Chapter – IV

Peptide isolation and feeding response

4.1 INTRODUCTION

Human beings have benefited by the silkworm in various ways and scientists have been continuously trying to improve the techniques of silkworm rearing. Plants are considered as a richest resource of phytochemicals and these phytochemicals have been reported to manipulate the life cycle and activity of different insects (Rajashekaragouda *et al.,* 1997; Khyade, 2004). The effects of different types of dietary protein on silkworm growth were resolved by using semi-synthetic diets. Some workers have clearly described that protein acts as an essential ingredients in silkworm diet for their growth and silk production. Since, smaller proteins have also been considered as peptides, therefore it can be predicted that these peptides might also have significant effect on the growth and development of silkworm larvae.

Over the past decades, several natural peptides have been identified from number of organisms especially from nervous and non-neural endocrine systems of vertebrates and invertebrates. The lists of 40 identified neuropeptides from invertebrates are known but the physiological functions of these peptides in insects are yet to be under investigation (Greenberg and Price, 1985). Neuropeptides are being identified in insects and played a crucial role to control brain function as well as it can stimulate the production of juvenile hormones (JH) in insects. JH and peptides were responsible for silk gland formation and silk protein synthesis in non mulberry silkworm and JH as well as peptides could increase larval growth, cocoon weight and ultimately silk production (Unni *et al.,* 2008). Application of juvenile hormone, Methoprene, a juvenile hormone analogue and juvenile hormone activating peptide could increase larval growth, cocoon weight and silk production of muga silkworm *Anthereae assama* (Saikia, 2001).

Several antimicrobial peptides also were isolated from insects. Antimicrobial peptides appear to be multipotent components of inner immune defense system of both the animal and plants (Boman, 1995; Broekaert *et al.,* 1995; Ganz and Weiss, 1997). Numerous antimicrobial peptides namely plant defensins, γ-thionins (crambin) was isolated from plant and diptericin, apidaecin peptides were isolated from insects (Ganz, 2003).

But the effects of plant peptides on metabolism, reproduction and production of insects are not yet reported. In present study, a scientific attempt was made to figure out the effect of peptide(s) of two different molecular weight ranges (0.5-3 kDa and 3-10kDa) isolated from different mulberry leaves on silkworm growth and silk production. For peptides isolation, those mulberry cultivars were selected which had better feeding response by larvae and most stress tolerant. To make comparative

findings, one susceptible mulberry cultivar with poor feeding preference was also selected for peptide isolation.

4.2 MATERIAL AND METHODS

Mulberry leaves of S1, S1635, V1 and Dudhiya cultivars were collected from sericulture farm of Malda, West Bengal, India at same season and same time. Leaves were selected at different maturity status e.g. young, mature and senescence leaves and was weighed out (1 kg each). Young, mature and senescence leaves were selected on the basis of the biological (i.e. chlorophyll content and protein content) and the morphological attributes of the leaves (i.e. length and breadth).

4.2.1 Isolation and purification of low molecular weight peptide(s)

4.2.1.1 Preparation of extraction

One kg of leaves of each set were washed separately and thoroughly under tap water and cut into pieces. After repeated wash, sets were treated with 0.2% Sodium hypochlorite solution to avoid excessive contamination and finally washed with distilled water. The leaf pieces were separately crushed for peptides isolation in presence of liquid nitrogen by a grinder and extracted with chilled measured amount of distilled water by blender at 4°C in cold room. To remove the unwanted materials, the extract was centrifuged at 10,000 rpm for 30 min using protease inhibitor PMSF at 4°C. The supernatant was collected and stored in deep freeze (-20°C).

4.2.1.2 Ether washes

The extracts were subjected to ether wash at acidic pH to remove endogenous hormonal impurities, fats, lipids and oil as impurities.

4.2.1.3 Ion exchange chromatography

The extracts were passed through separate cation exchanger resin (900 meq-Sigma Chemical Co. USA filled in glass column 60 cm × 2.9 cm, 1.6 meq/mL) to get anionic hormone free solution, like indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA_3). Solution of each sample held up some basic compound like cytokinin, amino acids, peptide and related compounds after coming from cation exchange resin column. The resin was taken out from column and was neutralized with ammonia with constant shaking to avoid exothermic reaction and again reloaded it in the column, further eluted with 3 (N) NH_4OH in cold. The total liquid was dried in freeze with liquid N_2 trap to remove ammonia. The sample obtained after cation resin processing was taken in water with little

acidic pH and again passed through anion resin (700 meq-Sigma Chemical Co. USA filled in glass column 60 cm × 2.9 cm, 1.6 meq/mL). Again it was washed, neutralised and eluted with 1 (N) HCl. After passing through this column, basic compounds and cytokinins were removed from solution. As a result, liquid samples were free from electrolytes, only amphoteric compounds, peptides and amino acids were present in each collected sample. Then concentrated aqueous acidic solutions were washed 4 times with equal volume of peroxide free ether to remove traces of IAA, ABA, and GA.

4.2.1.4 Ultra-filtration

After discarding of anionic hormones, the extracts were filtered through Millipore ultra filtration system with Amicon filters 10 kDa (YM 10), 3 kDa (YM3) and 0.5 kDa (YC 05) cut off with 1.5 kg/cm^2 N_2 gas pressure. The samples were repetitively filtered and finally purified and dry extracts were obtained which were semi-solid. The obtained peptide extract was dissolved in 50 mL distilled water and stored in freeze at -20˚C for further analysis.

4.2.2 Rearing of silkworm larvae

4.2.2.1 Experimental insect and rearing method

Mention in section 2.2.2.1

4.2.2.2 Leaf treatment

Peptide(s) isolated from young (Py), mature (Pm), and senescence (Ps) leaves in both range 0.5-3 kDa and 3-10 kDa was 20 times diluted by distilled water. Leaves were soaked in peptide(s) for 30 min before feeding them to the larvae and air-dried for 15 min and given to silkworm. Six separate groups, with 15-20 larvae were kept and fed by different peptide(s) treated leaves in separate plastic trays.

4.2.2.3 Rearing bed maintenance procedure and data collection

Mention in section 2.2.2.1

4.3 RESULT AND DISCUSSION

Essential nutrients in exact ratio are required to improve the growth and development of *B. mori* (Kanafi *et al.,* 2007). Sarker (1993) noted significant improvement of silkworm larval growth upon feeding them with mulberry leaves supplemented with different nutrients. In our present study 5^{th} instar larval growth rate pattern was found to be improved under the influence of different peptide(s) treatment. Consumption rate of the larvae under peptide(s) treatment was increased significantly over control. Maximum larval growth rate was observed between 72-96 h after 4^{th} moulting in both

molecular weight range of peptide(s) treatment and as well as in control set. The 5th instar silkworm larvae showed no significant difference in maintaining larval duration even in peptide(s) treatment. Similar result was obtained with folic acid administration where larval growth was influenced by folic acid (from 24 h onwards) and folic acid inserted no difference in larval duration of the 5th instar silkworm (Rahmathulla *et al.,* 2007).

Separate experimental setup was maintained for each peptide treatment in respect of control. After nourishment with S1 peptide, highest larval growth rate was recorded at 96 h in S1Y LMW (Low molecular weight 0.5 to 3 kDa, peptide isolated from S1 young leaves) followed by S1Y HMW (High molecular weight-3 to 10 kDa, peptide isolated from S1 young leaves), S1M LMW, S1S LMW, S1M HMW, S1S HMW and control (Figure 4.1 and 4.2). But in case of S1635, V1 and Dudhiya peptide treatment, peptide isolated from mature leaves (S1635M, V1M and DM respectively) exhibited better growth rate in the comparison with peptides isolated from young and senescence leaves (Figure 4.3-4.8). Low molecular weight peptides (0.5-3 kDa) exhibited better performance in comparison with high molecular range (3-10 kDa) peptides in all treatments. Hence, it was evident that peptide(s) treatment elicited better growth potential for silkworm larvae.

Paralytic peptides of silkworm larvae had *in vitro* effects on both in hematopoietic regulation and silkworm larval hemocyte immune reaction (Nakahara *et al.,* 2003). Literature survey revealed that peptide had a role on larval growth and metabolic activity (Unni *et al.,* 2008). But until now, it was not clear about whether the effect of peptides on larvae was dependent on nature or source of peptides. To clarify this knowledge gap, in the present study four sources of peptides was selected and also compared their role on larval growth and silk production. A comparative statistical work was conducted for each treatment with their respective control to evaluate the rate of increase or decrease of each economical attributes of silkworm rearing (Figure 4.9, 4.14, 4.19 and 4.24). These studies revealed that all S1 peptide (S1Y, S1M and S1S) influenced the larval growth at each molecular weight range and other economical attributes of silkworm rearing system. But when we compared the effects of S1635, V1 and Dudhia peptides on larvae, it was found that these three peptides showed comparatively reduced effects on larval growth than S1. Only nourishment with LMW peptides of S1635M, V1M and DM increased 56.41%, 33.17% and 35.53 % larval growth rate respectively, while the growth rate after application of HMW peptides from those cultivars was insignificant. On the other hand, treatment with peptides of S1Y, S1M and SS in both low and high molecular range increased growth rate significantly (Figure 4.9).

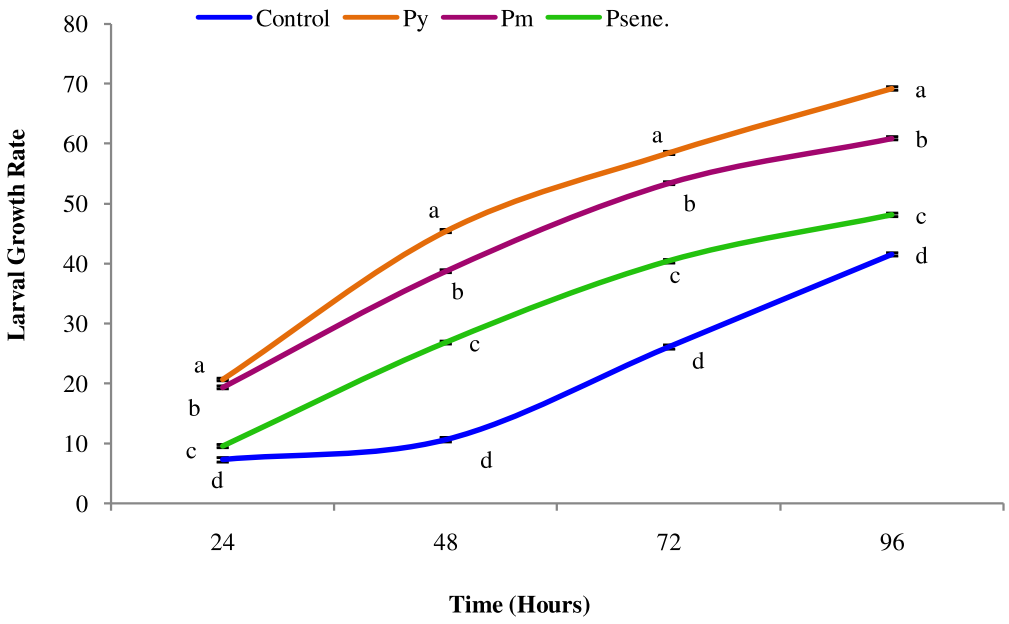


Figure 4.1: Larval growth rate after feeding S1 leaves, elicited by 0.5-3 kDa peptide isolated at different maturity stages of leaves (S1) and in control (without peptide elicitation).
Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

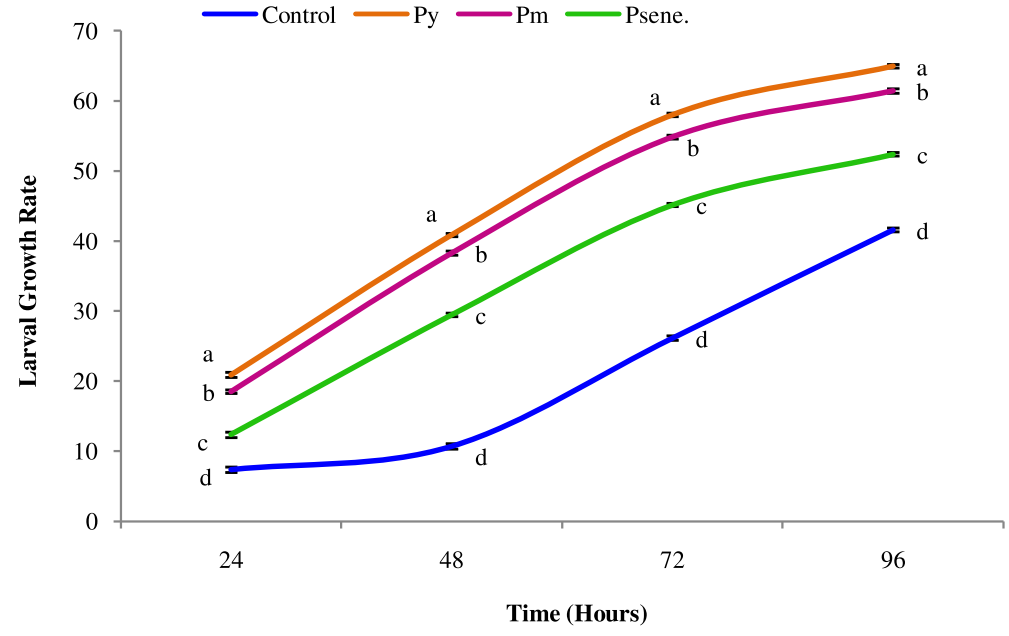


Figure 4.2: Larval growth rate after feeding S1 leaves, elicited by 3-10 kDa peptide isolated at different maturity stages of leaves (S1) and in control (without peptide elicitation).
Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

Abb. Used: Py, Pm & Psene: peptide isolated from young, mature & senescence leaves respectively.

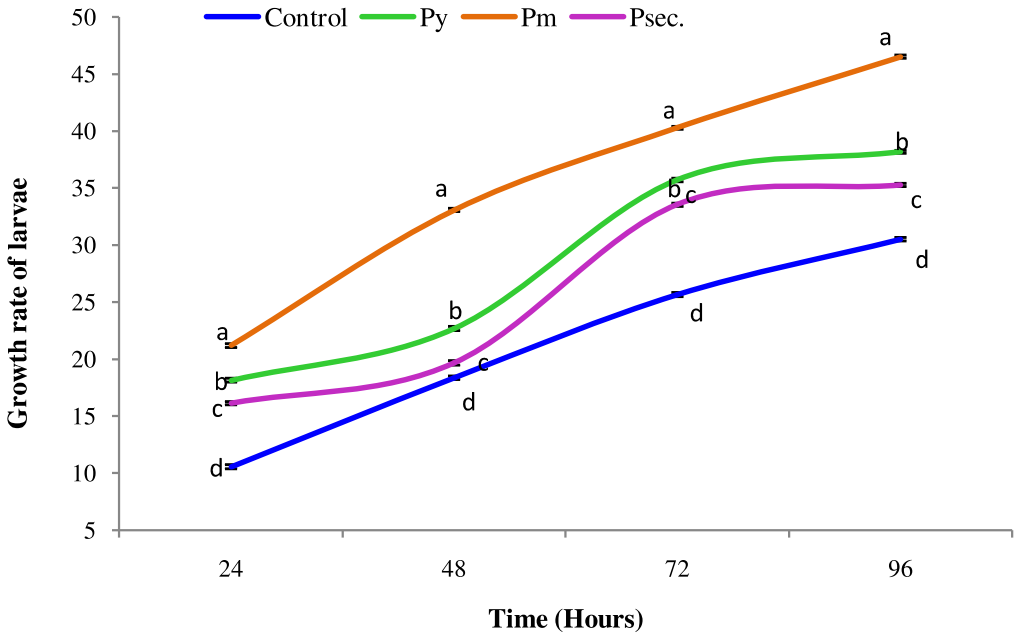


Figure 4.3: Larval growth rate after feeding S1 leaves, elicited by 0.5-3 kDa peptide isolated at different maturity stages of leaves (S1635) and in control (without peptide elicitation). Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly (P < 0.05) different from each other by Duncan's multiple range test (DMRT).

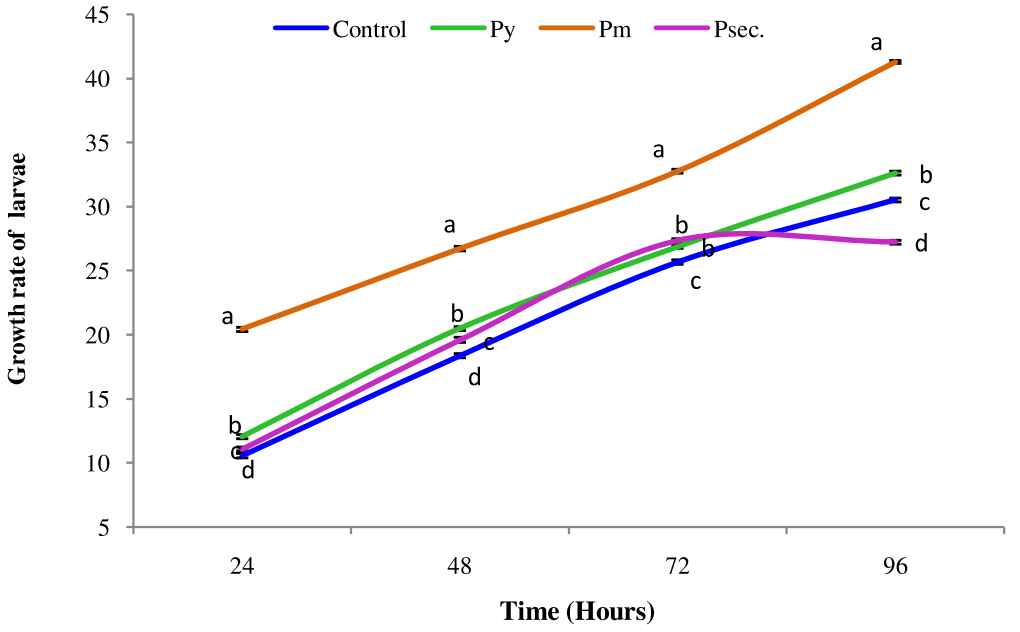


Figure 4.4: Larval growth rate after feeding S1 leaves, elicited by 3-10 kDa peptide isolated at different maturity stages of leaves (S1635) and in control (without peptide elicitation). Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly (P < 0.05) different from each other by Duncan's multiple range test (DMRT).

Abb. Used: Py, Pm & Psec: peptide isolated from young, mature & senescence leaves respectively.

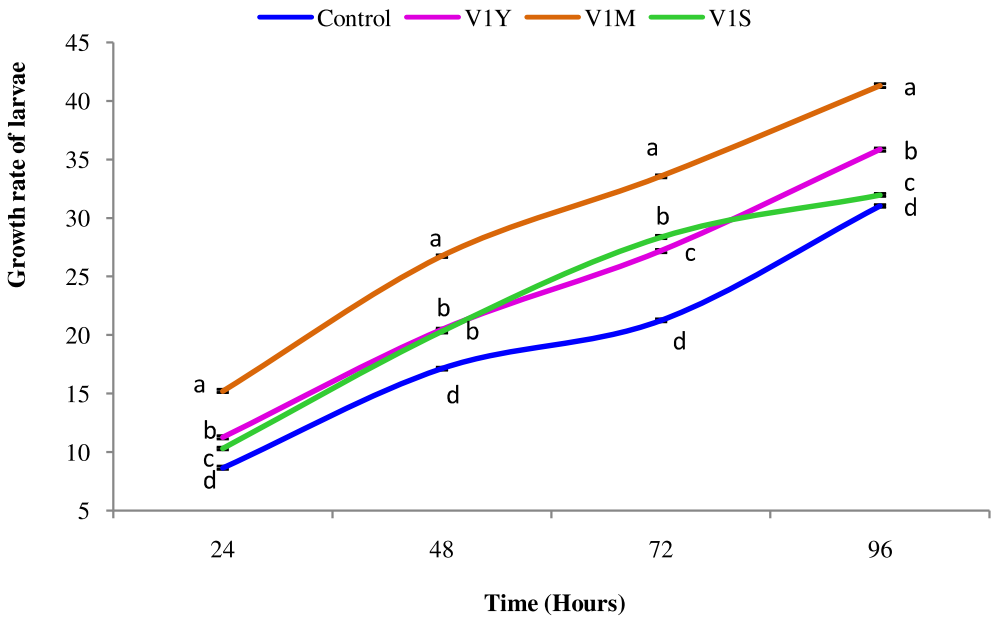


Figure 4.5: Larval growth rate after feeding S1 leaves, elicited by 0.5-3 kDa peptide isolated at different maturity stages of leaves (V1) and in control (without peptide elicitation)
Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

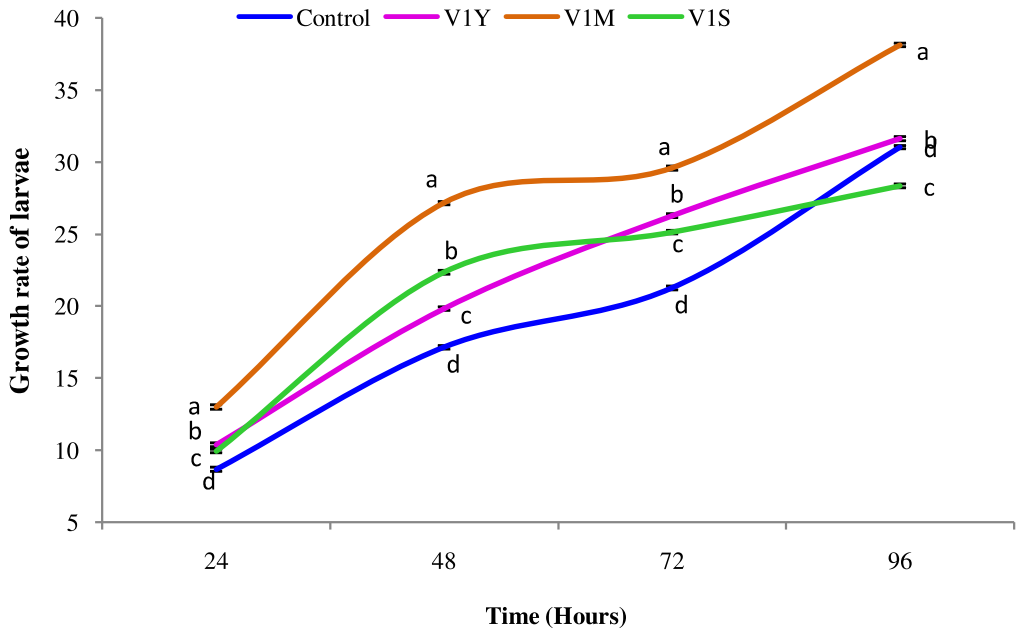


Figure 4.6: Larval growth rate after feeding S1 leaves, elicited by 3-10 kDa peptide isolated at different maturity stages of leaves (V1) and in control (without peptide elicitation).
Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

Abb. Used: V1Y, V1M & V1S: peptide isolated from young, mature & senescence V1 mulberry leaves respectively.

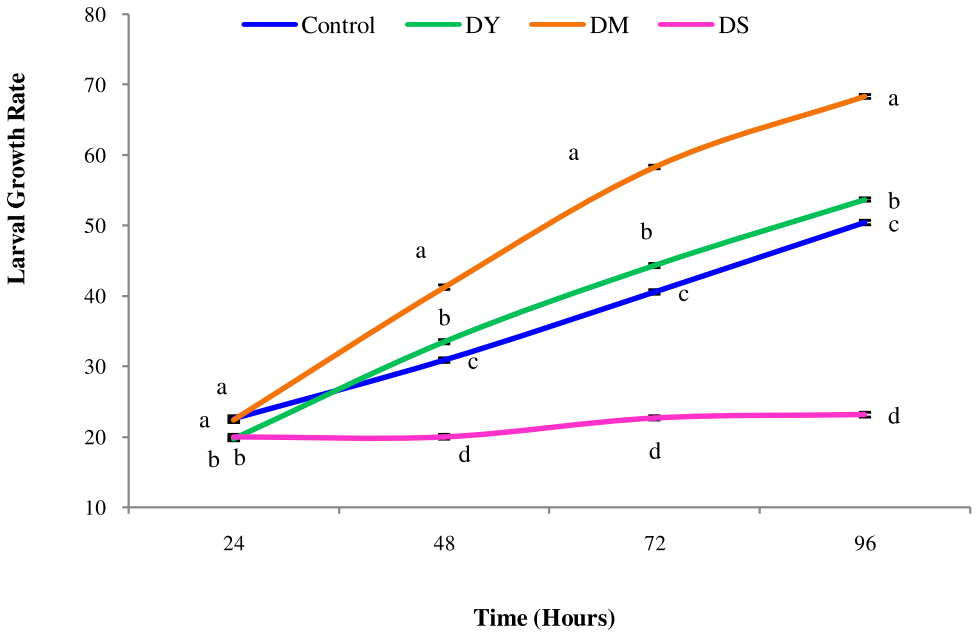


Figure 4.7: Larval growth rate after feeding S1 leaves, elicited by 0.5-3 kDa peptide isolated at different maturity stages of leaves (Dudhiya) and in control (without peptide elicitation). Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

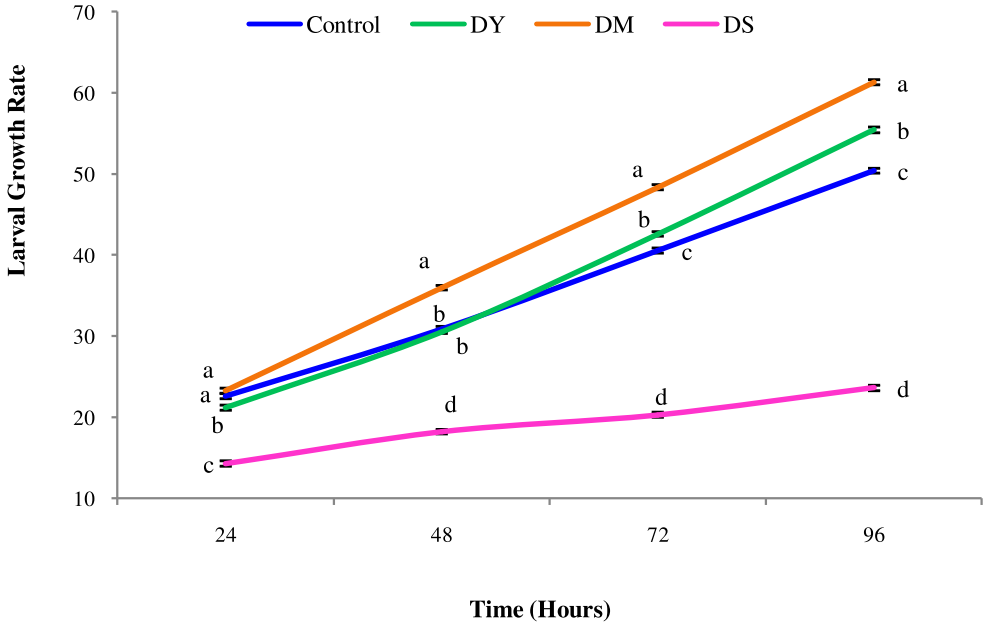


Figure 4.8: Larval growth rate after feeding S1 leaves, elicited by 3-10 kDa peptide isolated at different maturity stages of leaves (Dudhiya) and in control (without peptide elicitation). Results are represented as mean ± SEM, n = 15. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

Abb. Used: DY, DM & DS: peptide isolated from young, mature & senescence Dudhiya mulberry leaves respectively.

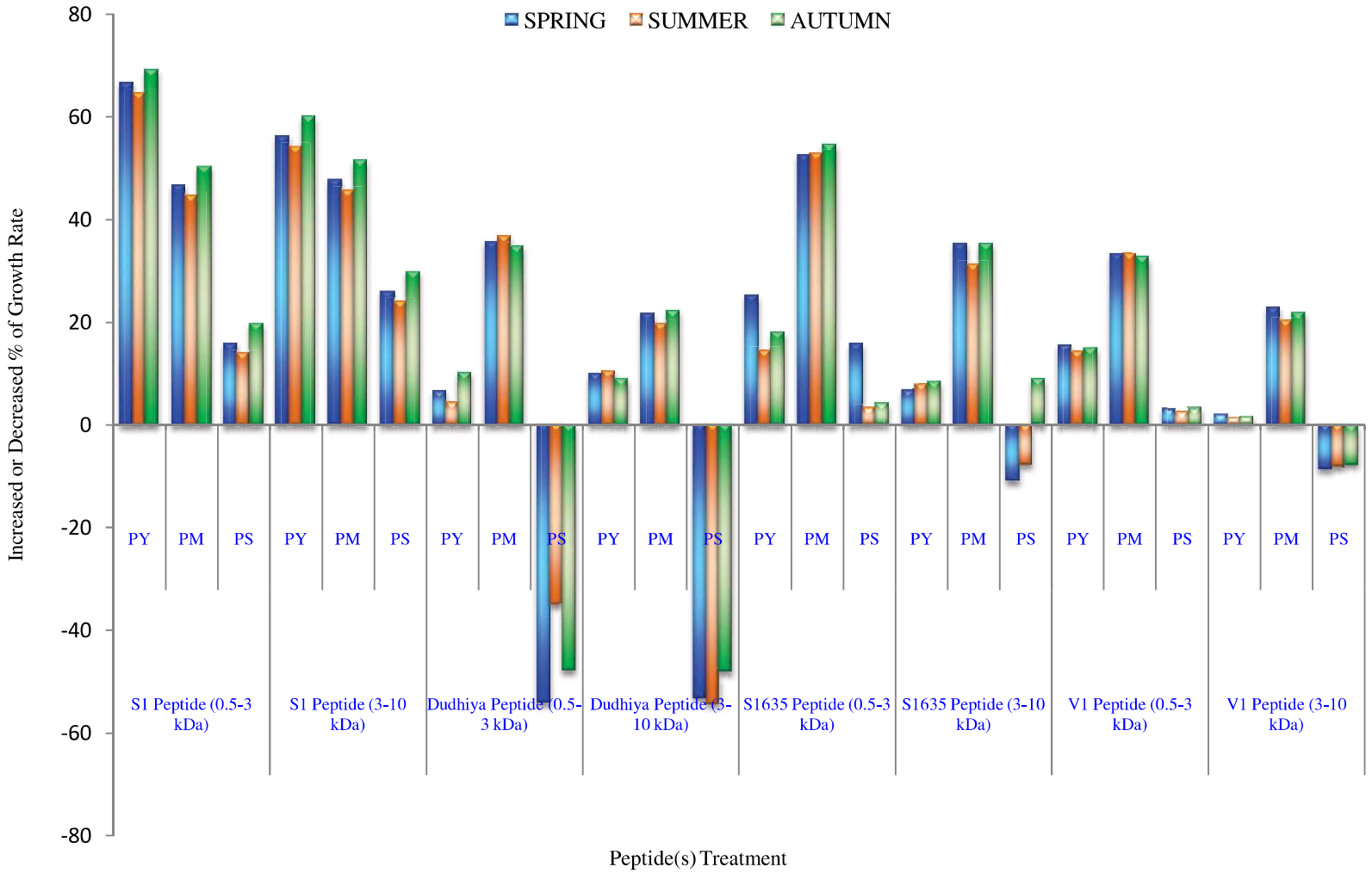


Figure 4.9: Increase or decrease (%) of weight of single cocoon (WSC) over control under peptides treatment

Abb. Used: PY, PM & PS: peptide isolated from young, mature & senescence leaves respectively.

Cocoon weight is considered as an important commercial character because it is used to determine approximate amount of the raw silk but shell weight cannot be used as commercial feature because it damages the cocoon (Nguku *et al.,* 2007). The cocoon production depends on larval growth and the nutritional value of supplied food for silkworm rearing. Cocoon weight was highly improved by feeding the silkworm larvae with mulberry peptide(s). Weight of single cocoon (WSC) was highly increased in S1635 peptide treatment followed by S1, V1 and Dudhiya in respect to their respective control (Figure 4.14). Only S1635M exhibited considerable enhancement of WSC, whereas S1Y, S1M and S1S each showed significant increase of WSC in respect to their control. Highest WSC was recorded under S1Y peptide treatment followed by S1M, S1S and control (Figure 4.10). Effects of S1635, V1 and Dudhiya peptides on WSC were shown in Figure 4.11-4.13. LMW peptide had improved effects on WSC when compared with HMW peptide treatment.

Shell weight and shell ratio are considered as vital attributes to determine the production rate and the silk quality in sericulture. Maximum single shell weight (g) was recorded under S1Y peptide treatment followed by S1635, V1 and Dudhiya in both molecular weight range (Figure 4.19). Peptides isolated from young leaves exhibited higher WSS in S1 and Dudhiya (Figures 4.15 and 4.18) whereas peptides from mature leaves of S1635 and V1 showed better response than others (Figures 4.16 and 4.17). Present work revealed that significant difference in the shell ratio (%) occurred with S1 peptide(s) application followed by S1635, V1 and Dudhiya (Figure 4.24). Peptides had considerable effects on SR% of silkworm rearing at LMW ranges (0.5-3 kDa) than HMW (Figure 4.20-4.23). Therefore, LMW peptides were selected from this study for further experiment. Another commercial character ERR% (Effective Rearing Rate) was calculated from cocoon weight (Figure 4.25-4.28). Supplementing the diet with protein ingredients might increase the digestibility and improvement of the larval growth and cocoon production (Ullal, 1978).

Similar experimental setup was conducted in different seasons. The effects of peptides on silkworm rearing system were more or less same in all season (Figure 4.9, 4.14, 4.19 and 4.24). Previously, it was recorded that larval growth rate and other economic parameters was affected in different seasons due to environmental stress condition. But the application of peptides helped to maintain larval growth and all economical attributes under various environmental circumstances. Oligopeptide segments of 0.5 -3 kDa and 3-10 kDa proteins can stimulate a complex mechanism in mulberry leaves which helps to maintain different metabolic pathway in proper way throughout all environmental situation.

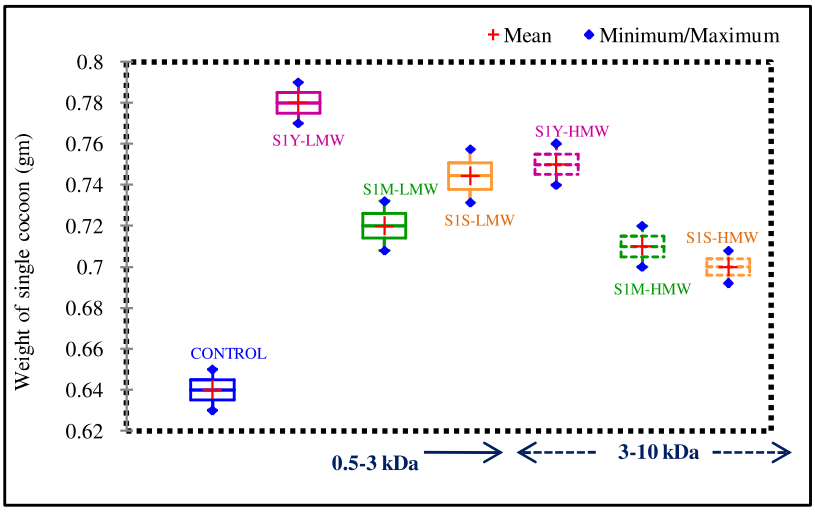


Figure 4.10: Weight of single cocoon under S1 peptide treatment and respective control set

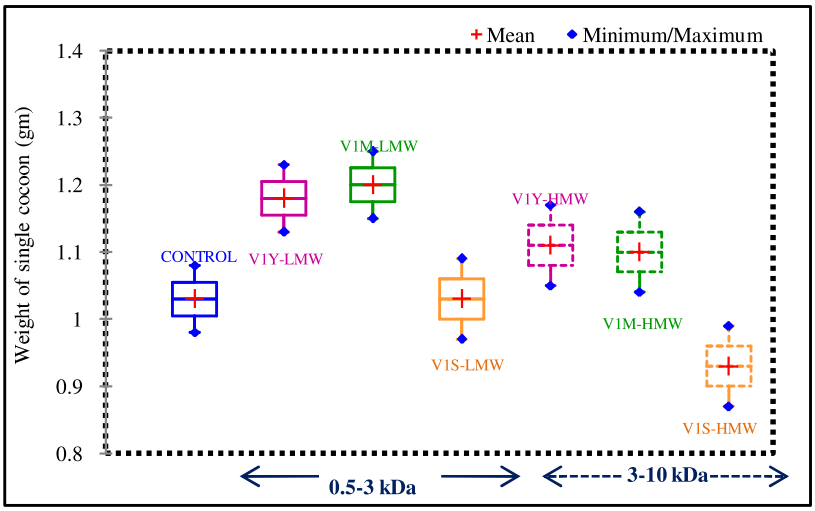


Figure 4.11: Weight of single cocoon under V1 peptide treatment and respective control set

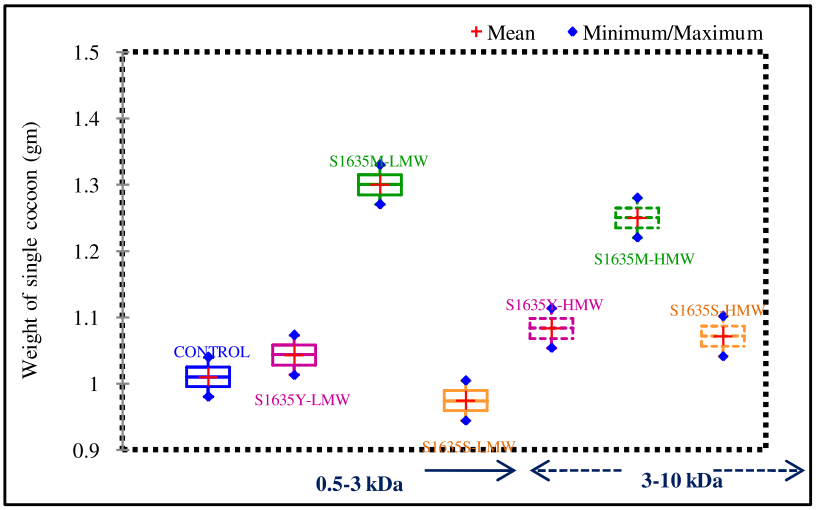


Figure 4.12: Weight of single cocoon under S1635 peptide treatment and respective control set

Abb. Used: Y, M & S: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

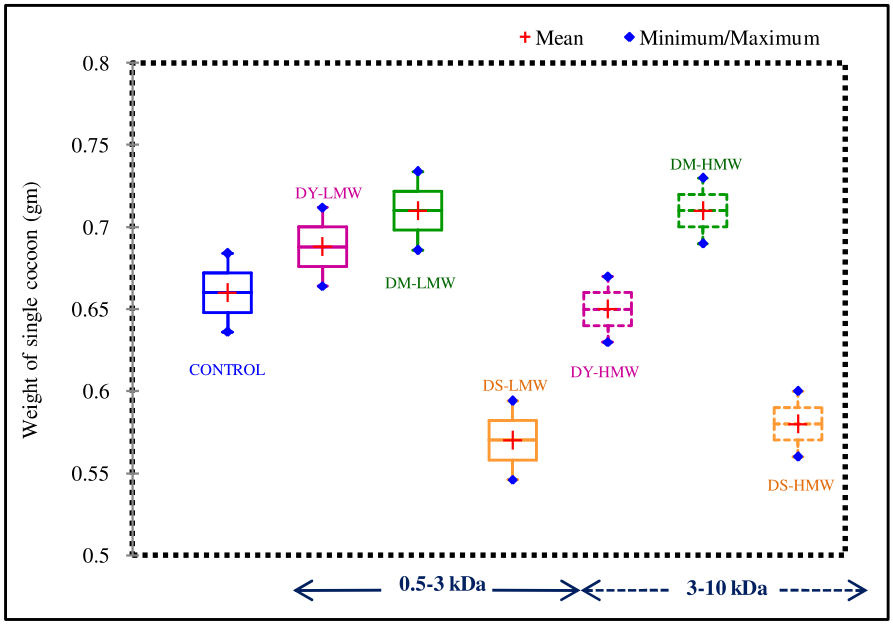


Figure 4.13: Weight of single cocoon under Dudhiya peptide treatment and respective control set

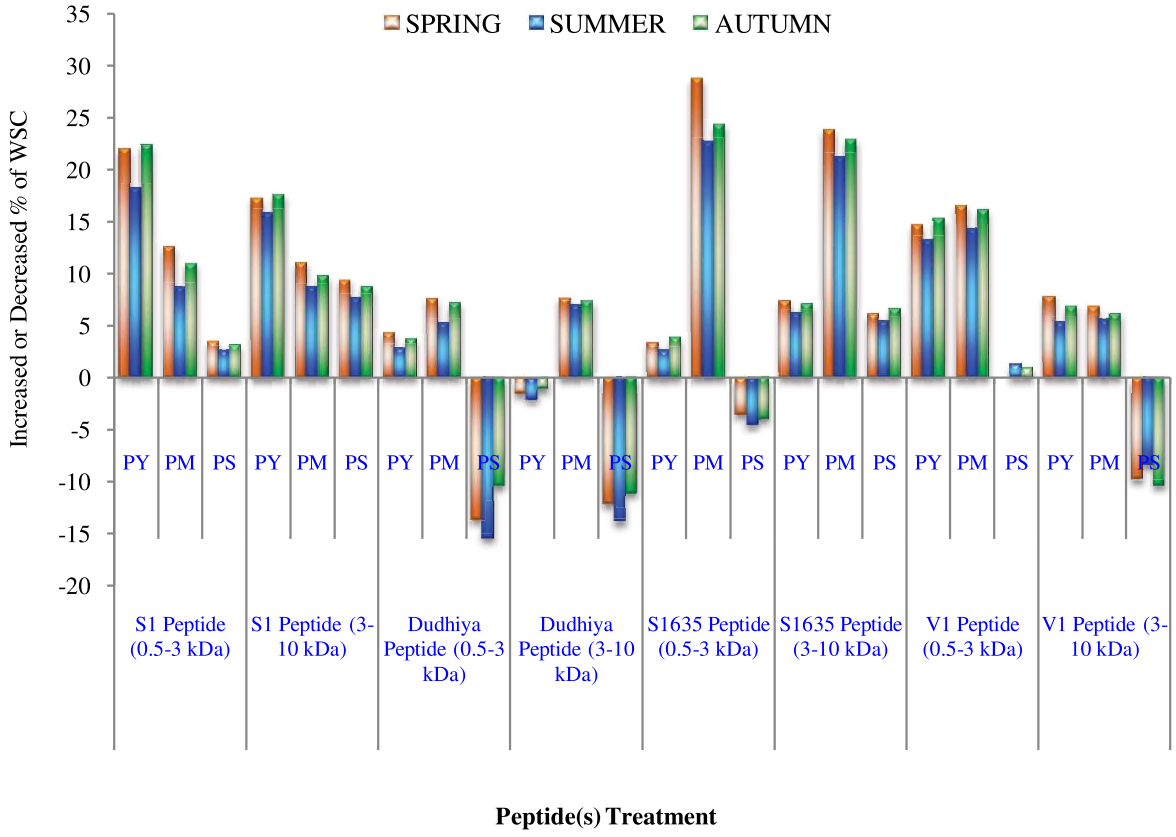


Figure 4.14: Increase or decrease (%) of weight of single cocoon (WSC) over control under peptides treatment

Abb. Used: PY, PM & PS: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

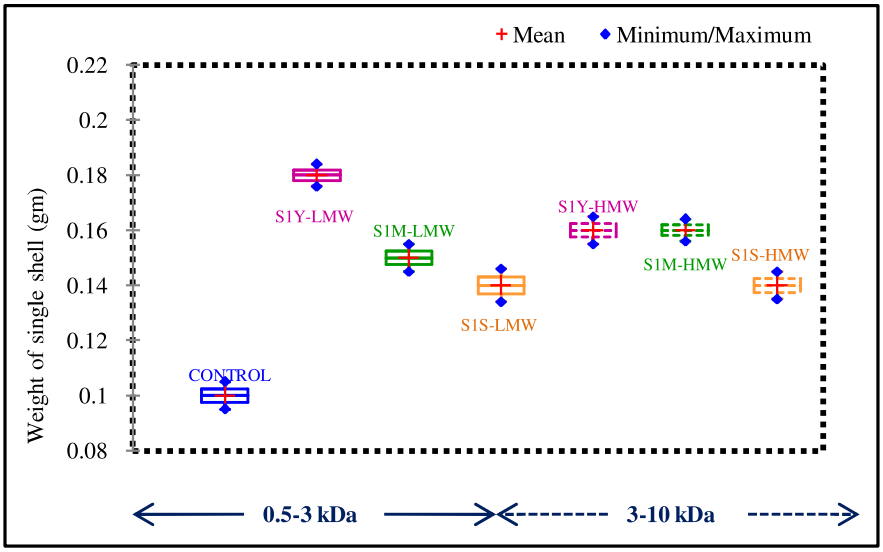


Figure 4.15: Weight of single shell under S1 peptide treatment and respective control set

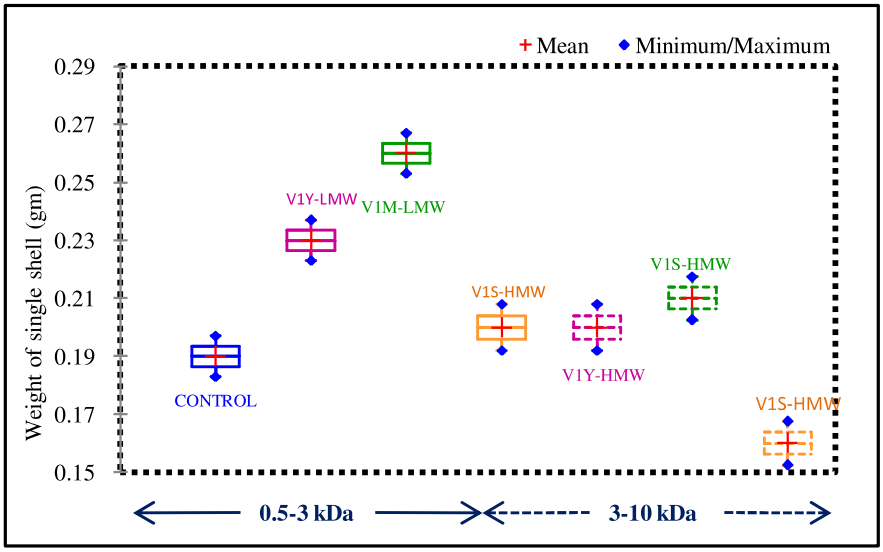


Figure 4.16: Weight of single shell under V1 peptide treatment and respective control set

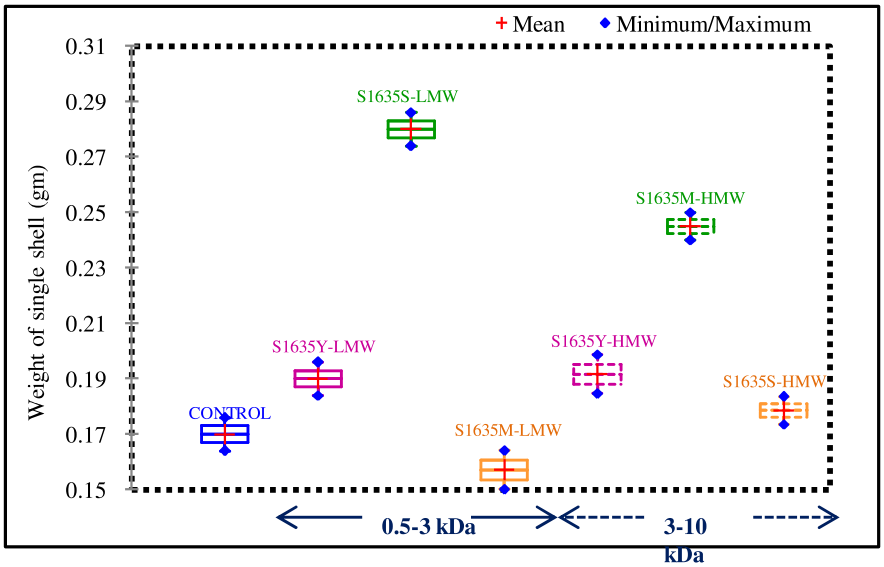


Figure 4.17: Weight of single shell under S1635 peptide treatment and respective control set

Abb. Used: Y, M & S: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

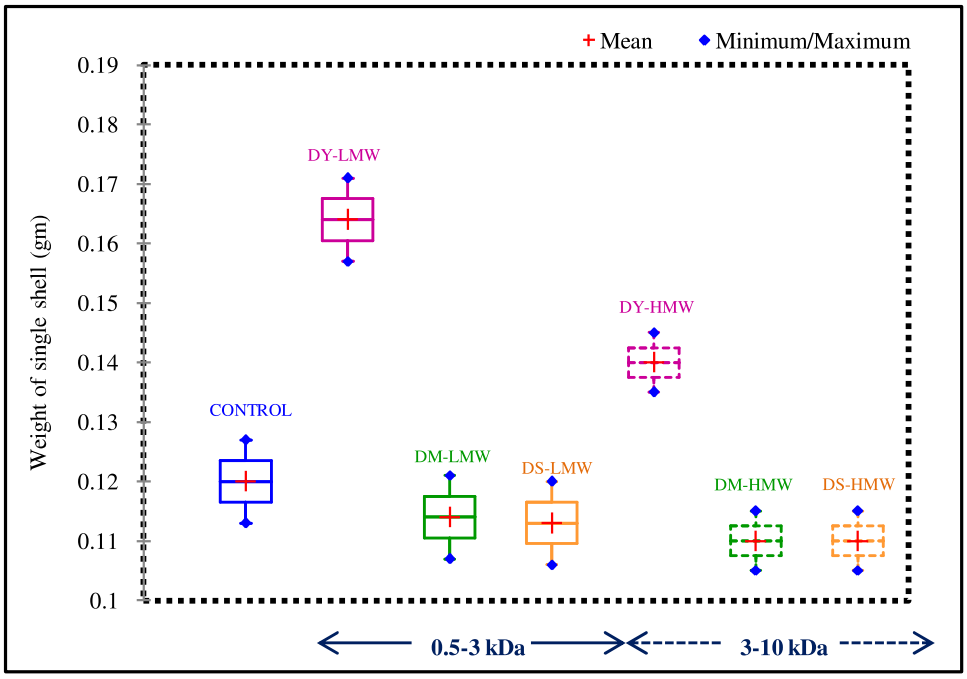


Figure 4.18: Weight of single shell under Dudhiya peptide treatment and respective control set

Abb. Used: Y, M & S: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

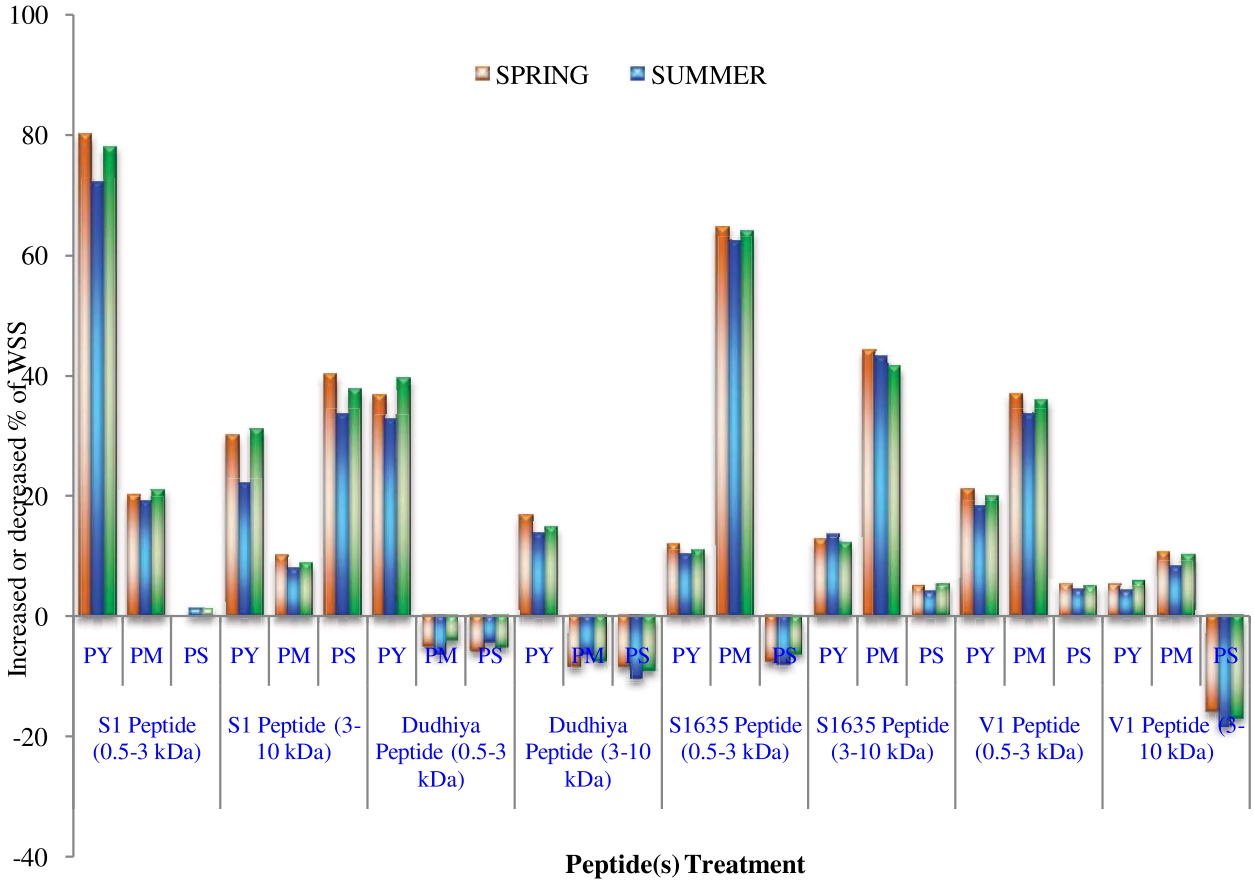


Figure 4.19: Increase or decrease (%) of weight of single shell (WSS) over control under peptides treatment

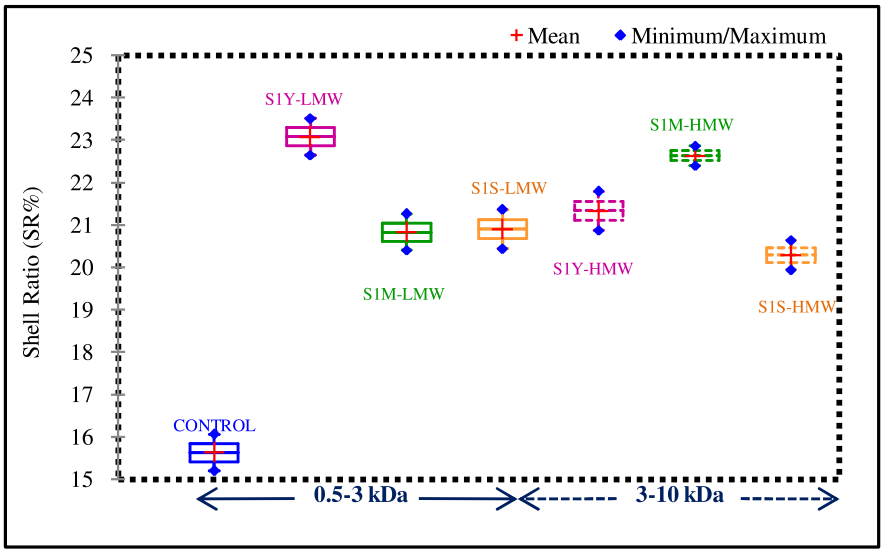


Figure 4.20: Shell ratio under S1 peptide treatment and respective control set

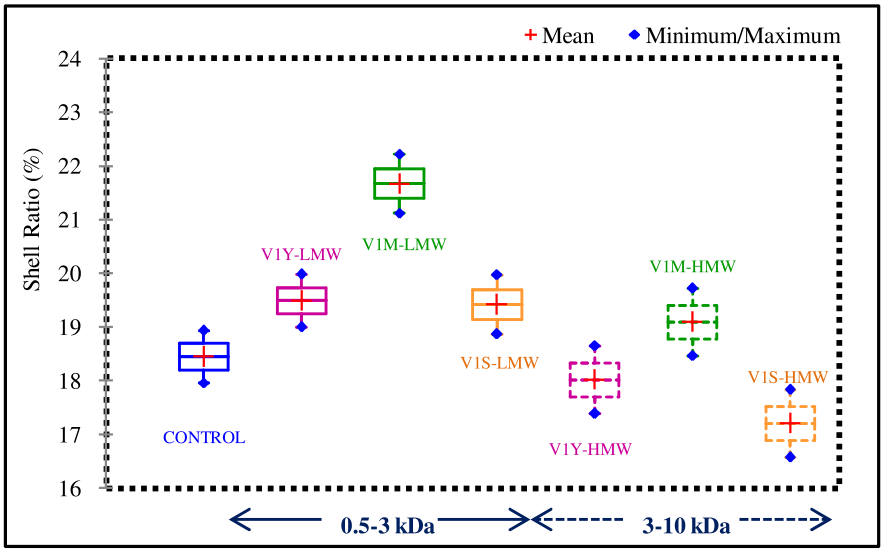


Figure 4.21: Shell ratio under V1 peptide treatment and respective control set

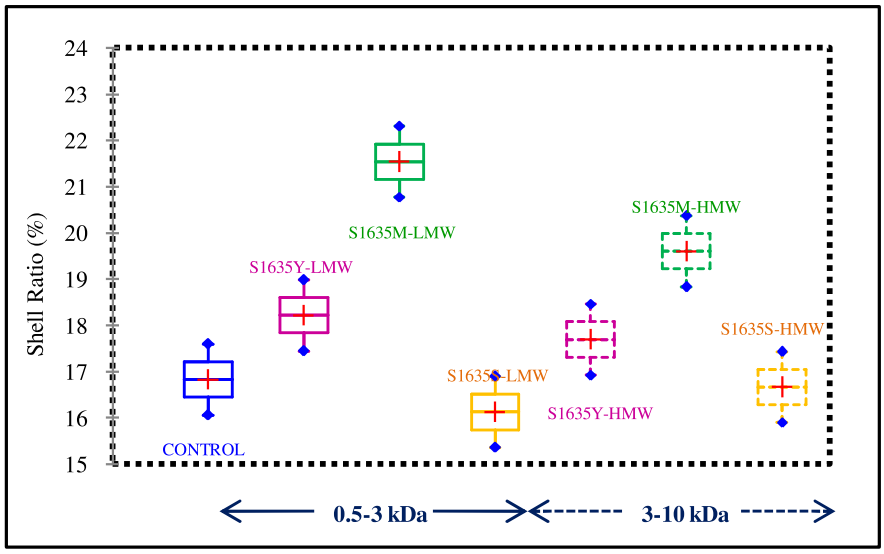


Figure 4.22: Shell ratio under S1635 peptide treatment and respective control set

Abb. Used: Y, M & S: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

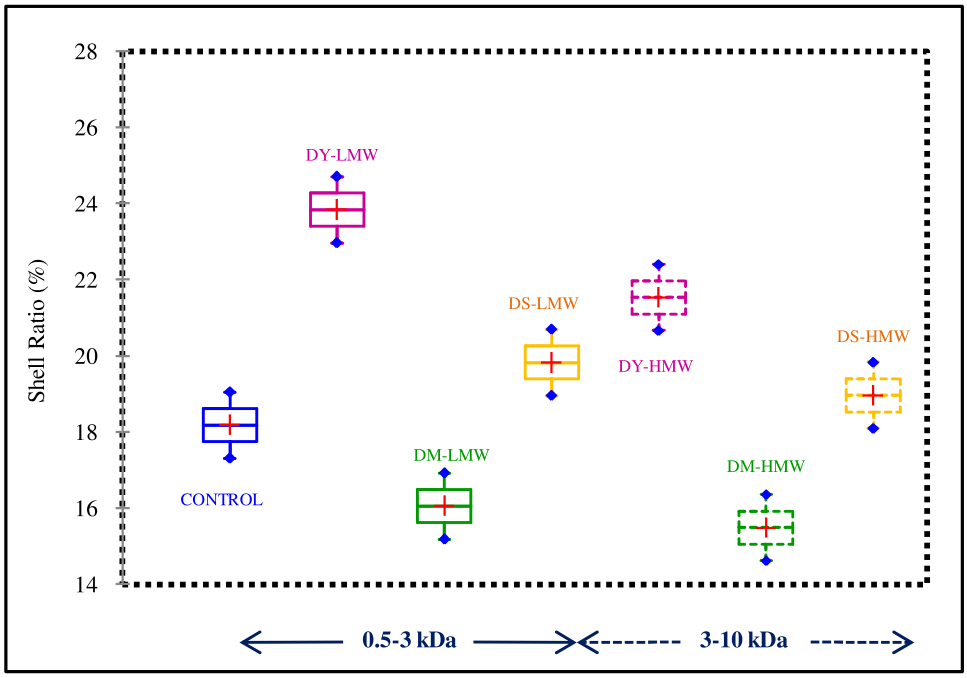


Figure 4.23: Shell ratio under Dudhiya peptide treatment and respective control set

Abb. Used: Y, M & S: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

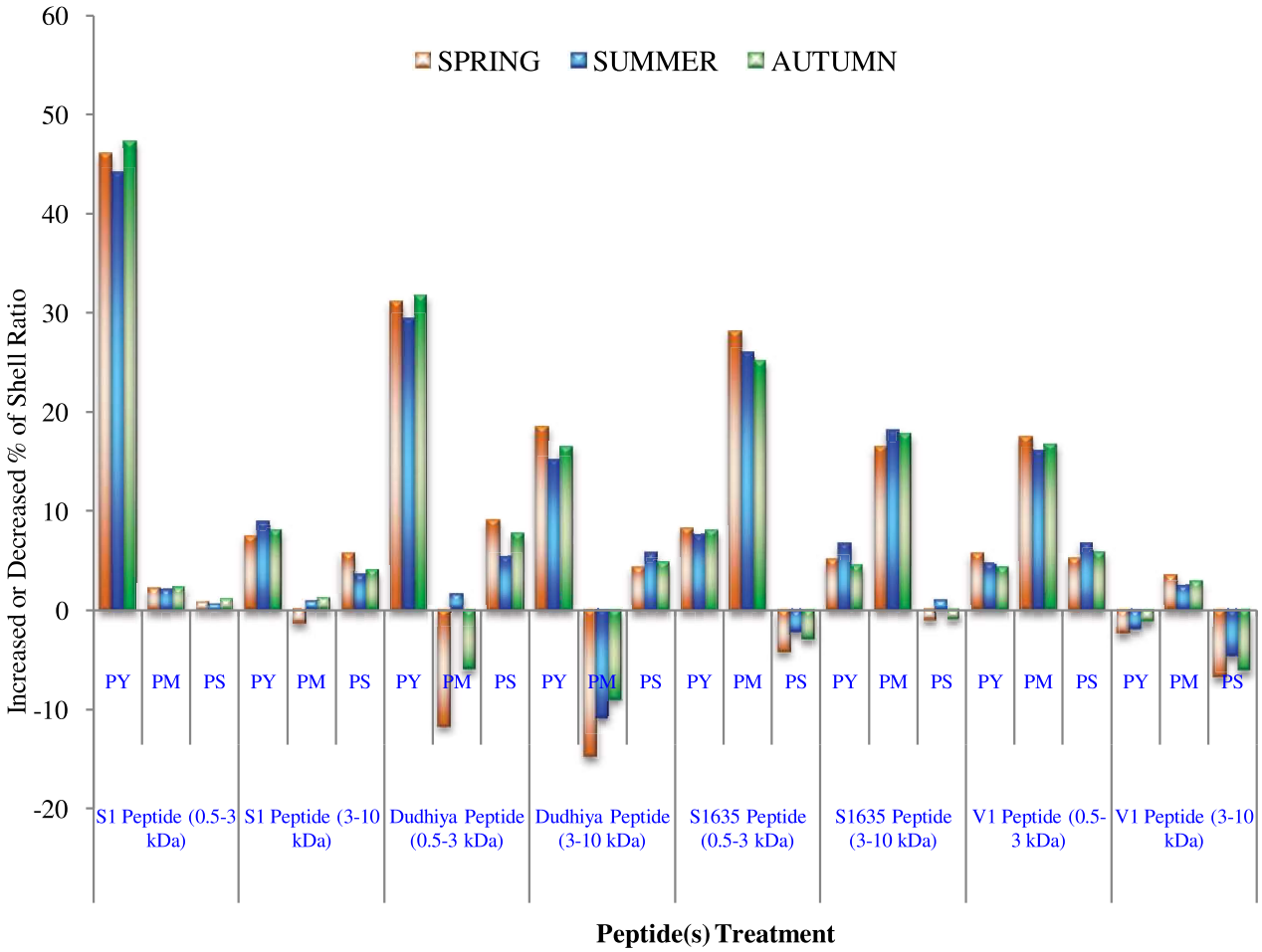
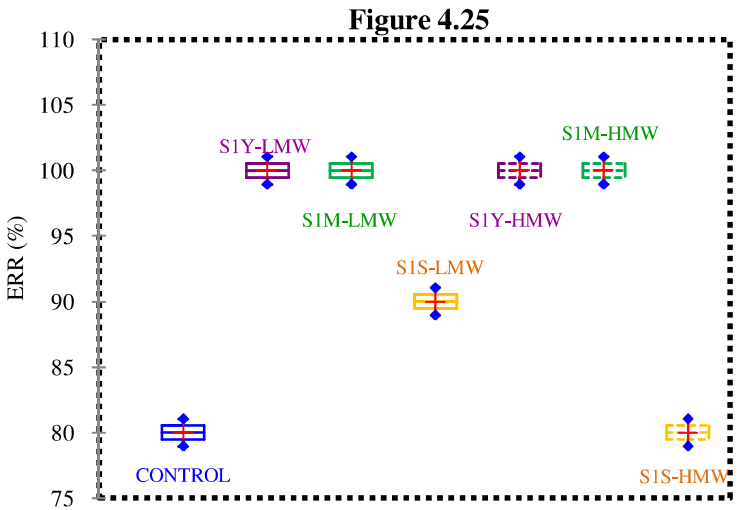
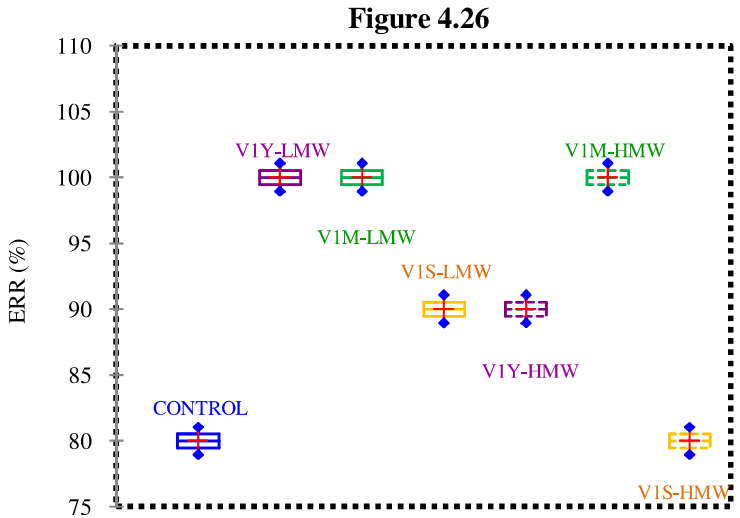
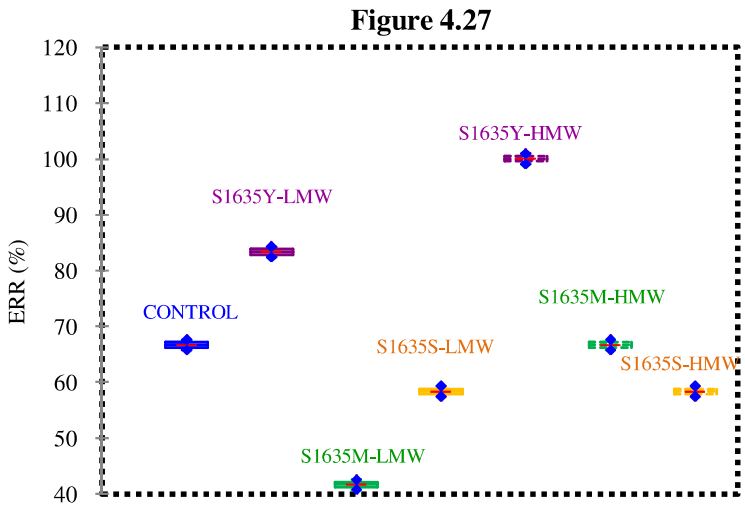


Figure 4.24: Increase or decrease (%) of shell ratio over control under peptides treatment







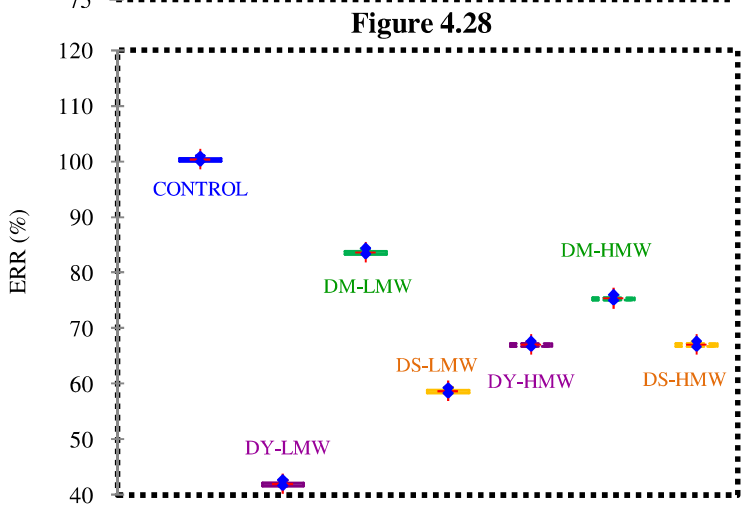


Figure 4.25-4.28: Effective Rearing Rate (%) under S1 (Figure 4.25); V1 (Figure 4.26); S1635 (Figure 4.27); and Dudhiya (Figure 4.28) peptides treatment respectively

Abb. Used: Y, M & S: peptide isolated from young, mature & senescence mulberry leaves; LMW: low molecular weight (0.5-3 kDa); HMW: high molecular weight peptides (3-10 kDa).

Table 4.1: Two-way ANOVA analysis of growth rate and economical attributes of silkworm rearing under peptide(s) treatment with seasonal variation

Source of Variation	*df*	*F crit*	GR				WSC			
			SS	*MS*	*F*	*P-value*	*SS*	*MS*	*F*	*P-value*
Peptide(s) treatment	23	1.767	56723.77	2466.25	234.76	3.11E-40*	7011.31	304.84	306.89	6.99E-43*
Seasons	2	3.200	92.07	46.033	4.38	0.0181**	37.19	18.60	18.72	1.13E-06*
Error	46		483.25	10.51			45.69	0.993		
Total	71		57299.08				7094.19			

*Significant at p<0.01 , **Significant at p<0.05 level

Table 4.2: Two-way ANOVA analysis of different economical attributes of silkworm larvae under peptides treatment with seasonal variation

Source of Variation	*df*	*F crit*	WSS				SR			
			SS	*MS*	*F*	*P-value*	*SS*	*MS*	*F*	*P-value*
Peptide(s) treatment	23	1.767	37503.09	1630.57	659.48	1.79E-50*	11310.39	491.76	149.27	8.83E-36*
Seasons	2	3.200	59.28	29.64	11.99	6.448E-05*	4.94	2.47	0.750	0.478162
Error	46		113.74	2.47			151.54	3.29		
Total	71		37676.10				11466.87			

*Significant at p<0.01

Abbr. used: GR- Growth Rate; WSC- Weight of Single Cocoon; WSS- Weight of Single Shell; SR- Shell Ratio

It may be possible that, oligopeptides can help to develop a defense network in mulberry leaves under different environmental circumstances. Oligopeptide elicitors can bind with plant cell membrane and exceed into larval body through mulberry leaf and trigger the larval metabolic activity, increased the juvenile hormones biosynthesis and ultimately increased the silk production. Similarly, Nurnberger *et al.* (1994) reported that an oligopeptide of 42 kDa glycoprotein elicitor can stimulate the defense system in parsley cells and also binds with plant cell membrane. On the other hand, Unni *et al.* (2008) reported that the peptides can stimulate the biosynthesis of juvenile hormone.

It was reported that the dietary protein on mulberry leaves is about 30% (Hamano and Okano, 1989). Though it was earlier observed that mulberry leaves alone does not always complement the entire dietary protein requirement of silkworm larvae, as a result of which cocoon production was affected (Takahashi, 2001). Protein supplemented mulberry leaf have significant effect on larval growth and different economical parameters of silkworm (Amala-Rani *et al.,* 2011). Application of peptides (more particularly juvenile hormone activating peptide) had optimistic effects on larval growth, cocoon weight, shell ratio and silk weight of muga silkworm: *Anthereae assama* (Saikia, 2001). Different nutrient formulation affects the cost benefit ratio in sericulture directly or indirectly.

Two-way ANOVA analysis was performed to find out the interaction of this two variable, one is the effect of peptide(s) treatment, and another is an effect of seasons on larval growth rate and different economic attributes of silkworm rearing at various seasons. Growth rate of larvae, weight of single cocoon and weight of single shell was greatly influenced by different peptide(s) treatment and also affected by seasonal changes. The effects of both variants were significant at $p< 0.01$ and 0.05 levels (Table: 4.1 and 4.2). Imperative attributes of rearing was shell ratio for determining the silk production rate, which was not affected by seasonal changes. ANOVA analysis reveals that peptides had a significant ($p<0.01$ levels) effect on shell ratio throughout all seasons.

4.4 CONCLUSION

In the present work, peptide(s) isolated from mulberry leaves have a beneficial effect on the silkworm rearing system. LMW peptide(s) supplemented with mulberry leaf have a significant role on larval growth, cocoon weight, and ultimately silk production. Elicitation with bioactive peptides helps to sustain nutrition in mulberry leaf throughout all environmental changes. Peptides when introduced directly or indirectly into larval body might influence silkworm larval growth. Application of peptides

triggered the juvenile hormone biosynthesis which ultimately influenced the growth, development and ultimately the production. From the present experiment, effective low molecular weight peptides (S1Y, S1635M, V1M and DM) were selected for peptides characterization.