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Studies on the Effect of Mulberry Leaves of Lune Variety on Silkworm (*Bombyx Mori l*) Rearing in Polluted Environment of Gangetic Plains of West Bengal, India

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Abstract

Silkworm (Bombyx mori L), is a monophagous insect. It has a great economic importance for its golden natural fibre. But environmental pollution due to automobile emissions and other man made factors in the ambient air of Gangetic West Bengal has a great impact on silkworm rearing. Generally two mulberry varieties, viz, S1 and S1635 are used as food for silkworm in Gangetic West Bengal. But Lune40, a Chinese variety exhibits better results in the same macro-environment in respect of leaf fresh weight (g), leaf area (cm2), leaf yield / plant (kg) shoot yield / per plant (kg) etc. The physio-biochemical analysis of mulberry leaves of above mentioned varieties exhibit that Lune 40 variety has higher leaf quality in similar agro climate and ambient environment than S1 and S1635 variety. Rearing of silkworm with Lune 40 help silkworm for their growth and higher vigor in final instar. Silkworm's intake less amount of Lune 40 leaves to produce more cocoon weight and filament length than the other two. Silkworms reared with Lune40 are less susceptible to diseases. So it could be propagated for better crop harvest in the polluted environment of Gangetic West Bengal by replacing S1 and S1635 variety.

Key words: *Silkworm, Mulberry leaves, poly nuclear hydrocarbons (PAHs), Lune40, S1 and S1635 mulberry variety, ambient environment, rearing of silkworm.*

Introduction

Silkworm, a lepidopteron insect has a great economic importance for its golden natural fibers[1].The quality and quantity of mulberry leaves play an important role in growth and development of silkworm (*Bombyx mori* L), particularly during adult larval stage ,which in turn influence the expression of cocoon productivity traits. This also leads to the increase in body size and dry weight of cellular mass which are dependent on the rate of metabolism, absorption of nutrients, and stage of development[2]. In recent times, there has been a remarkable improvement in the production of silk by domestic silkworms. In order to make this progress even more effective, it is necessary to study the physiological characteristics of silkworm (*Bombyx mori L*) and mulberry *(Morus* sp) as the mulberry leaf protein, which is taken as food by the silkworms, gets effectively converted into silk protein[3s]. So, it is necessary to improve the physiological conditions of the silkworms in order to have a higher production of the cocoons. But environmental pollution is a constraint against a full-fledged improvement of the silkworms. Even though, the mulberry leaves, the only food for silkworms are polluted by different air pollutants, the effect of pollution on different mulberry varieties in the same environment is different. From this

study, it is experimentally proved that, the Lune 40, a Chinese mulberry variety has shown better leaf quality than S1 and S1635 and silkworm feeding on Lune 40, produce better quality cocoons and silk in the same environment.

Outline of the study: The study included the leaf production of S1 S1635 and Lune 40 varieties of mulberry plants and rearing of silkworm (*Bombyx mori* L) at the same macro-environment of Sericulture training institute (STI), Berhampore, West Bengal, situated very near to the National Highway-34. The silkworm rearing was conducted in four different seasons from October to April (dry spell), utilizing mulberry leaves of S1, S1635 and Lune40 varieties. Mulberry leaves of above varieties were collected separately and their physio-biochemical properties were studied thoroughly to identify Polynuclear aromatic hydrocarbons (PAHs) in their extract, which acts as a marker of automobile pollution. Detailed record of the worms, which fed on the mulberry leaves of abovementioned varieties, were kept along with the characterization of the quality of their cocoon production. Air samples were collected mostly at an ambient condition to study the particulate density of ambient air. Further, the air samples were analysed for their characters in respect to the carbon composition and Polynuclear Aromatic Hydrocarbon (PAH) content. The protein level of the different varieties of mulberry leaves was also measured.

Material and Methods

Respiratory dust sampler of M/S Envirotech, Glass fiber filter paper (Grade-GF/A) of Whatman make ,all materials used in mulberry cultivation and silkworm rearing, small plastic container, compound microscope, spectrophotometer, centrifuge machine, chemicals etc were used for the experiment.

Sampling of Ambient Air: The particulate matter in the ambient air was sampled employing a respiratory dust sampler of M/S Envirotech. Ambient air laden with suspended particles enters the system through inlet pipe under suction. As the air passes through the cyclone coarse, non- respirable dust particles (particulate matter of diameter more than 10µm) are separated from the air stream by centrifugal force acting on the solid particles. These separated particles fall through the cyclone´s conical hopper and are collected in the sampling bottle fitted at its bottom. The fine dust forming the respirable fraction of TSP (Total Suspended Particles) passes through the cyclone and is carried by the air stream through the filter paper clamped between the top cover fitter adapter assemblies. The respirable dust (RSP) is retained by the filter paper, while the carried air is exhausted from the system through the blower.

Glass fiber filter papers (Grade-GF/A) of Whatman make were employed and kept in desiccators for conditioning for at least 24 hours. After conditioning the filter papers were taken out and weighed on an electronic balance. These filter papers were then setup on the particular positions of the air sampler. The sampling was carried out for about 12 hours. When sampling was completed the filter papers were taken out, folded and placed in the respective envelope. The time of introduction, removal time, duration of sampling, total volume of air sucked and the average airflow rate all these information's were recorded. Back to the laboratory, the filter papers were placed in desiccators and allowed to be conditioned overnight. After complete conditioning the filter paper were weighed again and the difference in weight was noted.

Total organic/Inorganic Carbon Analysis: Organic and Inorganic forms of carbon content in the sampled air particulates were analyzed by the Shimadzu total organic carbon analyser model toc

vcph according to the methodology prescribed by the manufacturer. The analysis was done from filter paper cut outs and the result was expressed in terms of amount of organic and inorganic carbon per unit mass of the particulate sample collected.

Polynuclear Aromatic Hydrocarbon Analysis: Altogether sixteen (16) Polynuclear Aromatic Hydrocarbons (PAH) has been identified by the USEPA to be present in the air which predominantly originates from automobile engines burning liquid fossil fuel. The mulberry leaves as well as the filter papers with particulate matters were collected. The method of analysis followed was Method 8310 of the USEPA. The instrument employed was the waters make High Performance Liquid Chromatograph system equipped with two M1 solvent delivery pumps and M2487 Dual Wavelength Absorbance Detector. The detection of the PAHs was made at an absorbance of 254nm. The standards for estimations of PAHs were purchased from Sigma Chemicals Company, USA and solutions of the standards were prepared as per the instructions of the manufacturer.

Plantation of mulberry: Well rooted saplings of six months of three improved mulberry varieties, viz, S1, S1635 and Lune40 were planted under 60cmx60cm spacing between plant to plant and row to row in randomized block design (RBD) with four replications after monsoon[4]. Data of various aspects like leaf fresh weight (g), leaf dry wt (g), leaf area (cm^2), specific leaf weight (g/m^2), leaf yield /plant (kg), shoot yield /plant (kg), moisture content (%), and moisture retention capacity (MRC) up to six hours, were recorded on 15 plants/ replication of each mulberry genotype.

Total soluble protein was determined in fresh leaves as described by Lowry et al[5]. Total soluble sugar, chlorophyll and phenol content were measured as per Morris[6], Arnon[7], Bray and Thorp[8] respectively. All the biochemical constituents were determined in triplicate and repeated twice in fresh 4-5 leaves of mulberry by leaving the top glossy leaves on 60th day after pruning.

Silkworm Rearing: The silkworm of multivoltine race (Nistari-M) were reared as per the specification and schedule maintained in FAO Manual, Volume – II with mulberry leaves of S1, S1635 and Lune40 varieties separately and named as Test T1 (rearing with S1 leaves), T2 (rearing with S1635 leaves) and T3 (rearing with Lune 40 leaves). Each test group was subdivided into three replications, viz, R1, R2 and R3. After third instars of silkworm rearing, 600 (six hundred) worms for each test batch (200 in each replications) were kept in every crop for late age rearing. Some amount of mulberry leaves were used for feeding worms of each test batch.

Statistical Analysis: The observations of a factorial design can be described by the following model.

Yijk=μ+ αi+ βj+ (αβ)ij+ εijk (i=1,2,3; J=1,2,3,4; K=1,2,3) Where yijk be the observed data corresponding to i'th treatment of j th block of k th replications. (μ=overall population mean, α i = effect due to i'th treatment, βj = effect due to j'th block, (αβ)ij=effect of interaction between α i and βj)

The treatment effects are defined as deviation from overall mean so that

$$\sum_{i=1}^{3}\alpha_i = 0,\ \sum_{j=1}^{4}\beta_j = 0,\ \sum_{j=1}^{4}(\alpha\beta)_{ij} = \sum_{i=1}^{3}(\alpha\beta)_{ij} = 0$$

Now we want to test before comparing the treatment effect is that if treatment and block effects interact that is to test:H0: $(\alpha\beta)_{ij} = 0$ for all i,j

H1: $(\alpha\beta)_{ij} \neq 0$ for some i, j.

So, we can divide the total sum of squares into 4 parts.

SST=SSA+SSB+SSAB+SSE, where SST is total sum of square, SSA is deviation due to treatment effect SSB= is deviation due to block effect , SSAB

is deviation due to interaction between treatment effect and block effect, SSE is error sum of square with degrees of freedom 3*4*4-1,2,3,2*3,3*4*2. Mean square can be obtained by dividing each sum of square by their corresponding df.

The test statistic for testing the hypothesis is MSAB/MSE (=F)and it follows F distribution with df (6,24). So, if the observed value is less than the corresponding F value from the table , then the null hypothesis will be accepted.

After seeing that, there are no interactions term present in the data. Now we have to test whether Lune 40 is performing well than S1, S1635 in polluted area.
So here our null hypothesis is:
H0: $\alpha_3 > \alpha_2$ vs. H1: $\alpha_3 \leq \alpha_2$
And H0: $\alpha_3 > \alpha_1$ vs H1: $\alpha_3 \leq \alpha_1$
As, now the model becomes
Yijk=μ+ αi+ βj + εijk
(i=1,2,3; J=1,2,3,4; K=1,2,3)
So an unbiased estimate of αi can be given by
$\hat{\alpha}_i = \bar{y}_{i..} - \bar{y}_{...}$.

$y_{i_1,j,\bullet} - y_{i_2,j,\bullet} \overset{iid}{\sim}$ Normal $(\alpha_{i_1} - \alpha_{i_2}, \frac{2\sigma^2}{n})$ for all i1,i2 =1,2,3; J=1,2,3,4; $x_j = y_{i_1,j,\bullet} - y_{i_2,j,\bullet}$ And so the complete sufficient statistic for this distribution is $(\sum_{j=1}^{4} x_j , \sum_{j=1}^{4} x_j^2)$. As testing the null hypothesis, the UMPU (uniformly most powerful unbiased)test depends on the the complete sufficient statistic , the test is of the form

$$\phi(\underset{\sim}{x}) = 1 \quad \text{if } \sum_{i=1}^{n} X_i < c$$

[where c is dependent on $\sum_{i=1}^{n} X_i^2$) =0

Here we take the level of significance α=0.05, we can evaluate the value of c_0 from the equation $E_{H_0}(\phi(\underset{\sim}{x})) = \alpha$

As, $T(\underset{\sim}{x}) = (\frac{\sqrt{b-1}}{\sqrt{b}}) \quad \frac{\sum_{i=1}^{n} X_i}{\sqrt{\sum_{i=1}^{n} (X_i - \bar{X})^2}}$ follows t distribution with parameter b-1(=3 here)
Where c_0 = the α th quantile of t distribution with parameter 3.

Now we apply this theory on the given data set on 6 different characteristics .Firstly we want to show that there is no interaction effect.For the 6 observed dataset the corresponding test statistic F (defined earlier) value are calculated (table 9) and each of them is less than the upper 5% quantile of F' distribution function with parameter (6,24) (which is equal to 3.84) So, the null hypothesis under level of significance 0.05 is accepted and there is no interaction effect.

Now we have to show that the performance of Lun40 is better than the other 2 in polluted area.
So we have to test whether for 6 characteristics (except for characteristics 2,3) the corresponding mean (treatment effect) of Lune40 is greater than the others while in case of characteristics 2,3 the opposite and the corresponding test will change accordingly.

So, now the value of test statistic $(T(\underset{\sim}{x}) = (\frac{\sqrt{b-1}}{\sqrt{b}}) \frac{\sum_{i=1}^{n} X_i}{\sqrt{\sum_{i=1}^{n} (X_i - \bar{X})^2}})$ is calculated and compared to lower 5% quantile of t distribution function with df 3(=-3.18). And they are greater than -3.18. So the Null hypothesis is accepted that means Lune40 is better than the other two in polluted zone.

Results and Discussion

Table 1 provides the data for the particulate matter collected from sampling of ambient air at the Sericulture Training Institute Campus, Berhampore. The particulate concentration per unit volume of air was found to vary between 110 μg/m^3 and 95 μg/m^3 in the year 2005 and 150.6 μg/m^3 and 121.7 μg/m^3 in the year 2009. It also indicates the higher percentage of total carbon and organic carbon. Table 2 clearly indicates the percentage of different PAH (Polynuclear Aromatic Hydrocarbons) species associated with the particulates collected at STI Campus. The total concentration of PAHs in the sample was 8.8μg/m^3. In the year 2005, only seven types of PAHs out of sixteen types were isolated. But in the year 2009, all the sixteen types of PAHs were isolated. The physio-biochemical analysis of mulberry leaves of above mentioned varieties (table3) exhibited that Lune 40 variety have higher leaf quality in similar agro-climatic conditions and ambient environment. The ANOVA of data on different morphological characters [such as total protein (table 4), total sugar (table 5), chlorophyll content (table 6) and phenol content (table 7)] revealed the highly significant result that the leaf quality of Lune 40 variety is superior than other two varieties. Moreover, the ANOVA test has been performed with the help of parametric processes. Table 8 shows different parametric comparison between the three varieties. Except the Parameter 7, i.e., moisture retention capacity of three mulberry varieties, all other characters are significant in Lune 40. The f-values of almost all the four parameters (table 9) implicated that there were significant differences at about a level of 1%.

Characterization of the particulates collected from the ambient air, for the carbon content analysis clearly establishes overwhelming presence of carbon of organic nature in the location of experiment. This phenomenon is fairly consistent since almost same observation repeated itself after four clear years (first,in the year 2005 and then 2009). The data represented in table 2, shows the presence of 8.8μg/m^3 of total PAHs in the RSPM of experiment area (2009), which is considerably high in comparison with the WHO guideline standard of 1μg/m^3. Thus, our finding of a total PAH concentration of 8.8 μg/m^3 may be termed as "considerable" and establishes the presence of sufficient organic carbons[9] in the ambient air of the location of experiment that after deposition on any surface could cause toxicity to it in case the surface is that of a living being from plant or the animal kingdom. So, in an established polluted area, three varieties of mulberry plants were cultivated simultaneously in the same plot providing similar macro climatic conditions. After a thorough analysis of different characteristics of S1,S1635 and Lune40 strains/varieties and corresponding rearing characteristics, the leaves of Lune40 variety were found to be more effective in enhancing larval weight and SR% of *Bombyx mori L than* S1 and S1635(table 9 and 10). Comparatively low moisture retention capacity of Lune40 perhaps creates a suitable environment for the digestion and absorption of more proteins from the said mulberry leaves by the silkworm and helps silkworms for their growth in final instars[10]. Leaf yield per plant of Lune 40 is higher than that of the other two varieties. The total soluble protein (36.18mg g-1fw) and total soluble sugar (45.33mg g-1fw)[11] are also almost equal with S1635. The higher sugar content in mulberry leaves results in increase rate of digestion [12]. The gradual increase in the concentration of total sugar from mulberry leaves to the haemolymph of silkworm helps the worm get energetic in the final days of fifth instars[13]. So, higher energy helps the worm to release silk filaments more easily through its spinnerette and also helps to lay maximum numbers of eggs. Silkworms take fewer amounts of Lune 40 leaves to yield 1 kg cocoon. It means leaf: cocoon ratio is less in Lune40 (lower leaf: cocoon ratio indicates superior result). The S.R percentage of cocoon is also higher which were produced from Lune 40 leaves.

Conclusion

The experimental observations clearly indicates that the total chlorophyll contents (2.58 mg g-1fw) was higher in the leaves of Lune 40 than S1 (1.65mg g-1 f w) and S1635 (2.11mg g-1 f w) in the same eco-climate which have better influence in producing sugar (figure.1). Thus, the said Lune40 may be propagated /multiplied for better crop return in polluted zones of Gangetic West Bengal. There is a scope of further studies to observe silk qualities by applying the said mulberry varieties to the different types of mulberry silkworms.

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Table-1: Concentration of respirable suspended particulate matter (RSPM) in ambient air of Behrampore along with the total and organic carbon contents in the particulates. Data were collected during winter of years 2005 and 2009 respectively. 12 hrs day is 1200 hrs to 2400 hrs and 12 hrs night is 0000hrs to 1200 hrs.

Place	Duration	RSPM (mg/m3)		Wt% of total carbon in RSPM		Wt% of organic carbon in RSPM	
		2005	2009	2005	2009	2005	2009
Behrampore	12 hrs day	110.5	150.6	49.42	59.68	45.55	55.92
Behrampore	12 hrs night	95.7	121.7	64.6	60.74	59.35	56.55

Table-2: Polynuclear Aromatic Hydrocarbon concentration in the RSPM fraction of the ambient air sampled in Behrampore during winter of years 2005 and 2009. Day and night samples were analyzed separately in 2005, while the total sample, collected during 24 hours, were subjected to extraction and estimation in year 2009.

PAH Component	Year 2005		Year 2009
	12 hrs day	12 hrs night	(Day and night put together) expressed in ng/m3)
Naphthalene	Absent	Absent	BDL
Acenaphthylene	Absent	Absent	BDL
Acenaphthene	Absent	Absent	BDL
Fluorene	Absent	Absent	BDL
Phenanthrene	Absent	Absent	BDL
Anthracene	Present	Present	BDL
Fluoranthene	Absent	Present	2.5
Pyrene	Absent	Absent	1.1
Benzo(a)anthracene	Present	Present	0.7
Chrysene	Absent	Present	BDL
Benzo(b)fluoranthene	Absent	Absent	BDL
Benzo(k)fluoranthene	Present	Present	0.4
Benzo(a)pyrene	Present	Present	1.7
Dibenzo(ah)anthracene	Absent	Absent	BDL
Benzo(ghi)perylene	Present	Present	2.4
Indeno(1,2,3-cd)pyrene	Absent	Present	BDL
TOTAL PAH			8.8

NOTE: Parameter1=Phenol content (mg/g fw); Parameter 2=Total soluble protein (mg/g fw);Parameter3=Total soluble sugar(mg/g fw); Parameter4=Total chlorophyll(mg/g fw) of three mulberry variety.

Table-3: A comparative representation of different physio-biochemical characteristics between S1,S1635 and Lune40 variety(Mean values of four replications)

Variety	Parameter1	Parameter2	Parameter3	Parameter4
S1	0.16	30.42	41.39	1.66
S1635	0.210	37.22	46.13	2.110
Lune40	0.200	36.3	45.27	2.480
CD at5%	0.007	1.0	38.0	0.25

Table-4: Analysis of variance of total soluble protein

Source	DF	SS	MS	F	Significance	CDat5%
Variety	2	10890%	54.450	136.95	**	1.009
Error	9	358%	0.398			
Total	11	11248%				

Note: ** Result is highly significant

Table-5: Analysis of variance of total soluble sugar

Source	DF	SS	MS	F	Significance	CDat5%
Variety	2	5103%	25.516	460.98	**	0.376
Error	9	50%	0.055			
Total	11	5153%				

Note: ** Result is highly significant

Table-6: Analysis of variance of total chlorophyll

Source	DF	SS	MS	F	Significance	CDat5%
Variety	2	135%	0.675	26.08	**	0.257
Error	9	23%	0.026			
Total	11	158%				

Note:** Result is highly significant

Table-7: Analysis of variance of phenol content

Source	DF	SS	MS	F	Significance	CDat5%
Variety	2	0%	0.002	123.50	**	0.007
Error	9	0%	0.000			
Total	11	0%				

Note:** Result is highly significant

Table-8: Different parametric comparison of mean values within three mulberry varieties,viz.S1,S1635 and Lune40

Variety	Parameter1	Parameter2	Parameter3	Parameter4	Parameter5	Parameter6	Parameter7
S1	2.85	0.49	15256%	0.35	0.5	81.28	70.18
S1635	4.63	1.02	37572%	0.510	0.690	75.31	72.13
Lune40	4.75	1.03	45127%	0.540	0.720	72.54	71.52
CD	0.22	1.0	4446%	0.025	0.01	4.85	NS

Note: inParameter1= Fresh leaf weight(g);Parameter2=Leaf dry weight(g); Parameter3=Leaf area(cm2); Parameter4=Leaf yield/plant(kg); Parameter5=Shoot yield/plant(kg); Parameter6=Moisture%; Parameter7=Moisture retention capacity of three mulberry variety.

Table-9: Observed F value of different physical characters of silkworms and cocoons

Characteristic used	observed F value
Weight of ten mature larvae	1.32
Mortality due to diseases	0.57
Leaf used for 1Kg cocoon production	1.51
Good cocoons harvested out of 100 worms in each treatment	2.95
Shell ratio percentage	0.36
Filament length(metre)	0.3

Table-10: Null hypothesis of different physical characters of silkworms and cocoons following ($\alpha_1 > \alpha_3$)

Characteristic used	Null hypothesis	$\alpha_1 > \alpha_3$
1. Weight of ten mature larvae	$\alpha_3 > \alpha_2$	9.1899
	$\alpha_3 > \alpha_1$	15.5092
2. Mortality due to diseases	$\alpha_2 > \alpha_3$	5.9079
	$\alpha_1 > \alpha_3$	5.9492
3. Leaf used for 1Kg cocoon production	$\alpha_2 > \alpha_3$	7.4329
	$\alpha_1 > \alpha_3$	8.8941
4. Good cocoons harvested out of 100 worms in each treatment	$\alpha_3 > \alpha_2$	3.8422
	$\alpha_3 > \alpha_1$	2.7239
5.Shell ratio percentage	$\alpha_3 > \alpha_2$	9.3141
	$\alpha_3 > \alpha_1$	13.1034
6. Filament length(metre)	$\alpha_3 > \alpha_2$	18.263
	$\alpha_3 > \alpha_1$	12.1495

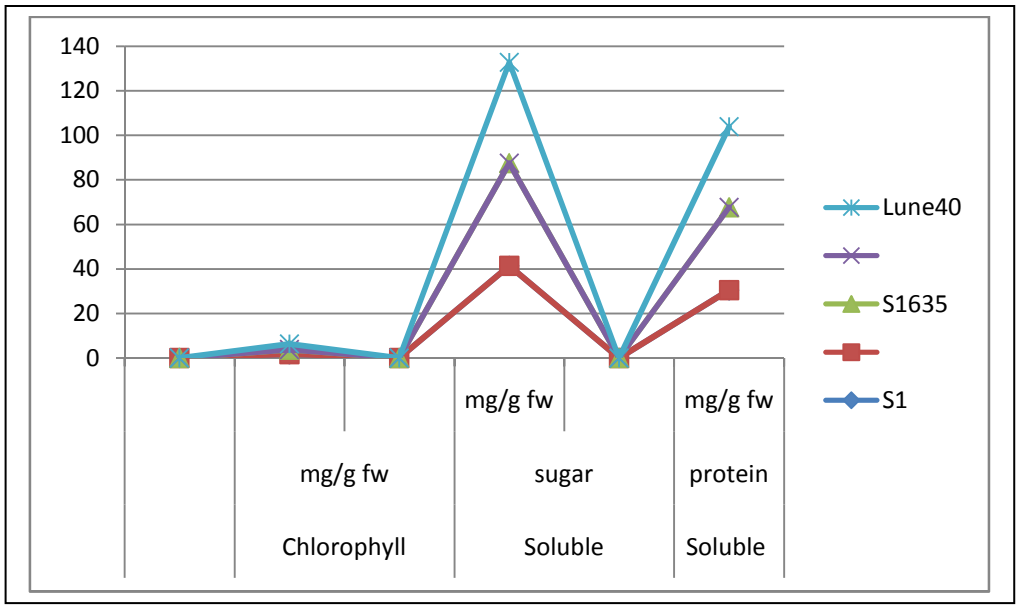

Figure-1: Comparative analysis of presence of chlorophyll, soluble sugar and soluble protein in S1,S1635 and Lune 40 variety