mulberry leaf determines the growth of *Bombyx mori* L. Polyamines (PA) were shown to be vital for growth, development, and stress tolerance in various plants. However, their presence and role in mulberry have not been studied yet. Experiments to examine the polyamines (PA) in four distinct cultivars of *M. alba* leaves by quantitative and qualitative analysis, revealed that PAs were found to be present in all four mulberry cultivars, and the cultivar V1 showed significantly higher PA levels when compared to the others. Thin-layer chromatography revealed several PA bands with different strengths among leaf varieties. The common polyamines found in all four cultivars were putrescine, spermidine, and spermine. The antioxidant potential of the V1 mulberry cultivar was significantly higher when compared to the others. V1 fed larvae of *B. mori* showed significant enhancement in the larval weight, cocoon weight, and length of the filament reeled. This study illustrates that higher endogenous PA levels of the V1 mulberry cultivar led to higher production of silk. © 2025 Association for Advancement of Entomology

KEYWORDS: Mulberry, silkworm, filament length, spermidine, antioxidants, silk production

INTRODUCTION

Morus alba L. is a commercially important plant utilized in sericulture, and pharmaceutical industries (Wang *et al.*, 2012; Budiman *et al.*, 2017). Its leaves serve as the primary food source for silkworms, *B. mori*, making mulberry cultivation essential for silk production. The nutritional quality and availability of its leaves play a crucial role in the growth of silkworms and the overall efficiency

of silk production in sericulture (Lalfelpui *et al.*, 2014). Mulberry cultivation faces significant challenges due to different abiotic and biotic stresses. Drought, salinity, temperature fluctuations, and pest infestations are major factors that effects its cultivation. Understanding the mechanisms underlying stress resistance in mulberry is essential for developing resilient varieties that can thrive under adverse conditions. Biochemical parameters and PAs have emerged as key players in plant stress

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responses, offering valuable insights into the physiological adaptations and defence mechanisms against different stress (Shao *et al.*, 2022; Tyagi *et al.*, 2023). Biochemical parameters such as antioxidants, enzymes, and osmolytes play roles in reducing the effects of stress by eliminating reactive oxygen species (ROS), and maintaining osmotic balance (Mehla *et al.*, 2017). PAs have been implicated in various stress responses, including regulation of gene expression, membrane stability, and ROS scavenging (Liu *et al.*, 2015).

PAs, putrescine (Put), spermidine (Spd), and spermine (Spm) are essential biogenic amines found abundantly in plants (Lenis *et al.*, 2017). They regulate diverse cellular functions, including DNA stabilization, protein synthesis, and stress tolerance, making them vital for, development, adaptation, and growth (Liu *et al.*, 2007). Put and Spd are the two vital PAs involved in cellular stress resistance. Put serves as a precursor for Spd and Spm synthesis. Spd, a three amino group PA, is particularly crucial for stress response, aiding in cellular mechanisms (Shao *et al.*, 2022). Cadaverine (Cad) has been correlated with root growth promotion and osmotic stress reduction in rice (Cassan *et al.*, 2009). Cad plays important physiological roles in plant growth, differentiation, and stress tolerance induced by biotic and abiotic factors (Jancewicz *et al.*, 2016; Ozmen *et al.*, 2023). Exogenous supplementation of Spd showed enhancement in growth and commercial properties of silk produced by *B. mori* (Kasa *et al.*, 2023; Yerra *et al.*, 2017). This study aimed to examine the levels of PAs and antioxidants in mulberry leaves from four different varieties to evaluate their impact on the growth of *B. mori* larvae.

MATERIALS AND METHODS

Mulberry leaf samples of Victory1 (V1), S13, Kosen (K), and Berhampur (B) cultivars were collected from a sericulture field located in Anandapuram, Visakhapatnam, Andhra Pradesh, India (Fig. S1). The varieties were selected based on the reported data with regard to their growth and stress tolerance characteristics (Table 1). Identification of leaves was done in the presence of a sericulture field officer, Government of Andhra Pradesh. Collected

samples were used for qualitative and quantitative analysis of PAs, antioxidant, and feeding experiments. Fresh leaves (100 mg) of different varieties were homogenised with 99 per cent ethanol, centrifuged, and the supernatant was used for estimation of antioxidants. Fresh leaves (100 mg) were weighed, and homogenised with 5 per cent Perchloric acid (PCA), and the supernatant was used for the estimation of PAs.

Quantification of PAs in different mulberry leaves: Derivatization of the PAs with dansyl chloride was carried out as described previously, with some modification (Rajan *et al.*, 2022b). Proline was added to stop the reaction and to remove unbound dansyl chloride. Dansylated PAs were extracted and used for quantitative and qualitative analysis. The benzene extracted dansylated PAs were diluted with DMSO, and fluorescent intensity was measured with the excitation at 350nm and emission at 495nm wavelengths using BioTek synergy H1 microplate reader. A standard curve was plotted using the Spd of different concentrations. The free PAs of samples were expressed in $\mu\text{g mg}^{-1}$. The experiment was performed in biological triplicates.

Qualitative PA analysis by thin layer chromatography (TLC): Dansylated PAs from the different leaf cultivars and standard containing known dansylated PAs; mixture of ornithine (Orn; RM057-25G), Put (RM5438-5G), Spd (RM443-5G), and Spm (RM7506-5G) were separated by loading on activated silica TLC plates. The loaded silica plate was placed in the glass chromatographic tank containing saturated ethyl acetate and n-hexane solvent (Lima *et al.*, 2023). Once the solvent front reached the desired distance, the TLC plates were removed from the tank and air-dried. The dried TLC plates were visualised under UV light. The intensity of the selected bands among different leaf cultivars was measured using Bio-Rad Image Lab 6.0 software.

Estimation of antioxidants: Antioxidants were tested by the TLC dot blot method following the protocol of (Huang *et al.*, 2005). Different concentrations of ascorbic acid and quercetin were used as standards. Ethanolic extracts of all the leaf

varieties were blotted on the activated TLC plate. Subsequently, 2% of 10 ml DPPH was blotted onto the sample. The change in colour from purple pink to yellow was observed which indicates the inhibition of DPPH radical by antioxidants present in the leaf sample.

Feeding different mulberry leaves to *B. mori*: *B. mori* larvae (CSR2 X CSR4) were procured from a sericulture farm located in Narsipatnam town, Anakapalle district, Andhra Pradesh, India. The larvae were divided into four groups of 10 larvae each ($n = 10$). Fresh, healthy, and mature leaves of four different varieties (V1, K, S13, and B) were fed to the *B. mori* larvae from day 1 to day 7 of the 5th instar stage. Feed was given to the worm three times a day. The leaves were evenly spread over the larvae in thin layers to ensure that all larvae could feed equally. Weights of the larvae (day 7) and cocoons were recorded from each group to assess the impact of the leaf variety on growth.

Economic parameter of the silk fibre: Cocoons from various groups were collected and submerged in hot water (65–75°C). A reeling device, Epprouvette, was used to extract the mono-cocoon fibre. The total fibre length was measured in meters, while its weight was recorded in grams (g). The filament denier was then calculated using the formula.

$$\text{Denier} = \frac{\text{weight of the filament}}{\text{length of the filament}} \times 9000$$

Statistical analysis was conducted using one-way ANOVA, and Student's t-test was performed with GraphPad software 10.2.3. among the different varieties and between two varieties of the mulberry leaves. The values superscripted with * are significant ($p < 0.05$) and with ** are highly significant ($p < 0.01$). The commercially important characters, namely length of silk filament, denier, cocoon weight, and larval weight, were correlated with PA content of different mulberry cultivars using a Pearson correlation matrix with pandas.DataFrame.corr function.

RESULTS

PAs show differences in abundance among the different mulberry varieties: Quantification of

PAs in different mulberry leaves: The quantity of PAs in cells is crucial for stress response because elevated levels have been shown to impact various cellular functions. Total free PA levels were checked for 100mg of leaf sample from each cultivar. Statistically elevated PA levels ($p < 0.01$) were observed in V1 ($1.08 \pm 0.13 \mu\text{g}$) followed by B ($0.52 \pm 0.048 \mu\text{g}$), S13 ($0.35 \pm 0.087 \mu\text{g}$), and K ($0.30 \pm 0.059 \mu\text{g}$), respectively. The pairwise comparison demonstrated that the S13, B, and K varieties showed significantly lower PA levels ($p < 0.05$) when compared to V1 (Fig. 1a, Table 2).

PAs show qualitative differences among the different mulberry varieties: Different PAs have specific affinities or activities that make them more suited for various cellular processes. Dansylated PAs were separated on TLC to check the differences in the number and their abundance among the selected varieties (Fig. 1b). The R_f values of the separated bands from samples and standards were calculated. R_f values of Band 1 (0.31), Band 2 (0.38), Band 3 (0.53) and Band 4 (0.61)

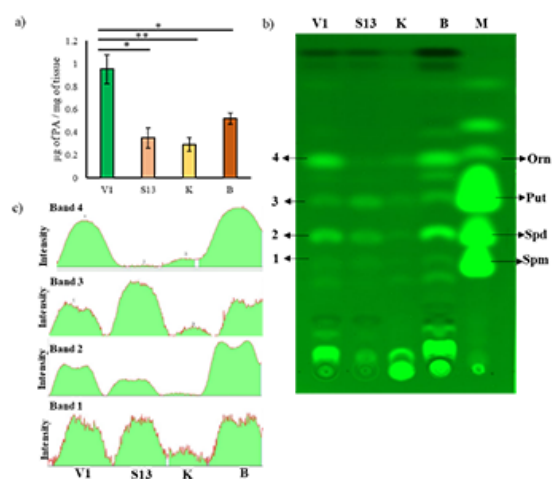


Fig. 1 Dansylated PAs profile in various mulberry cultivars. (a) Free PAs in 1mg of leaf samples. * Indicates significance difference ($p < 0.05$) and ** indicates highly significant difference ($p < 0.01$). (b) Separation of dansylated PAs from leaf samples along with dansylated standard PA mix on silica TLC. 'M' represents mixture of commercially available amines (Spm, Spd, Put, Ornithine), and numbers on the right side represent four selected bands. (c) Quantification of the selected bands was carried out by densitometry analysis using Bio-Rad Image Lab software

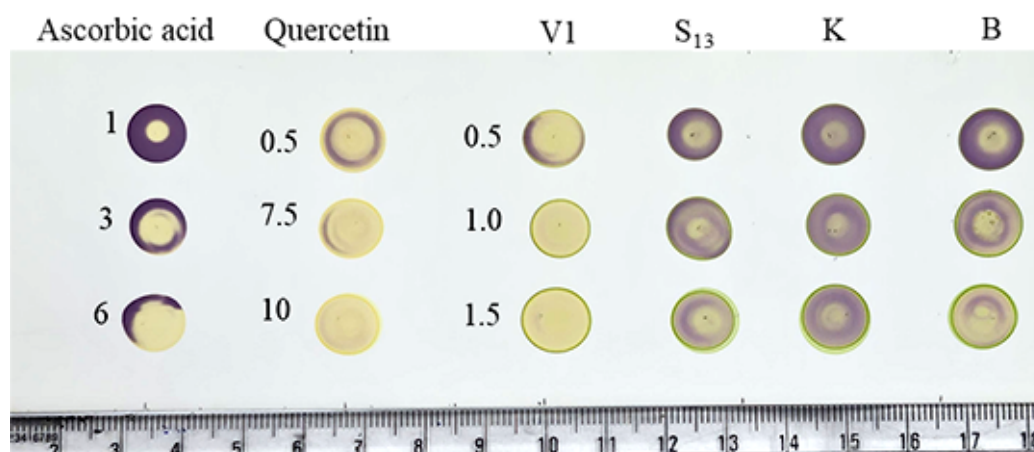


Fig. 2 Free radical scavenging activity of different mulberry cultivars by dot blot on TLC plate (n=3). The numbers given against standards (ascorbic acid and quercetin) represent concentration (μg). The numbers given against the mulberry cultivars represent the leaf weight (mg)

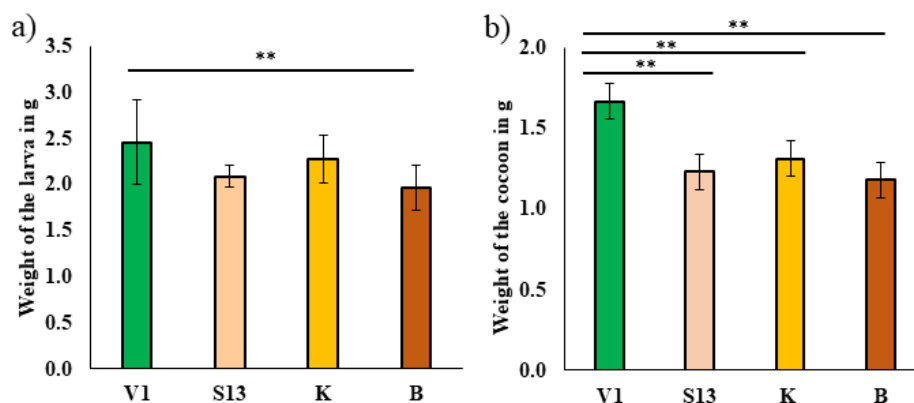


Fig. 3 Growth parameters of *B. mori* larvae fed with different varieties of mulberry leaves (a) Larval weight on day 7 (n=8) (b) Cocoon weight on day 15 (n=8)

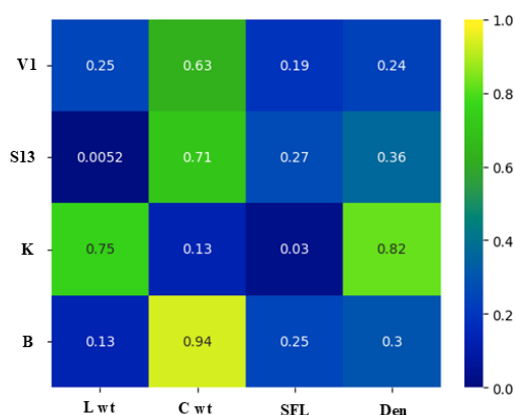


Fig. 4 Heat map representing correlation analysis of larval weight (L wt), cocoon weight (C wt), silk filament length (SFL), and denier (Den) against PA levels of different varieties of mulberry leaves. The colour intensity varies from blue to yellow, where blue signifies weak correlation and yellow signifies strong correlation

correspond to Spm (0.31), Spd (0.38), Put (0.53), and Orn (0.61) respectively. PA bands similar to Orn, Put, Spd, and Spm standards were detected with different intensities in all the varieties. Densitometric analysis showed high intensity of Orn in B, followed by V1, K, and S13. Put intensity was more in S13, followed by B, V1, and K. A highly intense Spd band was recorded in B, followed by V1, S13, and K. Spm recorded no significant difference among V1, S13, B, and K, however, it showed the least intensity in K variety (Fig. 1c).

Retention factor (Rf) values of dansylated marker bands and selected dansylated PA bands from mulberry leaves Spm (0.31), Spd (0.38), Put (0.53), Orn (0.61), Band 1 (0.31), Band 2 (0.38), Band 3(0.53) and Band 4 (0.61).

Table 1. High-yielding mulberry varieties used in the study

Variety	Region cultivated	Developed by	Characteristics
Victory-1 (V1)	South India, irrigated	India	Superior variety with high protein content
S-13	South India, rainfed	India	Drought resistant
Berhampore (B)	Irrigated regions of Eastern and Northeastern India	Philippines	Resistant to powdery mildew
Kosen (K)	Hills of Eastern and Northeastern India	Japan	Moderately tolerant to leaf spot disease

Table 2. Comparative analysis of polyamine levels among the different varieties of mulberry leaves (ANOVA) and pairwise comparison (t - test)

Variety	Mean	SD	F-test	t-test (p value)	
V1	1.08	0.13	52.69 p-value 0.000**	-	
S13	0.35	0.087		V1 vs S13	0.013*
K	0.30	0.059		V1 vs k	0.008*
B	0.52	0.048		V1 vs B	0.014*

** indicates ($p < 0.01$), and * indicates ($p < 0.05$) significant difference

Mulberry cultivars with high PA levels show elevated antioxidant potential: Antioxidants play a crucial role in scavenging and eliminating free radicals and are directly related with enhancing stress resistance in mulberry. The level of oxidative stress was checked with the help of DPPH dot blot. The results of the DPPH scavenging activity of mulberry leaves among four varieties showed notable differences in the antioxidant levels. Maximum scavenging activity was observed in V1, followed by S13, B, and K, respectively (Fig. 2). Increased PAs, Spd, and Put were known to play a role in the decrease of oxidative stress in mulberry leaves.

Mulberry cultivar with high PA levels shows enhanced growth parameters of silkworm: PA levels were shown to be directly related to the growth of the organisms by several studies. To assess the effect of PA levels in mulberry leaves on the larval weight of the *B. mori*, day 1 of 5th instar larvae were divided into four groups and fed with different varieties of leaves. Larval weights

were recorded on day 7, at the end of the feeding stage. A comparative analysis of larval weights between groups showed high significance with the highest larval weight in V1 (2.433 ± 0.31 g), followed by K (2.272 ± 0.26 g), S13 (2.092 ± 0.13 g), and B (1.972 ± 0.25 g), respectively. The pairwise comparison demonstrated that the S13 and B varieties had significantly ($p < 0.05$) decreased larval weight compared to the V1 varieties, and the K variety did not show a significant difference in larval weight when compared to the V1 (Fig. 3a, Table 3).

The larvae from different groups were allowed to form cocoons, and their weights were recorded on day 15. A comparative analysis of cocoon weights between the group showed highly significant difference ($p < 0.01$) with the highest cocoon weight in V1 (1.66 ± 0.21 g) followed by K (1.31 ± 0.36 g), S13 (1.23 ± 0.25 g) and B (1.18 ± 0.18 g) respectively. The pairwise comparison demonstrated that the S13, K, and B varieties had significantly ($p < 0.01$) decreased cocoon weight compared to the V1 variety (Fig. 3b, Table 4).

Table 3. Comparative analysis of larval weights on day 7 of the 5th instar among the different varieties (ANOVA) and pairwise comparison (t-test)

Variety	Mean	SD	F-test	t-test (p value)	
V1	2.461	0.46	2.58 p-value 0.089	-	
S13	2.092	0.13		V1 vs S13	0.051
K	2.272	0.26		V1 vs k	0.134
B	1.972	0.25		V1 vs B	0.006**

** indicates ($p < 0.01$) significant difference

Table 4. Comparative analysis of cocoon weights (g) among the different varieties (ANOVA) and pairwise comparison (t- test)

Variety	Mean	SD	F-test	t-test (p value)	
V1	1.66	0.21	5.65 p-value 0.003**		
S13	1.23	0.25		V1 vs S13	0.001**
K	1.31	0.36		V1 vs k	0.006**
B	1.18	0.18		V1 vs B	0.001**

** indicates highly significant difference ($p < 0.01$)

Mulberry cultivars with high PA levels show enhanced economic parameters of silk fibre:

Economic parameters of silk fibre were analysed by the length of the filament and denier. The length of the silk filament refers to the total continuous fibre length that can be reeled from a single cocoon. Intergroup comparison showed that the length of silk filament reeled was significantly higher in the V1 fed group ($p < 0.01$). The filament denier varies over the reelable filament's length. The quality of the yarn largely depends on denier. Silk is finer when the denier is less. The silk filament produced by the V1 and K variety fed silkworms exhibited lower denier values (Table 5).

Correlation analysis between PAs of mulberry cultivars and economic parameters

Correlation heat map analysis was carried out

Table 5. Comparative analysis of silk filament length (m) and denier among the different varieties (ANOVA) and pairwise comparison (t-test)

Variety	Mean	SD	F-test	t-test (p value)	
V1	814.45	58.18	30.45 p-value 0.000**		
S13	584.50	30.31		V1 vs S13	0.0001**
K	687.45	27.58		V1 vs k	0.0025**
B	657.13	46.87		V1 vs B	0.0012**

Denier

Variety	Mean	SD	F-test	t-test (p value)	
V1	3.158	0.222	8.109 p-value 0.000**		
S13	3.202	0.167		V1 vs S13	0.354
K	3.117	0.381		V1 vs K	0.419
B	3.845	0.359		V1 vs B	0.0015**

**indicates highly significant difference ($p < 0.01$)

between PAs and economic parameters (larval weight, cocoon weight, silk filament length, and denier). A positive correlation was observed between PA content and economic parameters. PAs showed a strong correlation with larval weight in K (0.75), cocoon weight in B (0.94), silk filament length in S13 (0.27), and lowest denier in V1 (0.24) mulberry varieties. PAs in V1 variety made a comprehensive contribution to every factor, making it the superior variety (Fig. 4)

DISCUSSION

PAs, known for their role in cellular homeostasis and stress adaptation, are essential for enhancing plant resilience to both abiotic and biotic stressors. Increased PA levels indicate a specialized adaptive mechanisms that help to mitigate stress-induced damage and optimize growth under adverse conditions (Sagar *et al.*, 2021). Previous studies have confirmed the presence of PAs in mulberry leaves (Hamana *et al.*, 1984). However, this study is the first to provide both quantitative and qualitative analyses of PAs in mulberry leaves.

Mulberry plants, the primary food source for *B. mori* in sericulture, hold significant economic importance. Several studies have demonstrated a direct relationship between PA content and cellular growth (Sagar *et al.*, 2021). Additionally, previous research has shown that Spd supplementation enhances silk production in mulberry silkworms (Lattala *et al.*, 2014). In this study, the V1 mulberry cultivar exhibited the highest PA levels. Earlier findings suggest that mulberry varieties with greater antioxidant potential, along with higher levels of Put, Spd, and Spm, demonstrate greater stress resistance (Chen *et al.*, 2023).

Qualitative analysis of PAs and antioxidants highlights the strong capacity of the V1 cultivar to regulate stress responses and withstand environmental challenges. Previous research has also emphasized the role of PAs in the development of *B. mori* larvae (Rajan *et al.*, 2022a). Our findings reveal that larvae fed on V1 leaves, which have high PA content, exhibited greater larval weight. Economic parameter analysis further showed that the V1-fed group produced longer

filament length with a lower denier. Among the cultivars studied, V1 exhibited the highest antioxidant activity, followed by B, S13, and K. A positive correlation between PAs and economic parameters of silk was observed in the present study. Attempts should be made to enhance the mulberry leaf components for providing a nutritionally enriched diet to silkworms. Such a diet can help the silkworms to grow better and also provide stress tolerance to them, which eventually helps in the enhancement of silk production. Higher levels of PAs and antioxidants in the V1 mulberry variety could have helped in enhanced larval and cocoon weights of the silkworm *B. mori*. The study is the first of its kind correlating PA content with the growth of the silkworm, *B. mori*.

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