DETERMINATION OF MINIMUM EFFECTIVE CONCENTRATION OF ALFALFA FOR OPTIMAL GROWTH, METABOLISM AND SILK PRODUCTION IN THE SILKWORM, *BOMBYX MORI*

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ABSTRACT

The sericultural productivity could be effectively modulated by enriching the silkworm diet with exogenous factors like alfalfa, a widely used homeo medicine. The minimum effective concentration (MEC) that promotes optimal larval growth, activates metabolism and improves silk production has been determined in *Bombyxmori, by a* step- down process starting from higher concentration (5%) to a lower concentration (1%) by running a parallel zero-dose control. The MEC for larval growth was determined by analyzing changes in the body weight and that of metabolism and silk production by assaying protein levels of the fat body, silk gland and haemolymph. The findings were meaningfully interpreted in terms of compound periodical growth rates. The study demonstrates that the MEC of alfalfa differs from tissue to tissue. While 2% alfalfa evokes optimal response in body growth and silk protein synthesis, 1% alfalfa triggers optimal protein synthesis in the fat body. Hence, 5% alfalfa in distilled water is recommended as the MEC for positive improvements in the larval growth and silk production and 1% alfalfa for modulating metabolism in the fat body of silkworm.

Keywords:*Alfalfa, Bombyxmori, Growth, Minimum effective concentration, Proteins*

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INTRODUCTION

The growth and development of silkworm depends on its nutritional status (Kanafi*et al.,* 2007). Most of the studies focused on the impact analysis of nutrients on its economic parameters. The approach in such studies included the modification of nutritional composition of silkworm diet by enriching it with the desired nutrients. In mulberry sericulture, the practice of enriching the mulberry leaves with exogenous nutrients has been viewed as balanced dietary approach for improving sericultural output (Laskar and Datta, 2000; Sanappa*et al.,* 2002; Etebari and Matindoost, 2005; Bhattacharya and Kaliwal, 2005a, 2005b, 2005c;Chakarabarty and Kaliwal, 2011; Kavitha*et al.,* 2012). Such nutritional-enrichment studies were based largely on indiscriminate application of commercial and expensive nutrients.TheapplicationofSBL Alfalfa, ahomeo-medicine and a product of barley in sericulture, has not been explored. Alfalfa is a potent energy stimulant enriched with~60 essential nutrients including vitamins (vitamin C, thiamine, niacin), minerals, amino acids and potent herbs.The present study intends to determine the minimum effective concentration (MEC) of alfalfa and to recommend that concentration for testing its efficacy on the growth and silk production in ***Bombyxmori*.**

MATERIAL AND METHODS

The present investigation was carried out on Pure Mysore x CSR_2 hybrid strain of *Bombyx mori* reared under 28°C and 85% RH as per Krishnaswami (1986). After hatching, the worms were fed with M_5 variety of mulberry leavesat 6AM, 10AM, 2PM, 6PM and 10PM, under normal 12hr light and 12hr dark conditions. After the third moult, the fourth instar larvae were divided into five batches(one control and four experimental) of 100 worms each. While the control batch was fed normally, the

Experimental batches were fed with mulberry leaves dipped in different concentrations (viz., 5%, 3%, 2% and 1%) alfalfa at their 6 PM diet. Before feeding, the alfalfa -fortified mulberry leaves were dried under cool weather conditions. The minimum effectiveconcentration(MEC) of alfalfa was determined by step-down process, starting from a higher concentration of 5%to a lower concentration of 1%along with a parallel zero-dose control (Williams, 1971,modified by Li Jan, 2005; Kavitha*et al*., 2011). The MEC of alfalfa for larval growth was determined by recording the body weight of 25 randomly selected silkworms in an electronic balance (ELICO; MODEL BL-22OH) during fourth and fifth instarstagesand the same was expressed in grams.The total protein content was estimated in 1% homogenates of silk gland (SG) and fat body (FB) and 1:9 dilutedhaemolymph (HL)in distilled water by the method of Lowry *et al.,* (1951) and the same was expressed in mg protein /gram wet weight of tissue (or) mg/ml of haemolymph. The experimental data were statistically analyzed online by usingGraphpad(www.graph.pad.com/quickcalcs/indexctm)andpercentchange(www.percent-change.com)/index/php) packages and meaningfully interpreted by computing compound periodical growth rates (CPGR)as perSivaprasad(2012).

RESULTS AND DISCUSSION

Minimum effective concentration (MEC), the lowest concentration level with a response greater than that of the zero-dose control is a powerful tool for tracing the concentration-response relationship in growth related studies (Stewart and Ruberg, 2000; Li Jan, 2005; Kavitha*et al.,* 2011; Thulasi and Sivaprasad, 2014). The present investigation involving, a step down process with four different concentrations and a zero-dose control (ZDC) has revealed that the impact of alfalfa was more pronounced at the concentration of 5% in distilled water(Tables 1and 2; Figures 1).

Alfalfa versus larval body growth

Fourth instar larvae: During the fourth instar development, the larval body weight of the control batch showed an overall growth rate (OGR) of ~257% and a compound periodical growth rate (CPGR) of 52.86% (Table.1A). When the silkworm larvae were fed with 5% alfalfa-enriched mulberry diet, the OGR was elevated by ~271% with a CPGR of 54.87%, representing a hike of ~4% over ZDC. At the concentrations of both 3% and 2 % levels, the body weight recorded an OGR of ~264.3% and a CPGR of 53.87%, representing a 2% net increase over ZDC. At 1% level, the larval weight showed an OGR of ~257% and a CPGR of 52.86%, representing same growth level as that of the control (Table 1A and Fig. 1A).

Fifth Instar Larvae: During fifth instar development, the larval body weight of the control batch showed an OGR of ~270% and a CPGR of 24.38% (Table 1B). When fed with 5% alfalfa-enriched mulberry diet, the OGR of larval body was elevated by ~309% with a CPGR of 25.53%, representing an overall increase of 6% over ZDC. At 3% level, the body weight recorded an OGR of ~284% and a CPGR of 25.15%, representing an increase of 4% over ZDC. At 2 and 1% levels, the body weight recorded an OGR of ~272% and a CPGR of 24.47%, showing a marginal elevation (~0.47%) over ZDC(Table 1B and Fig.1A).

The CPGRs in the larval body weight under treatment conditions ranged from 52.86% to 54.87% during fourth instar and ~24.47% to ~25.53% during fifth instar and it seems to vary as a function of concentration of alfalfa. Clearly, 5% alfalfa caused greater elevation in the larval growth during fourth instar (~54.87%) and fifth (~25.53%) instar stages compared those of ZDC. 5% alfalfa enhanced the larval growth rate by ~4% in fourth instar and by ~6% in fifth instar (Table. 1A). At other concentrations, alfalfa showed either negative or positive impact. For instance, at 1% concentration, it retained the same growth rate (0% change) in fourth instar and elevated it by 0.47% in fifth instar (Table 1A and 1B).Thus, the potency of alfalfa has been demonstrated at 5% concentration level (i.e., 5ml of alfalfa in 100ml /100 worms) and this is strongly recommended as the minimum effective concentration (MEC) for promoting larval growth in *B. mori*. The study also highlights another point of significance. The growth rates show instar specificity in the silkworm, being faster in the fourth instar and slower in fifth instar. Significantly, the control batch recorded a CPGR of ~52.86% in the fourth instar and ~24.38% in the fifth instar (Table 1A). When the larvae were fed with 5% alfalfa the CPGR levels were elevated by ~54.87% in the fourth instar and by ~25.53% in the fifth instar. Clearly, the alfalfa positively reinforced the day- to- day larval growth rate by ~2.01 percentile points (54.87 - 52.86%) in fourth instar and by just 1.15 percentile points (25.53 - 24.38) in fifth instar (Table.1A and 1B). Thus, alfalfa accelerates larval growth rate specifically during fourth instar development and compared to that during fifth instar. Through the reason for the growth stimulating effect is not in sight, it is attributable to the phago-stimulant activity of vitamin C, it contains (Sarker*et al.,* 1995).

Alfalfa versus tissue proteins

The analysis of tissue-based protein profiles under treatment conditions could be considered as an index of metabolic rate in ***B.mori*** (Kavitha*et al.,* 2011). Obviously, the MEC of alfalfa that could positively impact the larval metabolism in the silkworm could be determined by analyzing the protein profiles of SG, FB and HL.

Silk Gland Proteins (SGP): The silk gland is the major site of silk proteins synthesis. Apart from two silk proteins (fibroin and sericin), it synthesizes and stores 91 other proteins involved in metabolism, immunity, heat-shock mechanism, cytoskeleton formation, protease inhibition, transport and transcription (Nirmala*et al.,* 2001; Jin*et al.,* 2004; Takasu*et al.,* 2005 ;Kyung *et al.,* 2006; Zang*et al.,*2006; Hou*et al.,* 2007a). In general, the SGP levels showedan upward trend during fifth instar development. Theirprofiles recorded ~317% OGR and ~104.16% CPGR in the control batch, ~449% OGR and 134.30% CPGR at 5% alfalfa level, ~380.6% OGR and 119.23% CPGR at 3% level, ~367% OGR and 116.10% CPGR at 2% level and ~285% OGR and 96.12% CPGR at 1% concentration level of alfalfa (Table 1C). Compared to ZDC, the protein levels were deviated by +32%, +15%, +12% and -8% at the alfalfa concentrations of 5%, 3%, 2% and 1% respectively (Fig.1B).Thoughthe reasons for their changes have not been examined in the current study, the positive impact of alfalfa on the protein content of silk gland is attributable to its mineral and vitamin composition (Etebari and Matindoost, 2004; Thulasi and Sivaprasad, 2013; Thulasi and Sivaprasad, 2014).

Fat Body Proteins (FBP): The insect fat body represents the chief site of protein synthesis and amino acid metabolism. Functionally, it is analogous to the liver and adipose tissue of higher vertebrates (Scott *et al.,* 2004). In silkworm, the FB synthesizes and stores over 177 proteins implicated in larval growth and development (Hak*et al.,* 2005; Hou*et al.,* 2007 b). The present study highlights the positive impact of alfalfa on FBP at its MEC (i.e., 5%) level.Thegrowth rates reflected ~57% OGR and 25.17% CPGR in FBP levels of the control batch. When the larvae were fed with alfalfa-enriched mulberry leaves, the corresponding OGRs and CPGRs were 76.7 and 32.94% at 5% level, 68and 29.63% at 3% level, 64 and 28.05% at 2% level and ~64 and 27.96% at 1% level of alfalfa respectively (Table 1C). When compared with ZDC, the protein levels were enhanced by 13% at 3% level, 7% at 3% level, and 4.7% at 2% level and by 4.5% at 1% level of alfalfa (Fig.1C). Thus, under its influence, the CPGR of FBP levels were elevated by 7.77 percentile points (32.94-25.17), resulting in a 13% hike over ZDC (Fig:1C;Table:1). Probably, the mineral salts of potassium, zinc present in alfalfa could have significantly contributed to the rise in the levels of FBP (Bhattacharya and Kaliwal, 2005a).

Haemolymph Proteins (HLP): The HL plays a dual role of transportation and storage. It stores and transports about 241 to 298 proteins involved in metamorphosis, ecdysis, chitin and haemocyte formation, growth of silk gland and reproductive organs (Lix*et al*., 2006; Chai *et al.,* 2008; Nakahara *et al.,* 2009). The total proteins levels in the circulating medium of haemolymph were elevated by ~31.8% during fifth instar development and recorded a CPGR of 14.81% during the same period. Under the influence of alfalfa, the HLP levels recorded 44.5% OGR and 20.23% CPGR at 5% level, 36.2% OGR and 19.97% CPGR at 3% level, 43.9% OGR and 18.71% CPGR at 2% level and 35% OGR and 16.19% CPGR at 1% level (Table 1E). Compared with ZDC, the HLP levels of experimental batches showed an increase of ~10% at 5% level, ~9% at 3% level, ~7% at 2% level and a low hike of ~2% at 1% level of alfalfa (Fig. 1D). The HLP levels are potentially modulated by 5% alfalfa, like those of SG and FB. At this concentration, the CPGR of HLP was elevated maximally by 5.42 additional percentile points (20.23 - 14.81) resulting in an overall hike of ~10% over the ZDC during fifth instar development. It is likely that the alfalfa-enriched mulberry leaves significantly altered the biochemical composition of HL and thereby contributed to the somatic growth of the larval body during metamorphosis. One such possibility is changes in the levels of bombyxin hormone whose role has been demonstrated in silkworm growth and metabolism (Satakeet *al.,* 2000; Nijhout and Grunert, 2002; Etebari and Matindoost, 2004).

The analysis of CPGRs of protein profiles of these three tissues during fifth instar has revealed that 5% alfalfa caused a steady positive growth rate of ~134.30% in SG (Table1C), ~32.94% in FB (Table 1D) and ~20.23% in HL (Table 1E). This culminated in an overall increase in the levels of proteins by ~32 % in SG, ~13% in FB and ~10% in HL. At 3% level, it caused a net positive impact of ~15% in SGP, ~7% in FBP and ~9% in HLP over ZDC. At 2% level it caused a net positive impact of 12% in SGP, ~5% in FBP and ~7% in HLP levels over the ZDC. At 1% concentration level, the alfalfa caused a net negative impact of ~8% was recorded in SGP; ~5% was recorded in FBP and ~2% in HLP levels compared to the ZDC. Though alfalfa has shown positive impact on tissue proteins at all concentrations, except at 1% level, maximal elevations in SGP, FBP and HLP levels were recorded under the concentration of 5% level only. This appears to be the minimum effective concentration for this exogenous factor.The comparative analysis of protein profiles in the three tissues indicates that proteins are not only synthesized in SG but also mobilized from the reserve pool of FB. More importantly under the influence of alfalfa, the protein reserves are mobilized from FB and transported to SG through the circulating medium of haemolymph (Kiran Kumar *et al.,* 1998).

CONCLUSIONS

SBL's alfalfa is a homeo-medicine, with its rich base of proteins, minerals, vitamins, carbohydrates and other nutrients has been recognized as an essential nutrient (Laskar and Datta, 2000). Its impact on ***B. mori*** varies as a function of its concentration and the type of tissue under study. It showed promising gains for profits in the sericulture industry at its minimum effective concentration (MEC) of 5% in distilled water. It significantly influences not only the larval growth, but also its metabolism. It has been identified as potent stimulator of protein synthesis, more particularly that of fibroin (silk) and sericin (floss). Despite its contribution to floss, the sericultural wastage, it could be recommended as a supplemental diet for silkworm due to its gainful impact on silk protein synthesis.

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Table. 1: *Effect of different concentrations (5%, 3%, 2%, 1%) of* ***Alfalfa*** *on the growth of body weight during fourth (A) and fifth instar (B) larval stages and total protein levels of silk gland (C), fat body (D) and haemolymph (E) in Bombyxmori during fifth instar larval stage.*

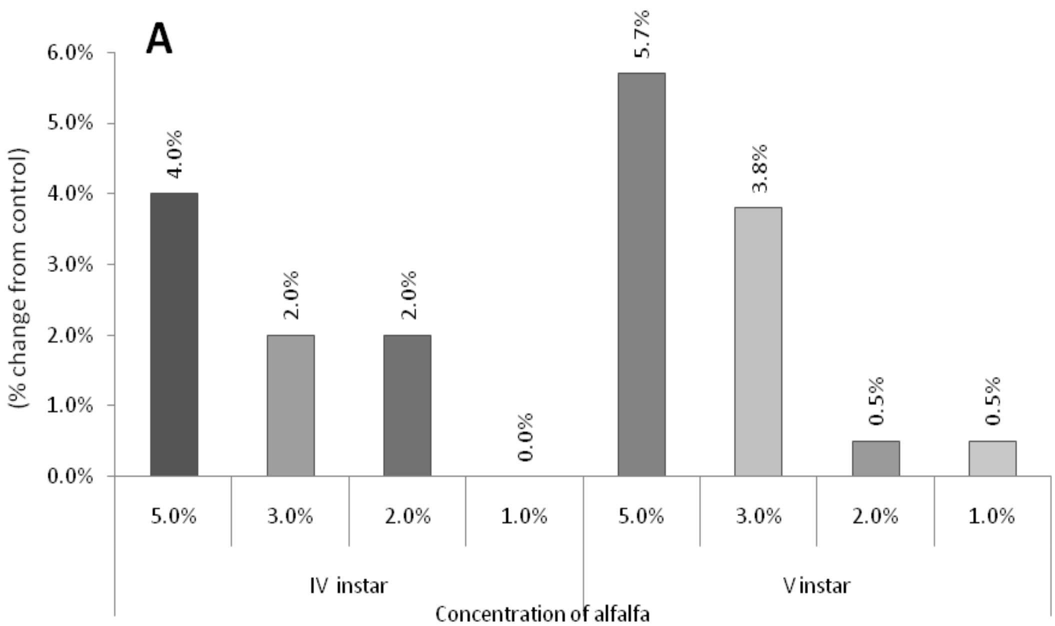
Day	Statistical tools	Control	Experimental (Concentration of alfalfa)			
			5%	3%	2%	1%
(A) Growth in Fourth Instar Larval Body Weight (mg/g)						
First (1)	Mean S.D	0.14 ±0.003	0.14 ±0.003	0.14 ±0.003	0.14 ±0.003	0.14 ±0.003
Last (4)	Mean S.D	0.50 ±0.01*	0.52 ±0.02*	0.51 ±0.02*	0.51 ±0.01*	0.50 ±0.01*
OGR (%)		(257.1)	(271.4)	(264.3)	(264.3)	(257.1)
CPGR (%)		52.86	54.87	53.87	53.87	52.86
(B) Growth in Fifth Instar Larval Body Weight (mg/g)						
First (1)	Mean S.D	0.57 ±0.01	0.57 ±0.01	0.57 ±0.01	0.57 ±0.01	0.57 ±0.01
Last (7)	Mean S.D	2.11 ±0.001*	2.23 ±0.06*	2.19 ±0.01*	2.12 ±0.001*	2.12 ±0.002*
OGR (%)		(270.2)	(308.8)	(284.2)	(272.0)	(272.0)
CPGR (%)		24.38	25.53	25.15	24.47	24.47
(C) Growth in Silk Gland Proteins (mg/g)						
First (1)	Mean S.D	19.11 ±0.07	19.11 ±0.07	19.11 ±0.07	19.11 ±0.07	19.11 ±0.07
Last (7)	Mean S.D	79.65 ±0.01*	104.91 ±1.18*	91.85 ±0.73*	89.24 ±0.47*	73.50 ±1.80*
OGR (%)		(316.8)	(449.0)	(380.6)	(367.0)	(284.6)
CPGR (%)		104.16	134.30	119.23	116.10	96.12
(D) Growth in Fat Body Proteins (mg/g)						
First (1)	Mean SD	28.79 ±1.45	28.79 ±1.45	28.79 ±1.45	28.79 ±1.45	28.79 ±1.45
Last (7)	Mean SD	45.11 ±0.83*	50.88 ±0.89*	48.38 ±0.28**	47.21 ±0.97**	47.14 ±0.28*
OGR%		(56.7)	(76.7)	(68.0)	(64.0)	(63.7)
CPGR (%)		25.17	32.94	29.63	28.05	27.96
(E) Growth in Haemolymph Proteins (mg/ml)						
First (1)	Mean SD	6.60 ±0.11	6.60 ±0.11	6.60 ±0.11	6.60 ±0.11	6.60 ±0.11
Last (7)	Mean SD	8.70 ±0.14*	9.54 ±0.04*	9.50 ±0.04*	9.30 ±0.009*	8.91 ±0.02*
OGR (%)		(31.8)	(44.5)	(36.2)	(43.9)	(35.0)
CPGR (%)		14.81	20.23	19.97	18.71	16.19

*Statistically significant; **statistically not significant

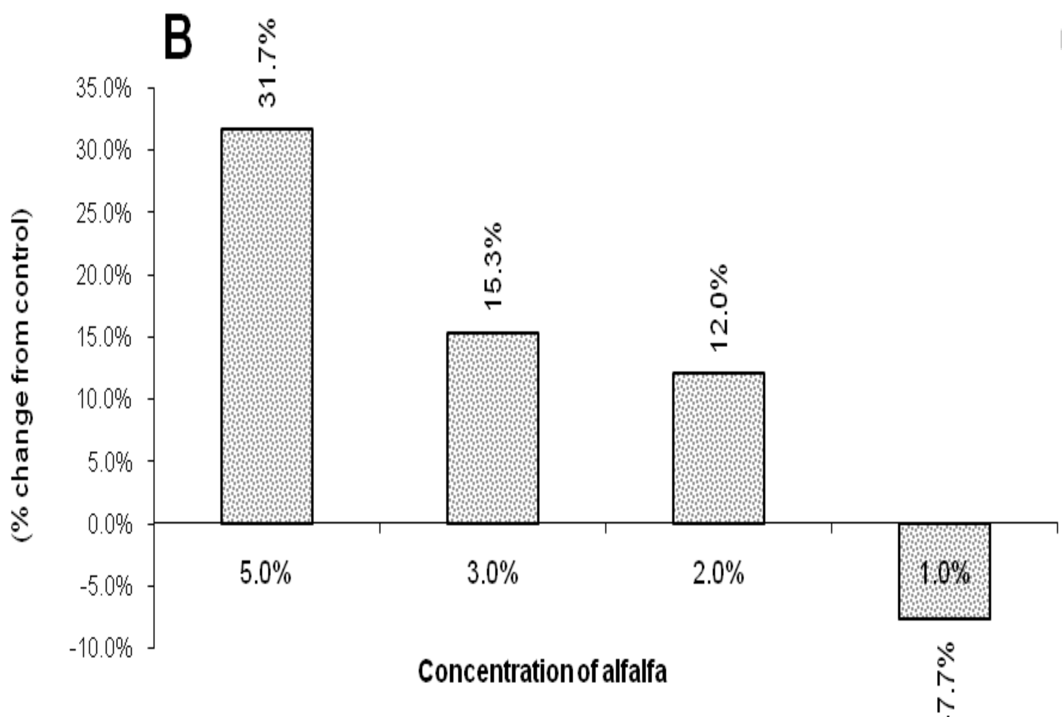
Each mean under body weight represents the average weight of 25 worms, expressed in grams. Each mean under proteins represent the mean, ± standard deviation of four individual observations, expressed in mg/g or mg/ml (P value < 0.001). The overall growth rates (OGRs) were calculated taking the control as the base value and the compound periodical growth rates (CPGR) were computed on the basis of first and last day values as per Sivaprasad, 2012.

Fig.1: *Effect of different concentrations (5%, 3%, 2%, 1%) of alfalfa on the growth of body weight during fourth and fifth instars larval stages. The values represent percent deviations from the zero-dose control.*

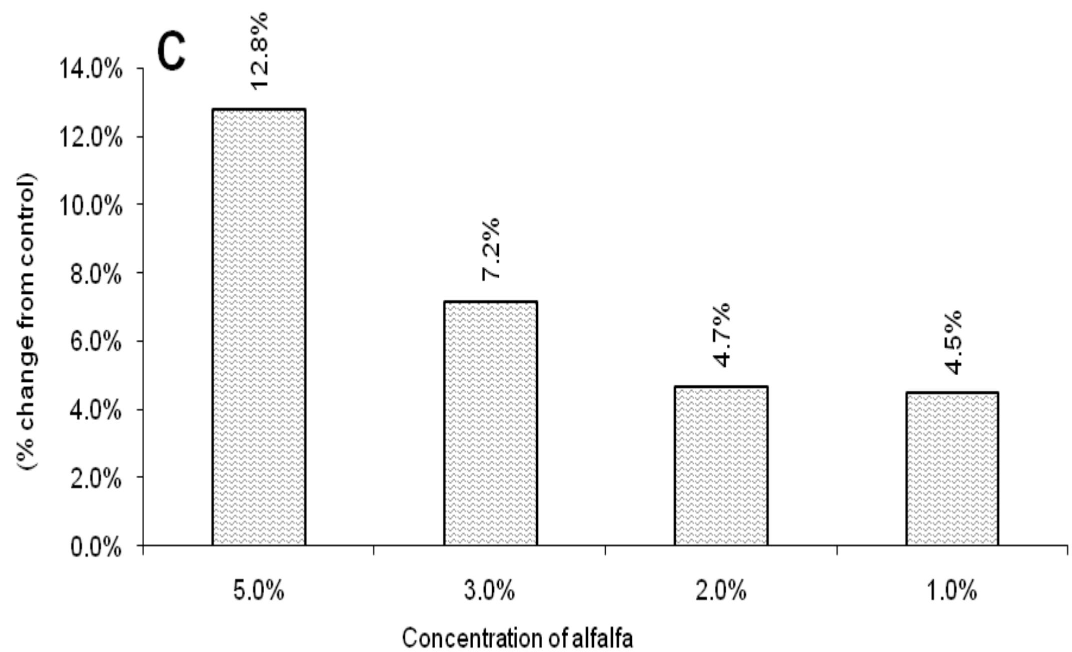
(A) Total proteins of silk gland



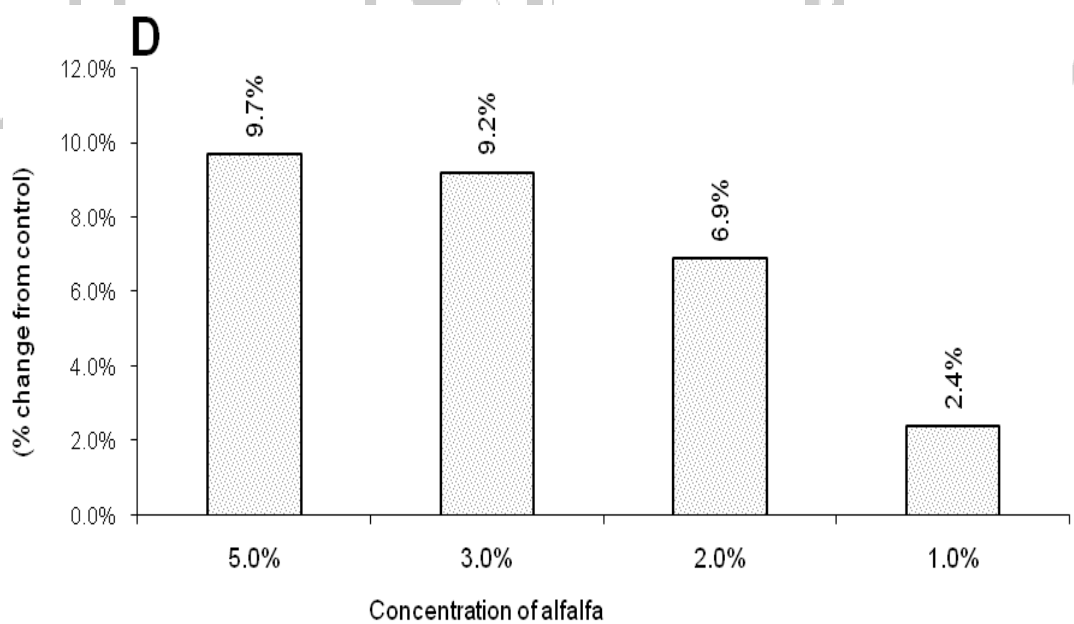
(B)Fat body



(C) Haemolymph



(D) During fifth instar larval stage in Bombyxmori



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