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(RESEARCH ARTICLE)

The effect of mulberry varieties on the protein patterns of the silkworm *Bombyx mori* L.

Rajkumar S. Bhonde *

Department of Zoology, C. J. Patel college, Tirora, Dist. Gondia, Maharashtra, India.

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Abstract

Four mulberry varieties— Kanva 2, Almora Local, China Peking China, and S-1096—were selected to study their effects on the protein banding patterns of fifth-instar larvae of *Bombyx mori*. The present results showed the level of haemolymph protein in different silkworm races PM x CSR2, CSR4 x CSR2, and CSR2 x CSR4 showed not much difference compared with each other, when fed on Kanva-2, Almora local MP and China Peking China mulberry varieties, only in S-1096 variety fed larvae was noticed less protein level as compared to other variety, it may due to poor leaf quality of S-1096. These findings suggest that although the general protein expression profile remains consistent across mulberry varieties, specific proteins are differentially expressed depending on the genotype of the host plant.

Keywords: Almora local; Kanva 2; China Peking China; S-1096; SDS-PAGE

1. Introduction

Mulberry is a perennial plant of considerable economic importance and constitutes the chief food for mulberry silkworm. Mulberry silkworm *Bombyx mori* draw its nutrition from its food plant *Morus Alba.* The silk threads of the cocoons of the silkworm *Bombyx mori* are composed of two major proteins (fibroin and sericin), produced by secretions of the silk glands. The silk gland is divided into three regions: anterior, central and posterior. The posterior silk gland secretes fibroin, while sericin, a glycoprotein which coats fibroin, is secreted by the central silk gland. Feeding trials conducted by several workers prove that the leaves levels of nutrient in different verities of mulberry have significant influence on the growth and development on silkworm and cocoon production (Kaffin 1960, Bari et.al., 1985, Macchi and Kataglori, 1991). Earlier the workers have reported the higher leaf potential of different varieties (Dandin, et.al, 2003, Kashiviswankathan, 1985). It is therefore possible that an increase in the protein level of mulberry leaves may lead to improvements in cocoon productivity. The nutritional richness of the diet influences the accumulation of storage proteins in the haemolymph of the silkworm larvae. Proteins are the building blocks of organisms therefore; proteins are important in all biological systems and play a wide variety of structural and functional roles. Proteins are also compounds of fundamental importance for all functions in the cell. The protein budget of the cell can be considered as an important analyte in evaluating the physiological standards of the cell. The high protein concentration is an indication of a greater metabolic activity of the tissue. So, improvement of the food value is probably determined by the protein quantity and quality (Li et al., 2012; Rajitha and Savithri, 2013; Madhu Babu et al., 2014). Seo *et al*. (1985) studied the protein patterns of the haemolymph and various tissues of final-instar larvae and pupae by electrophoresis: 15 protein bands were separated from the haemolymph during metamorphosis and were maintained at a high concentration during the final larval instar, but declined after pupation. Growth of silkworm larvae was improved significantly on feeding mulberry leaves supplemented with different nutrients including vitamins, sugar and other substances. Most methods used to evaluate the leaf quality or variations between different varieties are biochemical methods, phytochemical analysis or nutritive contents of different mulberry genotypes (Jyothi et al., 2014; Manjula and Kumari, 2015). On the other hand, the performance of the Bombyx mori is based on economic, biological characters and productivity. These assessments have been conducted to assess the effect of different mulberry cultivars on silkworm

* Corresponding author: Rajkumar S. Bhonde

rearing (Babu et al., 2014; Prieto-Abreu et al., 2016). However other studies on the biochemical components and bioassay of different varieties of mulberry leaves fed by silkworm in relation to silk production were done by Zannoon et al. (2008). Recent studies concerned with the protein quantification by SDS-PAGE gel electrophoresis were carried out to demonstrate the variability in protein profiles between different mulberry cultivars only (Madhu Babu et al., 2014; Jyothi et al., 2016), on the other hand, the comparative proteomic maps approach was applied to assess many factors that affect the silkworm haemolymph and posterior silk gland protein patterns (Li et al., 2012; Zhang et al., 2014; Dong et al., 2016). Meanwhile the effect of cultivated mulberry varieties on haemolymph protein patterns was investigated by El-Akkad et al. (2008).

Here we compare the effects of four varieties of mulberry leaves, Kanva 2, Almora Local, China Peking China, and S-1096 on the protein patterns in larval tissue of the 5th-instar larvae of silkworm races PM x CSR2, CSR4 x CSR2, and CSR2 x CSR4.

2. Materials and methods

The experimental plantation was established at the Centre for Sericulture and Biological Pest Management Research (CSBR), Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. Four mulberry varieties- Kanva 2, Almora Local, China Peking China, and S-1096 were planted in small plots, each consisting of 8–10 plants. A uniform spacing of 2 × 2 feet was maintained between plants to ensure proper growth and management.

2.1. Biochemical Estimation of Leaf Protein in Different Mulberry Varieties-

For biochemical analysis, two branches of equal height were selected from each mulberry variety (Kanva 2, Almora Local, China Peking China, and S-1096). From each branch, leaves positioned at nodes 1, 5, 10, and 15 were collected for protein estimation. Two leaf discs (1 × 1 cm) were excised from each selected leaf and homogenized in 5 ml of 0.6 N saline solution. The homogenate was centrifuged at 3000 rpm, and the resulting supernatant was used for protein estimation. A 10 µL aliquot of each sample was processed for total protein determination using the Bradford method (Bradford, 1976). Protein concentration was quantified spectrophotometrically using an Elico-171 spectrophotometer, and values were calculated for each sample across the different mulberry varieties.

2.2. Rearing of mulberry Silkworm *Bombyx mori* L-

The disease-free eggs laying (Dfls) of different hybrid races of mulberry silkworm, *Bombyx mori* (PM x CSR2, CSR4 x CSR2, CSR2 x CSR4) were obtained from National Silkworm Seed Organization, (NSSO), Central Silk Board, Bangalore. These different races were reared at the Centre for sericulture and Biological Pest Management Research (CSBR), RTM Nagpur University, Nagpur. Larvae were reared in plastic containers placed in a chamber where the temperature was at 25±1°c and the relative humidity was 75±5%, fed on leaves of different mulberry varieties from hatching to the fifth instar. The rearing schedule was followed as described by Dandin et al (2003). The larvae were brushed and rear up to third stage on Kanva-2/M5 mulberry variety. About 200 early IIIrd instars larvae of all races were selected, and distributed in four treys containing 50 larvae in each trey. The treys were coded as I, II, III and IV for every race of silkworm. The larvae of trey I of each race were fed on Kanva-2/M5 and treated as control. The larvae of all treys II, III and IV were given equal quantity of weighed mulberry leaves from the varieties such as Almora local MP, China Peking China S-1096, and respectively till spinning stage. The schedule of rearing was followed as suggested by Krishnaswami (1986).

2.3. Protein estimation in different races of silkworm *Bombyx mori* L

To estimate protein level in haemolymph, silk gland, and fat body, samples were collected from Vth instars larvae of each race of *B. mori* fed on different varieties of mulberry. Haemolymph, silk gland, and fat body of about two Vth instars larvae of each race fed on different varieties of mulberry were collected separately on alternate days in an eppendroff coated with Phenylthiourea. The silk gland, fat body was weighed and crushed in sample buffer (Arif etal-2001) & stored at -20°C. About 20µl of crushed sample were processes for estimation of total protein present in tissues. The estimation of protein was done using Bradford (1976) method. The amount of protein present in the sample was recorded on spectrophotometer (Elico-171).

2.4. Electrophoretic pattern of haemolymph, tissues of different races of silkworm *B. mori* and leaf protein of different varieties of mulberry

Characterization of protein of haemolymph using SDS phage (Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis) was done by using method described by Lamellae (1970).

The gel electrophoresis apparatus was cleaned and dried in an oven. The sides of glass plate of the apparatus were sealed using 1% agarose. A plastic comb was placed in between the glass plate and all the wells formed were marked using marker and the comb was removed. The prepared running gel was poured slowly between the glass plates, 1cm below the markings of wells. The distilled water was poured over the gel with the help of syringe and the gel was kept for an hour to polymerize. After polymerization of the gel the distilled water was removed and the gel surface was dried using filter paper. The prepared stacking gel was poured gently up to the top. The plastic comb was inserted in between the glass plates to make wells. After polymerization of the stacking gel the plastic comb was removed and the wells, which were formed, were washed with water and dried using filter paper. In the dried wells 1x Tank buffer was poured in both lower and upper tank. Samples containing at least 50-100µg equivalent protein were taken and mixed in equal volume of 5x Laemmeli buffer (sample buffer) (Laemmlae, 1970). The tubes containing samples were warmed in boiling water in a water bath for about 2-3 minutes in order to denature the protein present in the sample. The warmed samples were centrifuged in centrifuge machine and loaded in the wells. Lane 1 of the well was used for molecular marker while the samples were loaded in the wells from lane 2 onwards.

After loading the samples 50mA current was supplied and when the tracking dye crosses the stacking gel then the current was reduced to 25mA. The power supply was stopped when the tracking dye reached about 1cm above the bottom of the gel. The gel was removed and placed in a staining solution (Coomassie Brilliant Blue) on a gel rocker for about an hour and then kept at room temperature for overnight. Then the gel was transferred to destainer-I for an hour giving three changes after every 30 minutes. The gel again transferred to destainer-II and kept overnight. After distaining the background stain, the gel was preserved in 7% acetic acid solution

3. Results

3.1. Electrophoretic patterns of mulberry leaves proteins-

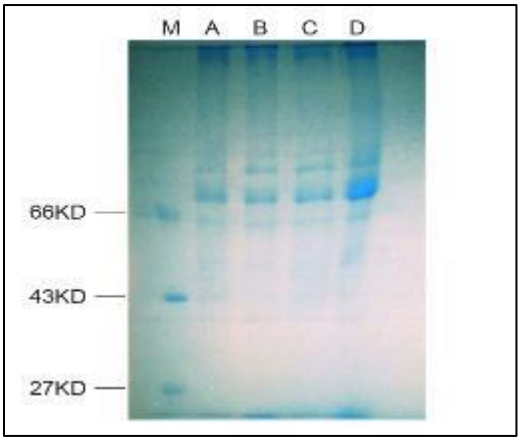
The banding pattern of leaf protein of different mulberry varieties Kanva 2, Almora local MP, China Peking China and S-1096, showed no significant change in the pattern when compared amongst each other. All the varieties having three bands appeared with molecular weight 66 kD in China Peking China and S-1096, 65 kD in Almora local MP where as in Kanva-2/M5 it was less 61 kD as compared to other three varieties and lower band appear between 45 kD-54 kD in all the varieties, it does not show significant difference in the banding pattern. (Fig.1 Table.1)

3.2. Electrophoretic patterns of Haemolymph Protein

The protein banding pattern of haemolymph of 5th day old larvae of three different silkworm races, PMxCSR2, CSR4 x CSR2 and CSR2 x CSR4 fed on Kanva-2/M5 mulberry variety showed 82 kD in PM x CSR2, where as in CSR4 x CSR2 and CSR2 x CSR4 was 71 kD and 50 kD respectively, it was less as compared to PM x CSR2 silkworm race. In all the races four bands appeared between 82 kD to 29 kD. The difference was noticed in the intensity of first band of CSR4 x CSR2 and CSR2 x CSR4 as compared to PMxCSR2 which was less. The haemolymph protein pattern on 5th day old larvae, when fed on Almora local MP mulberry variety showed maximum 6 bands between 95 kD to 29 kD in CSR4 x CSR2 race as compared to PM x CSR2 and CSR4 x CSR2. In PM x CSR2 only four bands appeared between 94 kD to 29 kD. The intensity of bands was appeared same in all the races (Table 2, 3, 4 and 5). The larvae fed on China Peking China and S-1096 mulberry variety showed less intensity of bands as compared to previous varieties, it was below 97 kD. In PM x CSR2 it was between 64-30 kD, CSR4 x CSR2, 61-30 kD and CSR2 x CSR4 57 kD to 30 kD in China Peking China and in S-1096 variety fed larvae it was 71 kD to 29 kD in PM x CSR2, 69-27 kD in CSR4 x CSR2 and 70 to 27KD in CSR2 x CSR4 silkworm races. (Fig 2 (A), (B), (C), (D), and Table 2,3,4,5)

3.3. Electrophoretic patterns of Fat body Proteins

In the fat body, protein pattern differed in the banding pattern about, 5 bands appeared in CSR4 x CSR2 and 4 bands in PMxCSR2 and CSR2 x CSR4. The intensity was more in CSR4 x CSR2 it was 81 kD and in PMxCSR2 and CSR2 x CSR4 it was 50 kD and 48 kD, in Kanva-2/M5 fed larvae. The maximum bands appeared in Almora local MP fed larvae, where observed in CSR4xCSR2 12 bands having weight between 85 kD to 29 kD followed by PM x CSR2 11 bands between 86 kD to 29 kD and 10 bands in CSR4 x CSR2 between 95 to 29 kD molecular weight whereas in China Peking China fed larvae showed 3 bands in PM x CSR2, and CSR4 x CSR2 and 6 band in CSR2 x CSR4 race. In the case of S-1096 fed larvae shows 4 bands in PM x CSR 2 in CSR4 x CSR2 and however only one band appeared in CSR2 x CSR4, as compared to Almora local MP and, Kanva-2 fed larvae (Fig 2 (Fig 2 (A), (B), (C), (D), and Table 2,3,4,5))



M-Molecular weight; A-Kanva-2; B-Almora Local; C-China Peking China; D-S-1096

Figure 1 Sodium Dodecyl Sulphate-Polyacralymide Gel electrophoretic pattern of leaf protein of Mulberry Silkworm host plant *Morus indica*.

Table 1 Molecular weight of the proteins separated on SDS-PAGE of leaf of different mulberry variety

No. of bands	**Molecular weight (kD)**				
	Molecular weight marker	**Kanva-2**	**Almora local MP**	**China Peking China**	**S-1096**
1	66,000	61,000	65000	66000	66000
2	43,000	60,000	61000	50000	63000
3	29,000	46,000	45000	45000	54000

Table 2 Molecular weight of the proteins separated on SDS-PAGE of 5 th day larvae fed on Kanva-2 Variety in silkworm *Bombyx mori* L.

No. of Bands	**Molecular weight (kD)**									
	Molecular weight marker	Haemolymph			Fat body			Silk gland		
		PMxCSR2	CSR4xCSR2	CSR4xCSR2	PMxCSR2	CSR4xCSR2	CSR4xCSR2	PMxCSR2	CSR4xCSR2	CSR4xCSR2
1	97000	82000	71000	50000	50000	81000	48000	47000	82000	84000
2	66000	46000	48000	46000	40000	47000	40000	39000	81000	75000
3	43000	39000	39000	39000	37000	37000	37000	37000	74000	74000
4	29000	29000	29000	29000	29000	31000	29000	29000	69000	69000
5						29000			67000	50000
6									50000	47000
7									47000	44000
8									44000	37000
9									37000	36000
10									36000	30000
11									30000	29000
12									29000	

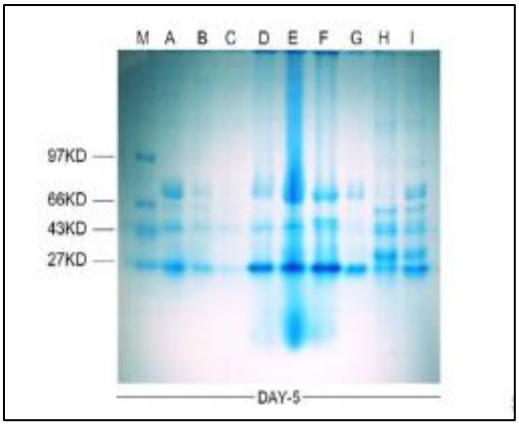


Fig-2 (A)

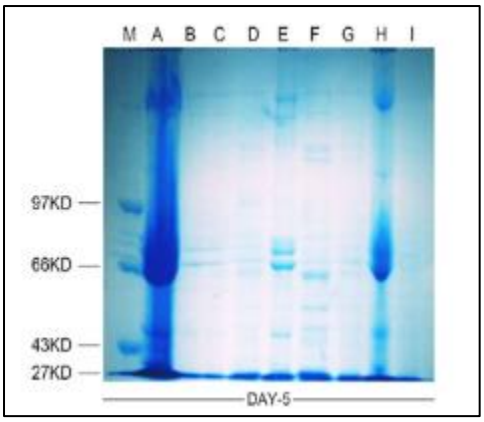


Fig-2 (B)

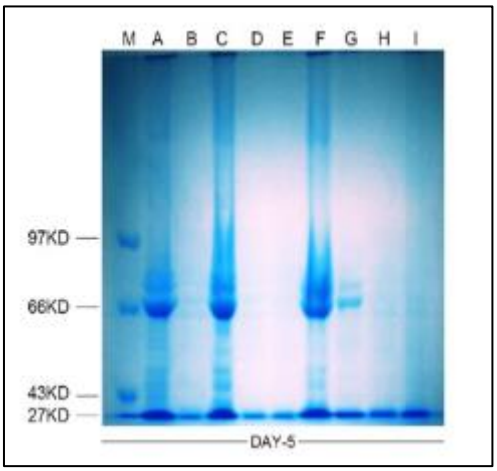


Fig-2 (C)

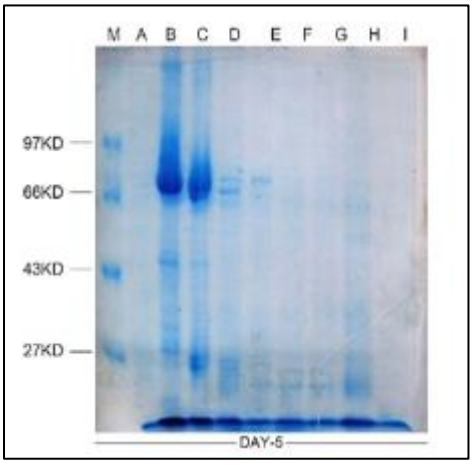


Fig-2 (D)

Figure 2 Sodium Dodecyl Sulphate-Polyacralymide Gel electrophoretic pattern of Haemolymph, Fatbody, Silkgland protein of different races of silkworm, Bombyx mori L fed on different varieties.

Fig- 2 (A) Kanva-2- M-Molecular Marker; Haemolymph A-PMxCSR2, B-CSR4xCSR2, C-CSR2xCSR4; Fat body- D- PMxCSR2, E-CSR4xCSR2, F-CSR2xCSR4; Silkgland-G- PMxCSR2, H-CSR4xCSR2, I-CSR2xCSR4; Fig- 2 (B) Almora Local –M-Molecular Marker; Haemolymph A-PMxCSR2, B-CSR4xCSR2, C-CSR2xCSR4; Fat body- D- PMxCSR2, E-CSR4xCSR2, F-CSR2xCSR4; Silkgland-G- PMxCSR2, H-CSR4xCSR2, I-CSR2xCSR4; Fig- 2 (C) China Peking China – M-Molecular Marker; Haemolymph A-PMxCSR2, B-CSR4xCSR2, C-CSR2xCSR4; Fat body- D- PMxCSR2, E-CSR4xCSR2, F-CSR2xCSR4; Silkgland-G- PMxCSR2, H-CSR4xCSR2, I-CSR2xCSR4; Fig- 2 (D) S-1096 – M-Molecular Marker; Haemolymph A-PMxCSR2, B-CSR4xCSR2, C-CSR2xCSR4; Fat body- D- PMxCSR2, E-CSR4xCSR2, F-CSR2xCSR4Silkgland-G- PMxCSR2, H-CSR4xCSR2, I-CSR2xCSR4

Table 3 Molecular weight of the proteins separated on SDS-PAGE of 5 th day larvae fed on Almora Local Variety in silkworm *Bombyx mori* L.

No. of Bands	**Molecular weight (kD)**									
	Molecular weight marker	Haemolymph			Fat body			Silk gland		
		PMxCSR2	CSR4xCSR2	CSR4xCSR2	PMxCSR2	CSR4xCSR2	CSR4xCSR2	PMxCSR2	CSR4xCSR2	CSR4xCSR2
1	97000	97000	95000	94000	86000	95000	85000	85000	94000	58000
2	66000	41000	93000	56000	84000	86000	84000	84000	47000	54000
3	43000	31000	54000	54000	73000	84000	81000	59000	31000	49000

4	29000	29000	50000	44000	69000	56000	78000	56000	29000	
5			31000	29000	59000	48000	69000	51000		
6			29000		55000	44000	64000	48000		
7					50000	38000	53000	38000		
8					46000	33000	50000	31000		
9					39000	29000	48000	29000		
10					34000	29000	38000			
11					29000		31000			
12							29000			

Table 4 Molecular weight of the proteins separated on SDS-PAGE of 5 th day larvae fed on China Peking China Variety in silkworm *Bombyx mori* L.

No. of Bands	**Molecular weight (kD)**									
	Molecular weight marker	**Haemolymph**			**Fat body**			**Silk gland**		
		PMxCSR2	**CSR4xCSR2**	**CSR4xCSR2**	**PMxCSR2**	**CSR4xCSR2**	**CSR4xCSR2**	**PMxCSR2**	**CSR4xCSR2**	**CSR4xCSR2**
1	97000	64000	61000	57000	60000	56000	56000	59000	50000	50000
2	66000	59000	56000	60000	56000	52000	53000	57000	30000	30000
3	43000	47000	53000	56000	30000	30000	48000	47000		
4	29000	44000	32000	49000			36000	30000		
5		40000	30000	47000			30000			
6		37000		40000						
7		36000		37000						
8		30000		32000						
9				30000						
10										
11										
12										

Table 5 Molecular weight of the proteins separated on SDS-PAGE of 5 th day larvae fed on S-1096 Variety in silkworm *Bombyx mori* L.

No. of Bands	**Molecular weight (kD)**									
		Haemolymph			**Fat body**			**Silkgland**		
	Molecular weight marker	**PMxCSR2**	**CSR4xCSR2**	**CSR4xCSR2**	**PMxCSR2**	**CSR4xCSR2**	**CSR4xCSR2**	**PMxCSR2**	**CSR4xCSR2**	**CSR4xCSR2**
1	97000	71000	69000	70000	73000	71000	57000	84000	70000	50000
2	66000	47000	50000	49000	70000	58000	33000	66000	30000	30000

3	43000	29000	49000	43000	58000	33000	46000	55000		
4	29000		42000	35000	29000	31000		36000		
5			36000	30000		29000		31000		
6			30000					29000		
7			29000							
8										
9										
10										
11										
12										

3.4. Electrophoretic patterns of Silk gland Proteins

The banding of silk gland protein in 5 days old silkworm larvae of different races fed on different mulberry variety shows maximum number of band in CSR4 x CSR2 having 82 kD to 29 kD mol weight and followed by CSR2 x CSR4 with11 band having 84 kD to 29 kD and followed by CSR2 x CSR4 with 11 bands and 84 kD to 29 kD whereas in PM x CSR2 only 4 bands appeared of 47 kD to 29 kD of molecular weight when fed with Kanva-2 variety when fed with Almora local MP larvae showed 9 bands in PM x CSR2 and only 3 to 4 in CSR2 x CSR4 and CSR4 x CSR2 races respectively. In case of China Peking China and S-1096 leaves fed larvae showed 4 and 6 bands in PM x CSR2 and in CSR4 x CSR2 and CSR2 x CSR4 showed only 2 bands each having molecular weight 50 to 30 kD. (Fig 2 (Fig 2 (A), (B), (C), (D), and Table 2,3,4,5)

4. Discussion

Protein and amino acid are particular importance for the silkworm larvae because of their active utilization even for the synthesis of silk proteins. Horie (1978, 1980) who studied the utilization of food stuff by silkworm, reported that the optimum level of 20-25% is required for better growth of silkworm larvae. In the present study, the highest value of protein level was observed in Almora local MP. The present results showed the level of haemolymph protein in different silkworm races PM x CSR2, CSR4 x CSR2, and CSR4 x CSR2 showed not much difference compared with each other, when fed on Kanva-2, Almora local MP and China Peking China mulberry varieties, only in S-1096 variety fed larvae was noticed less protein level as compared to other variety, it may due to poor leaf quality of S-1096. Similar results were noticed in all the silkworm races. Results are in agreement with that of Madhu Babu *et al.* (2014), who found that protein molecular weight of 48 kD and 55 kD were rather common in all the five examined mulberry varieties and the 44 kD protein was the major component of the leaf proteins. Results are in context with Jyothi *et al*. (2016) who detected 10-12 protein bands in leaves of four cultivars and explained the relationship between mulberry leaves and silkworm protein patterns. They added that mulberry leaves protein bands may belong to sericin and fibroin family, the main components of silk gland and silk fibres. The fat body protein level was high in Kanava-2/M5 and Almora local MP mulberry fed larvae as compared to China Peking China and S-1096. Similar results were noticed in the silk gland protein. The result obtained showed influence of environmental condition and seasonal changes on protein level of silk gland. The present results obtained showed there is no significant change in the banding pattern, only difference was noticed in the intensity of band in the different silkworm races, fed on all the mulberry variety In the fat body protein, maximum band was appeared in Almora local MP mulberry variety fed larvae as compared to other three varieties in all the silkworm races Similar trend were maintained in the silk gland protein pattern.

Although there are some differences in protein band numbers and abundance in the leaves of the four investigated mulberry varieties, the proteins pattern profile in haemolymph, fat body and silk gland of larvae fed on the leaves of all four investigated mulberry varieties were closely similar which may mean that the Almora local MP variety leaves can be used in silkworm *Bombyx mori* L. rearing system.

5. Conclusion

The present study highlights that although minor variations in protein band numbers and intensities were observed among silkworm races fed on different mulberry varieties, the overall protein profiles in haemolymph, fat body, and silk gland remained largely similar, with Almora local MP showing consistently higher protein levels compared to other

varieties. Poor leaf quality of S-1096 was associated with reduced protein levels, while Kanva-2 and Almora local MP supported better protein expression in fat body and silk gland. These findings confirm earlier reports linking mulberry leaf protein composition to silkworm growth and silk protein synthesis, emphasizing the importance of leaf quality and environmental conditions. In conclusion, Almora local MP emerges as a promising mulberry variety for silkworm rearing, and this study will benefit society by supporting sustainable sericulture practices and guiding future breeding programs for enhanced silk production.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Authors have declared that no Conflict of interests exist.

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Author's short biography

Dr Rajkumar S. Bhonde, HOD and Associate professor of Zoology Department at C.J. Patel college, Tirora, Dist.-Gondia, Maharashtra, affiliated to RTM Nagpur university, Nagpur. He has completed his Ph.D. from RTM Nagpur university, Nagpur. He has having, 14 years' experience of teaching Zoology subject, at undergraduate level. He has published 20 research papers in reputed journal, and also published various good quality books as per RTMNU syllabus. His areas of interest include Agricultural entomology, Mammalian physiology and Insect pest.