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EFFECT OF ENZYMATIC DEGUMMING ON THE PROPERTIES OF SILK FABRIC

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ABSTRACT

The silk fabric samples were degummed by using proteolytic enzymes viz., cellulase, papain, pepsin, trypsin and compared with traditional soap-soda method. The efficiency of degumming process was assessed through per cent gum loss, tensile strength, wetting time and Scanning Electron Microscope (SEM) measurements and dyeability with acid dye. Insignificant difference in gum loss was observed between papain enzymatic and soap-soda degummed fabrics. The tensile strength loss percentage in both warp and weft in the case of papain were found to be as 18.55 % and 14.20 % whereas they were 27.95 % and 26.37 % for soap-soda. The characterization through SEM showed distinct morphological changes in silk fabric. FTIR study showed no change in the molecular conformation of silk even after degumming which confirmed degumming as surface phenomenon. High colour yield (K/S value) with good colour fastness to washing and light was obtained when silk fabric degummed with papain was dyed with acid dye.

Key words: Degumming, FTIR, proteolytic enzymes, SEM, silk fabric.

INTRODUCTION

Silk fibre is one of the most familiar, as well as a very useful biopolymer and is universally acclaimed for most of the desirable properties of textile fibre: fineness, strength, elasticity, dyeability, softness, flexibility, smooth filling, luster, elegance, grace and high rating (Khan *et al.*, 2010, Metin *et al.*, 2012). Raw silk is composed of 30 % of sericin, 70 % of fibroin and traces of water and mineral salts. Fibroin is a single protein with a fibrillary structure (Kim *et al.*, 2005) that does not dissolve in water. As for sericin, it is available on the fibre and has an amorphous structure and this gummy protein dissolves in water much more than fibroin (Niyaz *et al.*, 2010, Padamwar and Pawar, 2004). Thus it is easy to remove this gum through various processes without causing any serious harm to the fibre (Mondal *et al.*, 2007, Farideh *et al.*, 2013). Sericin hides the brightness and whiteness of the silk as well as causing it to have a hard

handle. It also prevents its dyeability. Therefore, sericin existing on the silk fiber is made to dissolve by being hydrolyzed through different methods and with degumming agents (Teshome *et al.*, 2011, Freddi *et al.*, 2003). Some important degumming processes have been developed such as alkali boil, acid boil, soap-soda boil and Marseille soaping method, among which Marseille soaping method is the most effective. However, Marseille soap made out of olive soap is rather expensive and has to be imported; therefore, degumming is generally carried out with nonstandard native and home-made soaps based on sodium stearate. Soap-soda boiling method finds major commercial application in silk industries for its cost effectiveness, less time consumption and ease of application, but during highly hot, alkaline silk degumming, hydrolytic degradation of fibers is accompanied, leading to undesirable aesthetic and physical properties such as dull appearance, surface fibrillation and tensile strength loss. The presence of soap

and alkalis in the waste water makes this method a non-ecofriendly process too. As far as environment is concerned, the utilization of chemicals by most of the aforementioned methods induces serious pollution to the receiving waters (Xiang *et al.*, 2011). The use of enzymes in the silk industry is relatively unexplored and has generated a lot of interest owing to its ecologically safe and milder condition and much research is being carried out internationally. For this purpose, a special class of enzymes (proteolytic enzymes) called cellulase, papain, pepsin and trypsin which are specialized in hydrolyzing sericin seem to be an appropriate answer (Gowda *et al.*, 2007, Gulrajani and Gupta, 1995, Gulrajani, 1992).

In the present study, an attempt has been made to apply proteolytic enzymes *viz.*, cellulase, papain, pepsin and trypsin on silk fabric in order to evaluate the best suited enzyme for degumming in terms of gum loss, strength, K/S value, wetting time and dyeability. Finally, the performance of the same has been compared with the process of conventional degumming of silk using soap-soda.

MATERIALS AND METHODS

Raw Murshidabad silk fabric (1/1 plain, 62 ends/cm, warp count 22^d, 54 picks/cm, weft count 34^d, 60 g/m²) was used.

The recipe used for degumming of silk fabric using conventional soap-soda method is as follows (Krishnaveni, 2010):

Soap	- 0.6 %
Soda	- 0.2 %
Sodium silicate	- 0.1 %
pH	- 10.5
Time	- 2 h
Temperature	- 98 °C
MLR	- 1:20

Sodium silicate is commonly used as stabilizer in the silk industry where the use of soft water is strongly advocated in order to avoid the harsh feel, formation of

marks on the materials during degumming with hard water due to the possibility of deposition of calcium and/or magnesium silicates on silk fabric. Though, silicate free stabilizers have been developed and available in the market, it has been observed that the desired effects with regard to degumming including whiteness are not obtained with these silicate free stabilizers (Baehr *et al.*, 1990) when compared with silicate containing stabilizers.

Enzyme degumming (Krishnaveni, 2010) involves the proteolytic degradation of sericin using the specific proteins with negligible or no effect on fibroin in fibrous form. Before enzymatic degumming, the material should be pre-wetted or pre-treated. The object of pre-wetting is to soften and swell the sericin and ensure its easy removal from substrate in the subsequent stage. The recipes used for pre-wetting and treatment of the fabric with cellulase, papain, pepsin and trypsin are as given below:

Pre-wetting process

Wetting agent	- 1 %
Sodium carbonate	- 1 %
pH	- 8-9
Temperature	- 60 °C
Time	- 30 min
MLR	- 1:20

Degumming (with cellulase)

Cellulase	- 2, 4, 6, 8, 10 %
Sodium bicarbonate	- 7.5 %
pH	- 8.5
Temperature	- 60 °C
Time	- 2 h
MLR	- 1:20

Degumming (with papain)

Papain	- 2, 4, 6, 8, 10 %
Sodium Phosphate	- 1 %
Formic acid	- 1 %
pH	- 5.2-6
Temperature	- 70 °C
Time	- 1 h
MLR	- 1:20

Degumming (with pepsin)

Pepsin	- 2, 4, 6, 8, 10 %
Formic acid	- 1 %
Hydrochloric acid (dil.)	- 1 %

pH	- 2
Temperature	- 60 °C
Time	- 1 h
MLR	- 1:20

dried.

Gum loss which corresponds to the amount of sericin removed was calculated from the weight loss of silk fabric after degumming.

Degumming (with trypsin)

Trypsin	- 2, 4, 6, 8, 10 %
Sodium phosphate	- 1 %
Sodium Carbonate	- 1 %
pH	- 7.8-8
Temperature	- 50 °C
Time	- 1 h
MLR	- 1:20

$$\text{Gum loss (\%)} = [(W_1 - W_2) / W_1] \times 100$$

where, W_1 and W_2 are the weights of the sample before and after degumming.

The silk fabric samples were treated separately with different enzymes *viz.*, cellulase, papain, pepsin and trypsin at varying concentrations such as 2, 4, 6, 8 and 10 %. The effectiveness of different enzymes was estimated and compared by measuring the weight loss, strength loss, elongation, wetting time and K/S value. The best suited enzyme was selected as per the comparative performance in terms of above parameters.

Surface colour strength (K/S value indicating dye shade depth on surface) was calculated from the diffuse reflectance of the samples using an Ultraviolet-Visible (UV-Vis) spectrophotometer (Color iQC, Version 6) at 503 nm. The K/S values of dyed samples were determined using the Kubelka-Munk equation (Ibrahim *et al.*, 2007):

$$K/S = (1-R)^2 / 2R$$

To see the extent of sericin removed from silk, the ease by which the sample gets dyed was measured. The recipe used for dyeing of silk fabric with acid dye (C.I. Acid Orange 74) (Onar and Saruřik, 2004) is as follows:

where, K is the coefficient of absorption, S is the coefficient of scattering and R is the reflectance value of the dyed samples. To compensate for the colour of the undyed material, the reflectance of the un-dyed material was subtracted from the reflectance of the sample before the calculation of K/S. The K/S values were calculated from the four repetitive measurements.

Palatine Fast Orange GEN	- 3 %
(C. I. Acid Orange 74)	
Glauber's salt	- 7.5 %
Acetic acid	- 3 %
pH	- 4
Temperature	- 100 °C
Time	- 90 min
MLR	- 1:20

Tensile strength of the fabric in both warp and weft directions were measured on an Instron Tensile Strength Tester as per the British Standard (BS 2576) method. Ten fabric samples of each treatment were extended in a direction parallel to the warp and another ten parallel to the weft so that no two samples contain the same longitudinal threads. The specimens (fabric samples) were cut to a size of 60 mm x 300 mm and then frayed down in the width equally at both sides to give samples which were exactly 50 mm wide. This ensured that all the threads ran the full length of the sample contributing to the strength and also that the width was accurate. The rate of extension was set to 50 mm/min and the distance between the jaws (gauge length) was set to 200 mm. As per norms, any breaks that occurred within 5 mm of the jaws was rejected. The data of

The pH was adjusted to 4 with acetic acid. In the after treatment step, the dyed samples were treated with 0.2 % acid buffer (citric acid – disodium hydrogen phosphate buffer, pH 4.5-5.5) at 50°C, rinsed with warm water for 15 minutes, soaped with 0.2 % detergent (Iodet-T) at 50 °C for 15 minutes and finally rinsed with cold water and

each treatment was averaged from the data of ten fabric samples. For evaluating the wet ability, samples (1 in. x 3 in.) were cut from both warp and weft directions. On each sample, two lines were drawn 1 in. apart with an ink marker. With the help of a clamp stand, the samples were immersed in distilled water till the lower line. The spreading of colour showed the rise of water. The time taken by water to travel from lower mark to upper mark was recorded (Gulrajani *et al.*, 2000). The morphology of untreated and treated silk samples was examined with a Hitachi S-2380N Scanning Electron Microscopy (SEM) at 15 KV of acceleration voltage. Before placing the samples in the SEM chamber, the samples were mounted onto an aluminium stud and sputter coated with gold/palladium for 180s (E-1010 10N SPUTTER, Hitachi, Japan) to prevent charging. The secondary structure and conformation of the silk fibre were measured by using Fourier Transform Infrared (FTIR) Spectroscopy (Perkin-Elmer RX USA) with KBr discs keeping air as reference. In this analysis, resolution 4 cm^{-1} was chosen and data collection was in the range $1900 - 900\text{ cm}^{-1}$.

Wash and light fastness of the dyed samples were measured using Atlas Launderometer as per ISO: 9002 recommended and assessed as per the IS: 3361(1979) and Microsol light fastness tester MBTF fading lamp and assessed as per the IS: 2454 (1967). For evaluating the handle and lustre, the samples of equal size (2 in. x 2 in.) were mounted on black sheet and ten people were asked to evaluate these samples on the basis of handle and lustre. Mean ranking was taken.

RESULTS AND DISCUSSION

Figure 1 shows the effect of different enzymatic treatments under different concentrations (2-10 %) on efficiency of degumming process in terms of gum loss. It is observed from the figure that for all the proteolytic enzymes, with the increase in concentration, the gum loss increased significantly and slowly and thereafter the changes remained insignificant. The optimum gum loss percentage were found to be 16.92 % for 6 % cellulase,

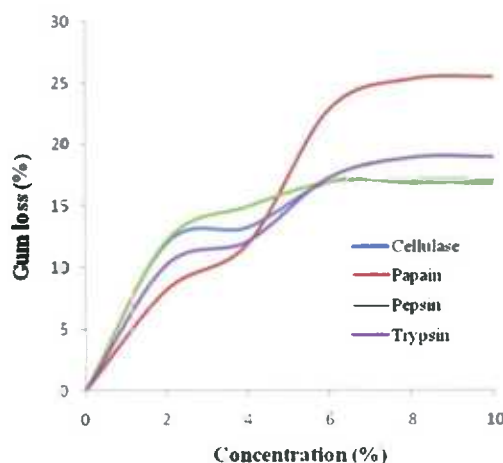


Figure 1: Effect of concentration of different enzymes on gum loss

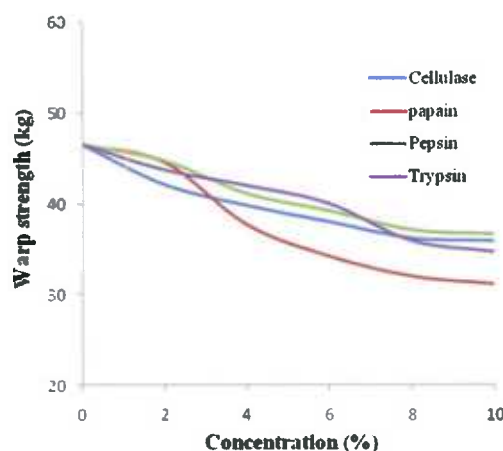


Figure 2: Effect of concentration of different enzymes on warp strength

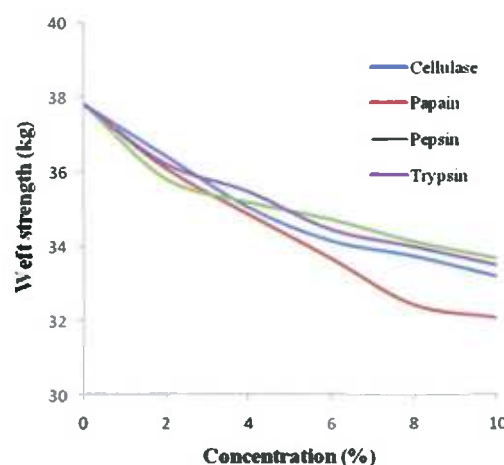


Figure 3: Effect of concentration of different enzymes on weft strength

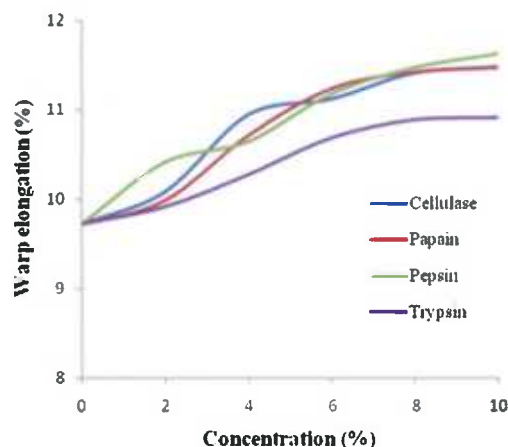


Figure 4: Effect of concentration of different enzymes on warp elongation

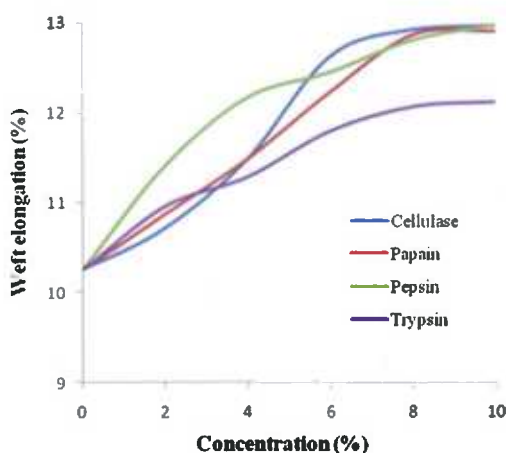


Figure 5: Effect of concentration of different enzymes on weft elongation

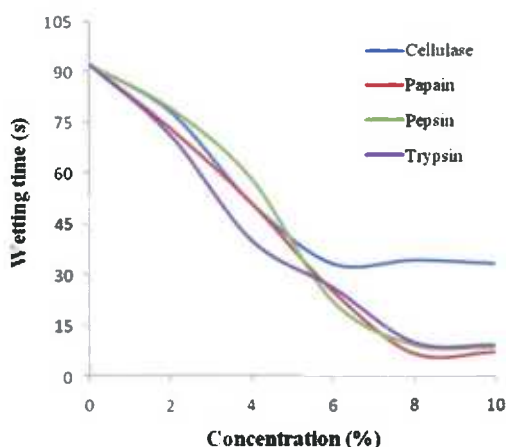


Figure 6: Effect of concentration of different enzymes on wetting time

25.29 % for 8 % papain, 17.11 % for 8 % pepsin and 18.90 % for 8 % trypsin concentration whereas, for conventional soap-soda process, it was found to be as 26.25 %. This is due to the specificity of the particular enzyme at particular concentration. At higher concentration (> 6 % for cellulase, > 8 % for papain, pepsin and trypsin), the gum loss did not increase significantly (Chopra *et al.*, 1996). In addition, at high enzyme concentration there is a saturation of the cleavage sites available on silk, so the rate of hydrolysis attains a plateau. Moreover, a kind of competition may occur between the sericin still on the fibre and the one accumulating in the degumming bath, which may be a more accessible substrate for the enzyme. The gum loss level at 8 % papain concentration was found to be almost at par (25.29 %) with conventional soap-soda process (26.25 %). Also, the high temperature (at 98 °C) and alkaline pH (10.5) used in conventional soap-soda process might cause degradation of fibroin (Xiang *et al.*, 2011). The enzymatic treatments, on the other hand are milder (temperature 50-70 °C and pH 2-8) and give fairly good gum loss (Gulrajani *et al.*, 2000).

The results of strength and elongation (both warp and weft directions) of silk fabric after different degumming processes using proteolytic enzymes at different concentrations (2-10 %) are shown in Figures 2-5. It appears that with the increase in the concentration of the enzymes, the strength of silk fabric decreased significantly up to 8 % concentration in the case of papain, pepsin and trypsin whereas, the strength diminished significantly up to 6 % concentration of cellulase and thereafter remained more or less constant. Among the different enzymes used, cellulase showed the lowest strength loss both in warp and weft directions (14.32 % and 9.70 %) and trypsin showed the highest strength loss (22.75 % in warp and 10.13 % in weft directions) against conventional soap-soda process in which the strength loss in warp and weft directions were 27.95 % and 26.37 %, respectively.

Since sericin is a polar, less crystalline protein with a relatively high lysine and arginine content, it is easily hydrolysed by proteolytic enzymes. On the other hand,

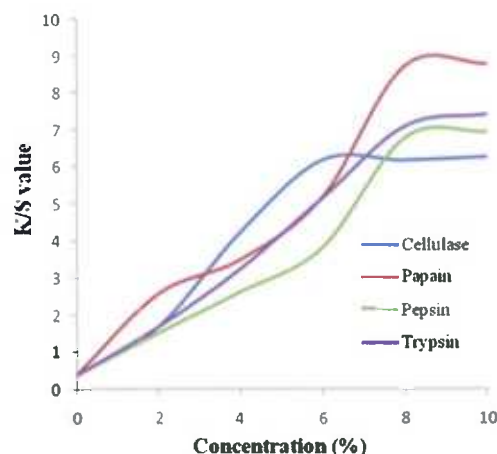
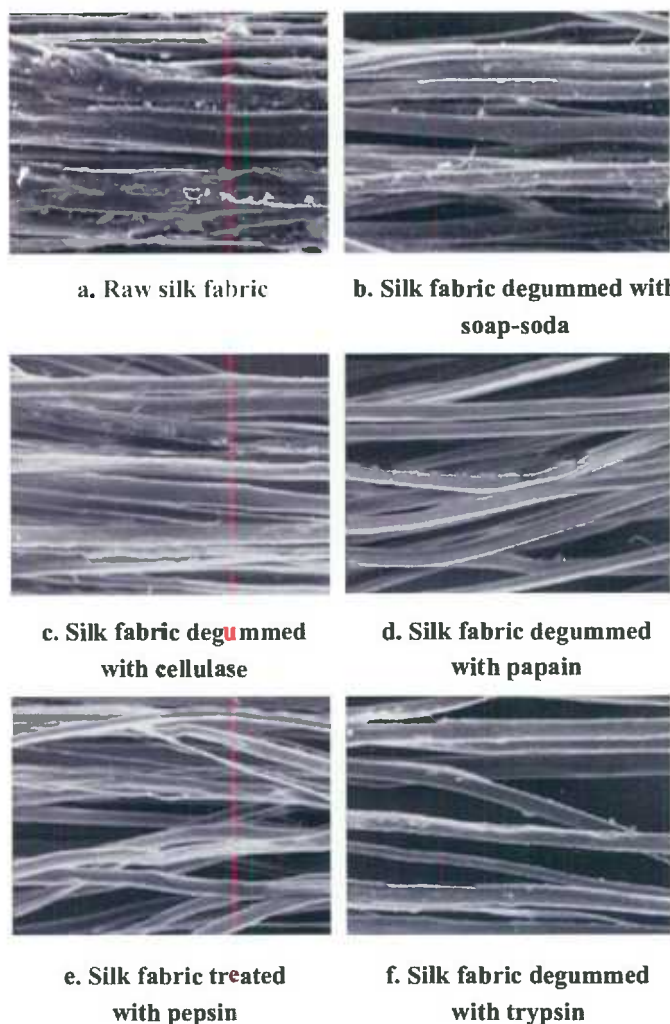
Table 1: Effect of different degumming agents on K/S value and fastness properties

Degumming agents	K/S value	Wash fastness	Light fastness
Soap-soda (Soap - 0.6 % Soda - 0.2 %)	9.5417 ± 0.3709	4	6
Cellulase (6 %)	6.1924 ± 0.2513	3	3-4
Papain(8 %)	8.7534 ± 0.3372	4	6
Pepsin (8 %)	7.1161 ± 0.3247	3	4
Trypsin (8 %)	6.8124 ± 0.2679	3	3-4

Table 2: Effect of different degumming agents on handle and lustre

Degumming agents	Silk fabric	
	Handle	Lustre
Soap-soda (Soap - 0.6 % Soda - 0.2 %)	1	4
Cellulase (6 %)	3	2
Papain(8 %)	2	1
Pepsin (8 %)	3	3
Trypsin (8 %)	2	1

proteolytic enzymes do not readily attack fibroin in fibrous form apparently due to lower proportion of the lysine and arginine present in its structure and also due to the denser packing of the protein chains without bulky side chains in the fibroin (Devi, 2012; Perez-Rigeiro *et al.*, 2002). Hence, no degradation of fibroin takes place during enzymatic degumming. Therefore, whatever the strength loss resulted after enzymatic degumming is due to the hydrolysis of sericin only and is found reasonable (Nakpathom, 2009). The variation in the performance of strength loss for different enzyme is due to variation in their activity profile (Chopra *et al.*, 1996). The lowest strength loss in the case of cellulase is due to its poor intensity of proteolysis activity owing to the less density of linker peptides present within the modules of endo - or exo-glucanases (Lenting and Warmoeskerken, 2001). On the other hand, the high proteolytic activity and degree of hydrolysis (DH) of trypsin (Trypsin_{activity} : 9.5×10^4 μ /g and Trypsin_{DH} : 15.1 ± 0.9) results in the highest strength loss of silk fabric during degumming as compared to

**Figure 7: Effect of concentration of different enzymes on K/S value****Figure 8: SEM images of silk fabric degummed with different degumming agents**

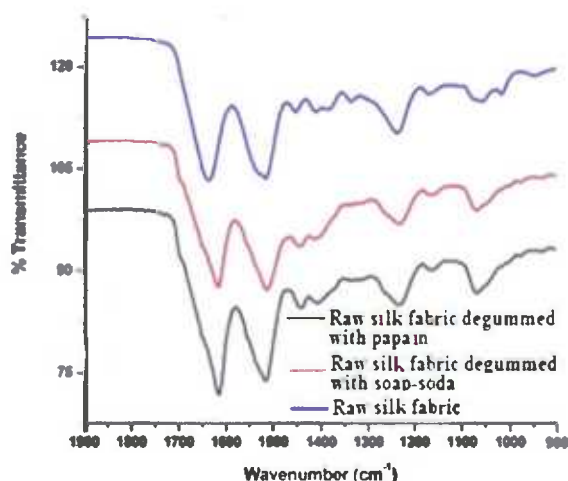


Figure 9: FTIR spectra of raw and degummed silk fabric

papain and pepsin (Papain_{activity} : 8.5×10^4 μ /g, Papain_{DH} : 9.5 ± 0.8 , Pepsin_{activity} : 4.5×10^4 , Pepsin_{DH} : 5.3 ± 0.3) (Ghambari *et al.*, 2012). The high strength loss observed in the case of conventional soap-soda degumming is not only due to hydrolysis of sericin but also due to degradation of fibroin at high temperature (98 °C) and alkaline pH (10.5) maintained during the degumming process (Xiang *et al.*, 2011). Though all the enzymes show improvement in elongation in both warp and weft directions, there is a decline in the elongation of enzymatically degummed silk fabric sample over soap-soda degummed sample.

From Figure 6, it is observed that the wetting time of silk fabric treated with different enzymatic treatments decreases significantly up to a certain concentration (6 % for cellulase and 8 % for papain, trypsin and pepsin). The wetting time of raw silk fabric (control sample) is found to be 92 s which decreases to 7.2 s for the conventional soap-soda process as against 33 s for cellulase, 6.4 s for papain, 9.7 s for trypsin and 8.8 s for pepsin enzymes. The improvement in wetting time is due to the better formation of capillaries during degumming which result in an increase in the absorptivity. The performance variation for different enzymes is due to the variation in their activity at particular concentration. Among the enzymes, papain showed the best result and cellulase showed the worst in respect of the wetting time of silk fabric.

Cellulase is an enzymatic hydrolytic system which consists of four different components such as endo-glucanases, exo-glucanases, β -glucosidase and linker peptide. Endo-glucanases randomly hydrolyse the 1, 4- β glycosidyl linkages within the water soluble cellulose chains. Exo-glucanases hydrolyse the 1, 4- β glycosidyl linkages at either the reducing or non-reducing ends of cellulose chains to form cellobiose (glucose dimer). β -glucosidase converts the water soluble cellobiose into two glucose residues. In a modular organized endo - or exo-glucanases, the modules are connected via linker peptide which is particularly sensitive to proteolysis. Probably this sensitivity is caused by the fact that these linkages are exposed to the water phase. These fungal linker peptides are rich in proline, threonine and serine and are smaller in size than bacterial ones. When cellulase is used to cleave internal glucosidic bonds of cellulose polymer chain and convert into soluble glucose monomers, the first three components (endo-glucanases, exo-glucanases and β - glucosidase) work synergistically and for this, the linker peptides are protected against proteolysis by o-glycosilation. Thus it behaves like a cellulolytic enzyme. When cellulase is not protected against linker peptides, the linker peptides become active and under this situation, it behaves like a proteolytic enzyme. Therefore, cellulase can act also as a proteolytic enzyme (Lenting and Warmoeskerken, 2001). But the degree of proteolysis (DP) is less than degree of cellulolysis (DC) as the linker peptides are only responsible for proteolysis whereas for cellulolysis the first three components work synergistically resulting in higher intensity. For this reason, cellulase when acts as proteolytic enzyme shows poor result.

Among various enzymes tested, papain is the fastest and the most efficient for proteolysis followed by pepsin and trypsin. Basically, the activity of enzyme to cleave peptide bonds depends on the enzyme/substrate ratio and accessibility of the enzyme to the substrate cleavage sites. Furthermore, the difference in degree of hydrolysis (DH) is a result of the difference in the total number of cleavage sites of the substrate. Therefore, it can be concluded that although silk is degradable by all proteases, the number of

available cleavage sites for papain is much higher than other proteases used. Being an endo-proteinase, the efficiency of papain also is in line within its broad specificity towards a protein substrate (Ghambari *et al.*, 2012). On the other hand, digestion with pepsin and trypsin always left room for further extensive action of papain. Papain digests proteins approximately three times faster than trypsin and pepsin without much strength loss (Fan *et al.*, 2012, Onar and Saruřik, 2004). This makes papain more suitable for degumming than trypsin and pepsin.

The silk fabric degummed with different enzymes at different concentrations showed an increase in K/S value (Figure 7) up to a certain concentration (6 % for cellulase and 8 % for papain, pepsin and trypsin) and thereafter remained more or less constant. Among the different enzymes used for degumming, papain showed the highest K/S value (8.7534) with good wash and light fastness to the tune of 4 and 6, respectively which is nearer and equal to the K/S value, wash and light fastness of silk fabric sample degummed with soap-soda method (Table 1) This is due to higher frequency of formation of capillaries and resulting increase in the absorptivity owing to the removal of gum like protein (sericin) from the surface of the substrate which is clearly visible from the surface morphology study carried out through SEM.

The scanning electron micrograph of raw silk fabric, silk fabric treated with soap-soda and silk fabric treated with different proteolytic enzymes *viz.*, cellulase, papain, pepsin and trypsin are shown in Figure 8. In the control sample (raw silk fabric), sericin appears as a non-uniform coating on the surface of fibers and various granules deposits are visible in the interstices between filaments within the fabric. However, photographs of degummed samples show smooth surface due to perfect degumming without any damage to the surface of silk filament. It is observed that the surface of papain treated sample is to some extent cleaner than that of the samples obtained on soap-soda treatment. But in the case of cellulase, large remnants of sericin were noticed which was

comparatively less in the case of pepsin and trypsin.

The data in Table 2 shows that soap-soda treated sample has been ranked first in the case of handle and fourth in the case of lustre. Good handle could be due to uniform dissolution of sericin by the action of alkali, resulting in a smooth surface. Excess of alkali present in soap-soda liquor diminishes the lustre. Best result in terms of lustre is obtained for the samples treated with papain but the sample ranked second in terms of handle as compared to soap-soda method.

FTIR investigates the changes in secondary structure in silk fibre with different agents. The spectral scan between 1900 cm^{-1} and 900 cm^{-1} was considered for the prediction of amide groups in silk protein. The silk is usually characterized by the β - sheet absorption peaks which is found around 1630 , 1530 and 1240 cm^{-1} and an α - helix absorption peak around 1655 cm^{-1} (Kundu *et al.*, 2011). The FTIR spectra of raw silk fabric, raw silk fabric degummed with soap-soda and raw silk fabric degummed with papain (Figure 9) lie in the range of 1625 - 1630 cm^{-1} (amide I), 1520 - 1530 cm^{-1} (amide II) and 1265 - 1270 cm^{-1} (amide III) which are the characteristic absorption peaks of β - sheet which confirms no shift or stretching of band or peak even after treatment. Moreover, finger-print regions remain unchanged. Thus, the molecular conformation of silk does not show any change even after different treatments of degumming. Therefore, it can be concluded that degumming is nothing but a surface phenomenon where only removal of sericin takes place.

Hence, papain enzyme can be used to improve the degumming of silk fabric. The papain enzyme treatment gives almost at par gum loss with a sacrifice of reasonable strength loss, better wettability and cleaner longitudinal surface as compared to soap-soda treatment. The treatment with papain gives better results in terms of lustre but samples are ranked lower in terms of handle as compared to soap soda treated samples. The papain enzyme treatment can be a useful replacement to the traditional method of treatment with soap-soda.

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Article scientifique

EFFET DU DECREUSAGE ENZYMATIQUE SUR LES PROPRIETES DES TISSUS DE SOIE

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R E S U M E

Les échantillons de tissus ont été décreusés en utilisant des enzymes protéolytiques, la cellulase, la papaïne, la pepsine et la trypsine et comparés avec ceux décreusés par la méthode au savon sodique traditionnelle. On a estimé l'efficacité du décreusage par le pourcentage de perte du grès, la force de tension, le temps d'égouttage et par Microscopie électronique à balayage (SEM) ainsi que la prise de teinture avec un colorant acide. Une différence non significative est observée pour la perte de grès entre les tissus décreusés à la papaïne et ceux au savon. La perte de tension pour la chaîne et la trame est de 18,55% et de 14,20% avec la papaïne et de 27,95% et 26,37% avec le savon. La SEM montre des changements morphologiques distincts dans les tissus. L'étude FTIR ne montre pas de modifications moléculaires de la soie après décreusage, ce qui confirme que le décreusage est un phénomène de surface. Une absorption élevée de couleur (valeur K/S) avec une bonne dureté au lavage et à la lumière est obtenue chez les tissus de soie décreusés à la papaïne teints avec un colorant acide.

Mots-clés: Décreusage, FTIR, enzymes protéolytiques, SEM, tissu de soie.



COMPARATIVE ANALYSIS OF THE BIOCHEMICAL COMPOSITION OF DIFFERENT MULBERRY FRUIT VARIETIES

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ABSTRACT

Mulberry fruits were proved to contain several substances with medical action. To understand the biochemical composition, nine representative mulberry fruit varieties were selected from the National Mulberry Orchard (Zhenjiang, PR China). The quantity of amino acids, protein, total flavonoids, anthocyanins, 1-deoxynojirimycin (DNJ), and polysaccharides was estimated in the fruits. The amino acid content ranged from 0.011 to 1.858 g/100 g among the nine varieties. Total flavonoids content varied from 28.63 to 91.30 mg, and anthocyanins from 3.373 to 20.673 mg/100 g. DNJ contents were as low as 0.0219 to 0.2087 %. Polysaccharides content was found to range from 4.2517 to 7.969 mg/100 g. Based on the results, variety S11 was identified to serve as a potential source of dietary supplements towards processing of fruit products.

Key words: Biochemical composition, comparison, mulberry, varieties.

INTRODUCTION

Of late, the importance of inclusion of natural products in the diet is being popularized in the society to alleviate various health discrepancies. Further, the consumer's interest is being widened due to well spread awareness and new research interventions indicating potential health benefits associated with consumption of plants and their functional components (Gossiau and Chen, 2004).

Mulberry fruit has a remarkable place in traditional Chinese wisdom and has been used in folk medicines to effectively treat sore throat, cure fever, protect liver from damage, strengthen the joints, facilitate discharge of urine and lower blood pressure (Liu *et al.*, 2004; Li *et al.*, 2009), treat tinnitus, dizziness, constipation in the elderly and anaemia (Li *et al.*, 2009; Chang *et al.*, 2011; Wang *et al.*, 2013). Its medicinal worth is attributed to the presence of active ingredients with notable therapeutic functions (Ercisli and Orhan, 2007).

Studies revealed that the mulberry fruits contain not only an appreciable amount of proteins, carbohydrates, fats, fibers, mineral contents and some vitamins, but also many bioactive components, such as anthocyanins, flavonoids, alkaloids, *etc* (Onose *et al.*, 2013). Earlier investigations showed that mulberry is rich in anthocyanins compounds, and the major anthocyanins identified in the fruit extract are cyanidin-3-glucoside and cyanidin-3-rutinoside (Ercisli and Orhan, 2007). Anthocyanins are reported to have therapeutic benefits and are responsible for the color attribute and biological characteristics such as antioxidant, antiproliferative, antimicrobial, anti-cancer and anti-neoplastic properties as well as neuro-protective and anti-inflammatory properties (Liang *et al.*, 2012). Flavonoids are a class of phytochemical that possess a wide range of biological activities (Gupta *et al.*, 2012). Flavonoids have found applications in food and pharmaceutical industries for their valuable properties. The pharmacological effect is due to their inhibitory action on certain enzymes and high

antioxidant activity. Mulberry has also been known to be rich in 1-deoxynojirimycin (DNJ) which inhibits postprandial hyperglycemia by inhibiting α -glucosidase in the small intestine (Asano *et al.*, 2001; Gui *et al.*, 2001). It has anti-virus (Durantel *et al.*, 2001; Lazar *et al.*, 2007) and anti-tumor (Lou *et al.*, 2011) activities, and modestly decreases serum triglyceride (TG) level in humans (Kojima *et al.*, 2010).

Nowadays, mulberry fruit is widely consumed both in fresh form and as processed products, such as mulberry jelly, jam, wine, and juice drinks in sericultural countries such as China, Korea, Japan, *etc* (Jiang *et al.*, 2013). Mulberry fruit products consumption has been rapidly increasing because of their good taste, nutritional value, and biological benefits (Aramwit *et al.*, 2010).

To select proper mulberry varieties to process the fruit products, the biochemical composition of different mulberry varieties is a vital information. In this paper, the main biochemical composition of nine mulberry fruit varieties in terms of anthocyanin, 1-deoxynojirimycin (DNJ), flavonoid, polysaccharides, amino acids, and protein contents was evaluated.

MATERIALS AND METHODS

Materials

Mulberry fruit (*Morus alba* L.) was harvested from the plantation of National Mulberry Orchard (Zhenjiang, PR China). Nine mulberry fruit varieties were selected at the ripe stage.

Chemicals

DNJ was purchased from Shanghai Shunbo Bioengineering Co., Ltd., Shanghai, China. Acetonitrile and 9-fluorenylmethyl (Fmoc-Cl) were procured from National Medicines Corporation Ltd., China. Cyanidin-3-glucoside (cy-3-glu) and Quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

Preparation of mulberry fruit

All nine mulberry fruit varieties were manually picked

at ripe stage and stored at -80 °C. Mulberry fruits were dried by freezing dryer (EYELA FDU-2100, Japan). The mulberry fruit powder was used for the determination of phytochemicals.

Determination of amino acids and protein

The Kjeldahl method was used for determination of protein contents in mulberry fruits. The amino acids of the fruits were analyzed with pre-column derivatization by phenyl isothiocyanate (Jiang and Nie, 2015; Morales-de la Peña *et al.*, 2012) using an amino acid analyzer (Agilent 1100, USA). Amino acids were determined quantitatively by comparing the peak areas of amino acids present in standard.

Determination of total anthocyanins

Total anthocyanin content was evaluated using the pH-differential method (Geetha *et al.*, 2011; Sutharat and Sudarat, 2012). One milligram of mulberry fruit powder was dissolved in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5), separately. These two buffers represent the conditions when anthocyanin would be the most and the least stable. The absorbance was measured using spectrophotometer at 520 and 700 nm. The absorbance (A) of the diluted sample was then calculated as follows:

$$A = (A_{520} - A_{700})_{\text{pH } 1.0} - (A_{520} - A_{700})_{\text{pH } 4.5}$$

Total anthocyanin content was calculated as cyanidin-3-glucoside, according to the following equation:

$$\text{Total anthocyanins (mg/l)} = A \times \text{MW} \times \text{DF} \times 1000 / (\epsilon \times l)$$

Where, MW (449.2 g/mol) is for cyanidin-3-glucoside; DF is the dilution factor; l is optical path length in cm; ϵ is a 26,900 molar extinction coefficient in l/mol/cm; 1000 is the factor to convert gram to milligram and A is absorbance.

Extraction of DNJ

DNJ content was extracted according to the method by Lou *et al.* (2011). About 1 g of mulberry fruit powder was dissolved in 0.05 mol/l HCl at 72.8 °C for 3 h and

centrifuged at 12000 rpm for 15 min at room temperature. The supernatant was saved and diluted into 25 ml with water and then used for subsequent derivatization.

Derivatization of DNJ

DNJ extraction and derivatization was carried out according to the procedure described by Jiang *et al.* (2010). Briefly, the broth powder was added to 0.05 M HCl (1:282, w/v) in bain marie for 3 h and 18 min at 72.9°C, and centrifuged at 10785 g for 10 min. The supernatant was vacuum-filtered and diluted to 200 ml with water (coordinated to pH 8.0). The diluted extract was utilized for further derivatization. Ten milliliters of DNJ standard solution or mulberry extract was mixed with 10 µl of 0.4 M potassium borate buffer (pH 8.5) in a 1.5 ml microtube. Twenty microliters of 5 mmol/l FMOCl (9-fluorenylmethyl chloroformate) was added to CH₃CN (Methyl cyanide) with immediate mixing and allowed to react at 20 °C for 20 min in a water circulator. After that, 10 µl of 0.1 mol/l glycine was added to stop the reaction by quenching the remaining FMOCl. The mixture was diluted with 950 µl of 0.1 % (v/v) aqueous acetic acid (17.5 mmol/l) to stabilize the DNJ-FMOCl, and filtered through a 0.45 µm syringe filter.

Determination of DNJ

The DNJ standard was weighed accurately and dissolved in a mixture of acetonitrile and water (50:50, v/v), and a stock solution containing 1 mg/ml of DNJ was prepared. A working solution was prepared in methanol and water (70:30, v/v) and diluted to provide a series of analytical standard as 0.25, 0.5, 1.25, 3.75 and 5 µg/ml for constructing calibration curves (Yatsunami *et al.*, 2008).

Mulberry fruit DNJ was determined using HPLC-FD system. This system consists of a Waters 515 pump (USA), a Waters 2475 fluorescence detector (USA), a Dionasil C18 column (USA) and an EC2000 Data Analysis System (China). The mobile phase consisted of acetonitrile and 0.1 % of aqueous acetic acid (40:60, v/v). The flow rate was adjusted to 1 ml/min and the column temperature was maintained at 25°C. DNJ was detected as an FMOCl derivative by fluorescence detector. The concentration of mulberry fruit DNJ was calculated from

the calibration curve for standard DNJ. All assays were run in triplicate.

Determination of total flavonoid

The total flavonoid content was measured by the aluminium chloride method (Sahu and Saxena, 2013), using quercetin as a standard. Briefly, 1 ml of extract solution (1 mg/ml) was diluted with 4 ml distilled water in a 10 ml test tube. Initially, 0.3 ml of 10 % NaNO₂ was added to each test tube; after 5 min, 0.3 ml of 10 % AlCl₃ was added followed by 2 ml of 1 M NaOH after 5 min. The absorbance was measured at 510 nm using UV-Visible spectrophotometer. The calibration curve of quercetin (20 to 100 µg/ml) was plotted by using the same procedure and total flavonoid contents were expressed as milligram quercetin equivalents (QE)/100 g (Wang *et al.*, 2013).

Determination of polysaccharides

The polysaccharide content was examined using phenol-sulfuric acid colorimetric method (Vazirian *et al.*, 2014) with glucose as a standard curve with different concentrations (0.01 to 0.07 mg/ml), and scanned with UV spectrophotometer (UV-2450, SHIMADZU) The standard plot curve of glucose was measured at 490 nm.

Data analysis

All assays were run in triplicate. The statistical significance was analyzed by Student's *t* test and regression analysis and the data were fitted by using the Expert Design 7.1.3 for Windows software (SPSS Inc., USA).

RESULTS AND DISCUSSION

Amino acid composition and proteins

The amino acid composition of nine mulberry fruit varieties are presented in Table 1. The highest contents of both essential amino acids and non-essential amino acids were observed in variety S6 (3.65 g/100 g and 7.289 g/100 g, respectively). But variety S12 possessed the lowest content, *i.e.*, 1.384 g/100 g and 4.060 g/100 g, respectively. Among essential amino acids, leucine (Leu) was of the

Table 1: Amino acid composition of nine mulberry fruit varieties

Amino acid	Content (g/100 g)								
	S2	S3	S6	S8	S11	S12	S19	S22	S31
Essential amino acid									
Histidine (His)	0.298	0.222	0.289	0.201	0.096	0.093	0.186	0.170	0.172
Acid isoleucine (Ile)	0.445	0.392	0.500	0.356	0.184	0.183	0.326	0.292	0.321
Leucine (Leu)	0.693	0.611	0.768	0.559	0.285	0.288	0.511	0.454	0.506
L-lysine (Lys)	0.508	0.401	0.559	0.375	0.244	0.245	0.336	0.323	0.434
Methionine (Met)	0.060	0.047	0.055	0.094	0.024	0.026	0.040	0.071	0.079
Acid phenylalanine (Phe)	0.451	0.393	0.505	0.355	0.177	0.173	0.346	0.313	0.321
Threonine (Thr)	0.352	0.281	0.361	0.247	0.144	0.146	0.228	0.216	0.246
Valine (Val)	0.543	0.485	0.613	0.465	0.233	0.230	0.402	0.381	0.428
Total	3.350	2.832	3.650	2.652	1.387	1.384	2.375	2.220	2.507
Non-essential amino acid									
Alanine (Ala)	0.489	0.383	0.503	0.345	0.211	0.199	0.331	0.319	0.365
Arginine (Arg)	1.512	1.130	1.187	1.060	0.585	0.552	0.948	0.808	0.703
Aspartic acid (Asp)	1.010	0.899	1.707	0.839	0.549	0.438	0.792	0.816	1.218
Cysteine (Cys)	0.038	0.032	0.037	0.036	0.011	0.011	0.023	0.021	0.030
Glutamic acid (Glu)	1.851	1.490	1.858	1.437	0.524	0.497	1.219	1.106	1.006
Glycine (Gly)	0.568	0.477	0.585	0.411	0.238	0.230	0.396	0.378	0.406
Proline (Pro)	0.660	0.646	0.681	0.633	0.462	0.487	0.554	0.694	0.418
Serine (Ser)	0.403	0.387	0.449	0.360	0.166	0.174	0.301	0.340	0.320
Tyrosine (Tyr)	0.253	0.291	0.282	0.212	0.095	0.089	0.193	0.180	0.187
Total	6.784	5.735	7.289	5.333	2.841	2.677	4.757	4.662	4.653

highest quantity (0.768 g/100 g in S6), and methionine (Met) was of the least amount (0.024 g/100 g in S11). Meanwhile, among non-essential amino acids, glutamic acid (Glu) was the highest (1.858 g/100 g in S6), and cysteine (Cys) was the least (0.011 g/100 g in S11 and S12). Consistent with amino acids, protein content showed the same trend, the highest being in S6, followed by that in S2, S3, S8, S31, S19, S22, S11 and S12 (Figure 1).

DNJ

Previous reports indicated the presence of 1-deoxynojirimycin (DNJ), a polyhydroxylated piperidine alkaloid in mulberry leaves and bark (Song *et al.*, 2009). It

plays as one of the most potent α -glycosidase inhibitors (Oku *et al.*, 2006) to suppress the elevation of postprandial blood glucose and secretion of insulin (Kimura *et al.*, 2007). In this study, DNJ in mulberry fruit is investigated from nine varieties for the first time. Results showed that DNJ content in various mulberry fruit varieties varied from 0.0219 (S12) to 0.2087 % (S2) as depicted in Figure 2. DNJ content in fruits of S2, S3, S6 and S19 varieties was found to be higher than that of corresponding mulberry leaf (about 0.1 %) as reported by Kimura *et al.* (2007).

Total flavonoids and anthocyanins

Anthocyanin is an important composition of total

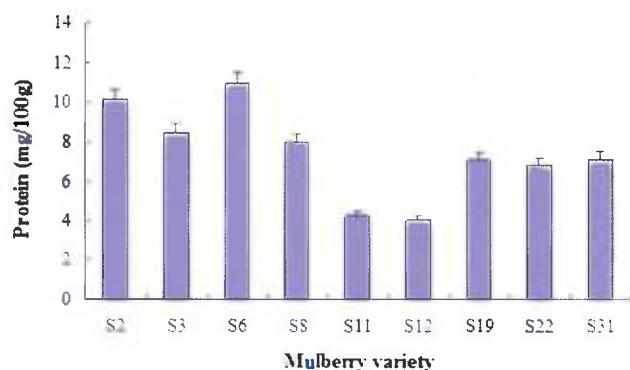


Figure 1: Protein content of nine mulberry fruit varieties

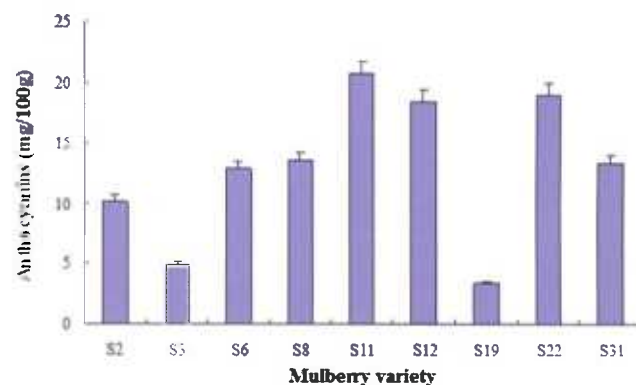


Figure 4: Anthocyanins content of nine mulberry fruit varieties

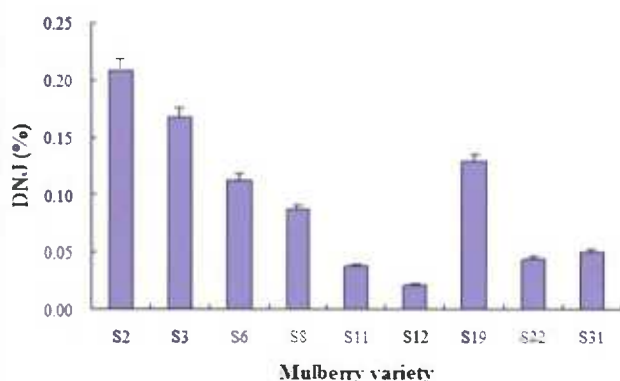


Figure 2: 1-Deoxynojirimycin content of nine mulberry fruit varieties

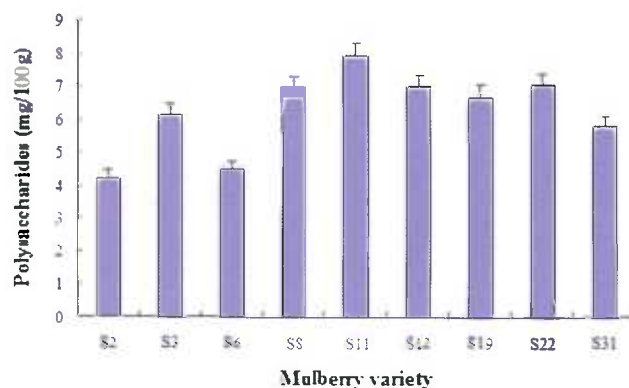


Figure 5: Polysaccharide content of nine mulberry fruit varieties

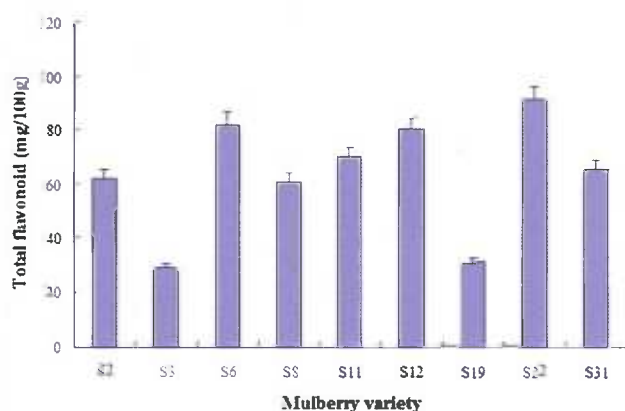


Figure 3: Total flavonoids content of nine mulberry fruit varieties

flavonoids. Chen *et al.* (2006) reported that cyanidin 3-rutinoside and cyanidin 3-glucoside obtained from *M. alba* fruit are of significant importance as antioxidants and in immunonutrition. Anthocyanin content of nine mulberry fruit varieties was evaluated as ranging from 3.373 to 20.673 mg/100 g. Figure 3 shows the highest value of 20.673 mg/100 g in S11 on dry weight basis. It is 5.5 folds of that of S19 (3.373 mg/100 g).

Total flavonoids in mulberry consist of flavone, flavonol, dihydro-flavonol and anthocyanins (Butt *et al.*, 2008). Ercisli and Orhan (2007) reported total flavonoids in fresh *M. alba* fruit as 29 mg/100 g (quercetin equivalent) on dry weight basis. Figure 4 indicates that the content of total flavonoid as ranging from 28.63 to 91.30 mg/100 g, the highest being in variety S22.

Polysaccharides

Polysaccharides from natural products is known as one of the immune modulator. It was reported that the polysaccharides of *M. alba* root bark potentially increases lymphocyte proliferation and decreases antibody production from B cells. Both these activities play an important role in its anti-inflammatory potential (Kim *et al.*, 2000). Present results showed the polysaccharides content varying from 4.2517 to 7.969 mg/100 g as shown in Figure 5. The highest content, 7.969 mg/100 g was found in S11.

Conclusion

Recently, *M. alba* L. has been highlighted in various scientific investigations, to explore its medicinal worth. Many mulberry products such as leaves tea, fruit juice, fruit wine, and fruit jam have been developed commercially in China, Korea, Japan, *etc.* Major interventions in the field of nutrition are focused on functional components of plants that hold therapeutic potential. Mulberry is rich in phytochemical compounds especially flavonoids and anthocyanins, and holds antimicrobial, antioxidant and immune modulate potential. It contains hypoglycemic agents such as DNJ and glycoproteins.

Mulberry fruits are a valuable horticultural product, and its high phytochemical profiles may be used as dietary supplements, in view of its rich and beneficial nutrient composition. In this study, the significant differences in the amounts of biochemical components *viz.*, amino acid, protein, DNJ, total flavonoid, anthocyanin, and polysaccharides contents among nine mulberry fruit varieties conclusively suggest S11 as a suitable source of fruit products.

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Article scientifique

ANALYSE COMPARATIVE DE LA COMPOSITION BIOCHIMIQUE DE DIFFERENTES VARIETES DE FRUITS DE MÛRIER

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R E S U M E

Il est prouvé que les fruits de mûrier contiennent plusieurs substances ayant une action médicinale. Pour appréhender la composition biochimique, neuf variétés de fruits de mûrier ont été sélectionnées au National Mulberry Orchard (Zhenjiang, RP de Chine). La quantité d'acides aminés, de protéines, de flavonoïdes, d'anthocyanes, de 1-déoxynojirimycine (DNJ) et de polysaccharides a été estimée dans les fruits. Le contenu en acides aminés se situe entre 0,011 et 1,858 g/100g selon la variété. Les flavonoïdes totaux varient de 28,63 à 91,30 mg et les anthocyanes entre 3,373 et 20,673 mg/100g. Les contenus en DNJ sont aussi bas que 0,0219 à 0,2087 %. Les polysaccharides se situent entre 4,2517 et 7,969 mg/100g. Selon nos résultats, la variété S11 représente une source potentielle de suppléments alimentaires.

Mots-clés: Composition biochimique, comparaison, mûrier, variétés.



APPLICATION OF IMAGE ANALYSIS METHODS FOR QUANTIFICATION OF FECUNDITY IN SILKWORM *BOMBYX MORI* L.

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ABSTRACT

The fecundity and hatching percentage are important economic parameters that have direct bearing on the cocoon yield traits in sericulture. The fecundity varies widely within and between geographic silkworm races depending on their genetic constitution, region, season, nutrition *etc.* Presently, manual counting of eggs in a sample lot of layings and extrapolation of the same to the entire lot / crop, is in vogue. This is a time consuming and tedious activity with no counter checks, in place. Automation of egg counting activity with acceptable accuracy would considerably reduce the manual effort of counting the eggs individually; improve accuracy of monitoring rearing space requirements for successful cocoon crop and validation of compiled cocoon yield parameters. This paper describes two methods namely "Unsupervised Learning Image Analysis" and "Supervised Learning Image Analysis" to estimate fecundity through the count of egg images in silkworm *Bombyx mori*. The initial results were promising and indicate egg count estimation accuracy of 91-95 % per disease free laying. The scope for improvement of accuracy as well as application of the method with multi-class classification parameters such as colour variation, texture *etc.*, for studies on related traits / parameters relevant to sericulture industry, are discussed.

Key words: *Bombyx mori*, fecundity, hatching percentage, image analysis.

INTRODUCTION

Life cycle of domesticated silkworm *Bombyx mori* L. encompasses egg, larva, pupa (cocoon) and moth stages. Kawamura (1988, 1990) reported that the egg size determining gene *Esd*, a unique morphological marker, is located on the W chromosome. The size of egg in silkworm races of different geographic origin varies from 1.3 to 1.4 mm in length and 0.87 to 1.27 mm in width. Thus, each egg is approximately of the size of a pin head, covered by egg shell called egg chorion. The oviposited non-diapausing polyvoltine (PV) and diapausing bivoltine (BV) eggs appear yellow in colour. Generally, BV and some pigmented non-diapausing (pnd) eggs turn brown in colour with progress in embryonic development and pass through "Pin head" and "Blue egg" stages before "Hatching" of larvae (Ants / Chawki). Fecundity

with regard to silkworm is the number of eggs oviposited by a fertilised female moth. Fecundity and hatching percentage are important economic parameters that have direct bearing on the cocoon yield traits in sericulture.

Fecundity varies widely within and between geographic silkworm races and hybrids depending on their genetic constitution, region, season, nutrition, toxic chemicals, larval & pupal weight, *etc* (Borah and Sengupta, 1991; Hosagoudar *et al.*, 2001; Rajanna *et al.*, 2012) Presently, the practice of manual counting of eggs in a sample lot of disease free layings (DFLs) and extrapolation of the same to the entire lot / crop, is in vogue. This is a time consuming and tedious activity with no counter checks. Automation of egg counting activity with acceptable accuracy would considerably reduce the manual effort of counting individually; improve accuracy

of monitoring rearing space requirements for successful crop and validation of compiled cocoon yield parameters of any cocoon crop harvested. This paper describes two image analysis (Rafael *et al.*, 2009.) techniques viz., “Unsupervised Learning Image Analysis”(ULIA) and “Supervised Learning Image Analysis”(SLIA) of egg images to quantify fecundity in silkworm *Bombyx mori*.

MATERIALS AND METHODS

The mulberry silkworm sheet eggs (DFLs) with diploid chromosome number (48), prepared at Silkworm Breeding & Genetics Laboratory, Karnataka State Sericulture Research and Development Institute (KSSR&DI), Bidadi were used as materials for the experiment. The first and second set of training & test images are of unhatched eggs (DFLs) and hatched eggs (DFLs), respectively.

Both schemes, ULIA and SLIA, were applied on each of the 2 sets of images of silkworm eggs. The first set (Image Set-1) of images contained 6 training and 2 test images of size 150x150 pixels and the second set (Image Set-2) contained 8 training and 6 test images of size 256x256 pixels. The Image Set-1 and Image Set-2 were obtained by arbitrarily cropping images of a given size from a set of larger images.

In ULIA scheme, the images were clustered using k-means algorithm to obtain multiple clusters, one of which represented the “eggs cluster”. Appropriate number of clusters (k) was determined after applying Cluster Validation techniques on the clustering results obtained experimentally using different k-values. We obtained k as 3. The area (number of pixels) of the eggs cluster in sample (training) images and the corresponding egg counts was used to arrive at average egg cluster area per egg *i.e.*, pixels per egg (PPE). The egg count in the test images is calculated by dividing the count of pixels in egg cluster in the test images by PPE obtained from sample images.

In SLIA scheme, the training and test images were annotated with red dots. The dot images were extracted

from annotated images. The actual count of eggs is obtained by counting red dots in the dot images. The input to the training was the sample egg images and the corresponding dot images. The image density function was learnt from the sample images (Lowe, 2004). The learnt function was applied on the test images to obtain the predicted/estimated egg count.

RESULTS

The training images and test images for Image Set-1 and Image Set-2 are shown in Figure 1(a), 1(b), 1(c) and 1(d), respectively. Figure 2 shows K-means Clustering input and output for 006egg.png (from Image Set-1). Figure 3 shows the same for image 014egg.png (from Image Set-2). Figures 4 and 5 show dot and original image from Image Set-1 and Image Set-2, respectively. Further, Table 1 shows the ULIA and SLIA scheme results for Image Set-1. Similarly, Table 2 shows the results of both schemes for Image Set-2.

Perusal of Tables 1 and 2 reveals that ULIA scheme showed an egg count accuracy of 89.18 % and 80 % on data sets Image Set-1 and Image Set-2, respectively. Interestingly, SLIA scheme showed an egg count accuracy of 91.15 % and 95 % on data sets Image Set-1 and Image Set - 2, respectively.

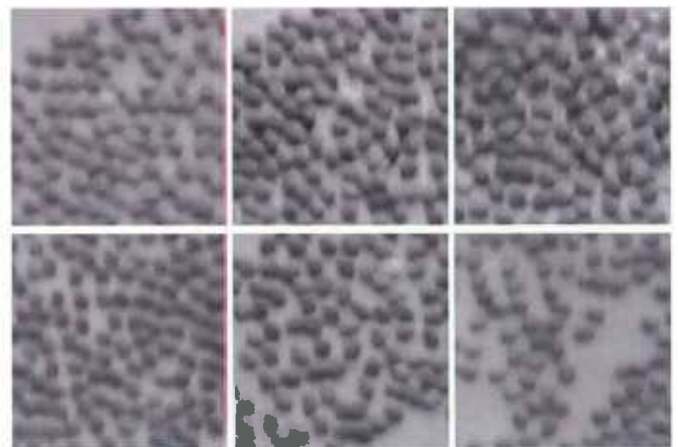


Figure 1(a): Training images (001egg.png to 006egg.png)
Image Set - 1

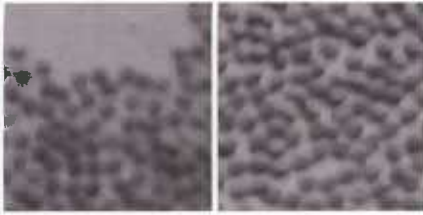


Figure 1(b): Test images (007egg.png to 008egg.png)
Image Set - 1

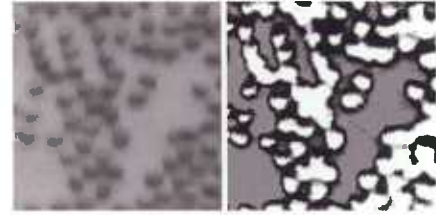


Figure 2: K-means clustering input and output for 006egg.png
(Image Set - 1). Number of clusters $K=3$

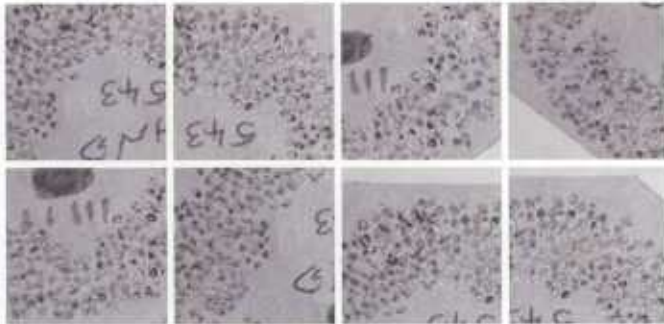


Figure 1(c): Training images (001egg.png to 008egg.png)
Image Set - 2

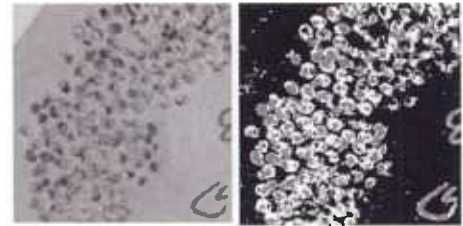


Figure 3: K-means clustering input and output for 014egg.png
(Image Set - 2). Number of clusters $K=3$.

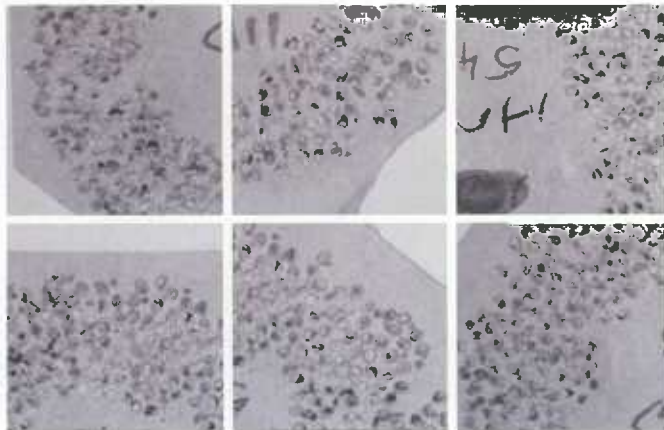


Figure 1(d): Test images (009egg.png to 014egg.png)
Image Set - 2



Figure 4: Dot and original image for 002egg.png (Image Set - 1)



Figure 5: Dot and original image for 007egg.png (Image Set - 2)

DISCUSSION

The fertility of an organism contributes greatly towards its evolutionary fitness. Fecundity is the reproductive rate of an organism or population and represents its fertility. Fecundity also contributes for maintenance of genetic diversity of species through genetic polymorphism (Haldane, 1927; Odum Eugene, 1959; Falconer, 1993; Hartl Daniel, 2007). Accordingly,

evolutionary fitness of mulberry silkworm is attributed to its higher fecundity and egg diapause in response to unfavourable environment, as two important contributing factors.

In any panmictic population, fecundity influences natural selection, genetic drift, mutation, recombination, gene flow and population structure (Sewal Wright, 1984). The low fecundity in highly domesticated silkworm

Table 1: Egg count estimation using both schemes – Image Set - 1

Image	ULIA scheme				SLIA scheme		
	Actual count of eggs	Estimated count of eggs	% Error	% Accuracy	Est mated count of eggs	% Error	% Accuracy
007egg.png	75	68.227	9.03	90.97	68.871	8.17	91.83
008egg.png	86	75.156	12.6	87.4	77.808	9.52	90.48
			Average	89.18		Average	91.15

Table 2: Egg count estimation using both schemes – Image Set - 2

Image	ULIA scheme				SLIA scheme		
	Actual count of eggs	Estimated count of eggs	% Error	% Accuracy	Estimated count of eggs	% Error	% Accuracy
009egg.png	108	83.693	22.51%	77.49	99.176	8.17	91.83
010egg.png	92	62.180	32.41%	67.59	83.242	9.52	90.48
011egg.png	105	78.871	24.88%	75.12	99.278	5.45	94.55
012egg.png	109	75.060	31.14%	68.8	105.435	3.27	96.73
013egg.png	108	103.518	4.15%	95.85	101.790	5.75	94.25
014egg.png	116	107.348	7.46%	92.54	115.093	0.78	99.22
			Average	80		Average	95

results in genetic drift leading to change in allele frequencies, caused by random sampling (Masel, 2011). Further, silkworm rearing temperature, humidity, nutrition, hormones *etc* influence the oviposition by the female moths over seasons in different geographic regions (Gohain and Borua, 1983; Nair *et al.*, 1998; Rahman *et al.*, 2004; Srivastava and Upadhyay, 2013). Therefore, maintenance of optimum population size in silkworm germplasm banks is very vital to ensure various genotypes to breed true over generations. Thus, precise and faster quantification of fecundity and hatching percentage of silkworm genotypes and hybrid crops contribute towards: 1. systematic maintenance of genotypes by practically harvesting and feeding required quantity of mulberry to the larvae on day-to-day basis and 2. providing optimum space to adult larvae as well as adequate mounting space to the spinning larvae both at the laboratory and field level. This will facilitate successful cocoon crop harvest as well as estimation of cocoon lots arrival in advance, to the cocoon markets.

In bacterial cell counting, Lempitsky and Zisserman

(2010) have obtained mean absolute error of about $3.6_{\pm 0.2}$ (Average number of cells in images $\approx 171_{\pm 64}$) *i.e.*, approximately 97.9 % accuracy. In the present image analysis by SLIA scheme, we have obtained silkworm egg count accuracy of 91.15 % and 95 % on data sets Image Set-1 and Image Set-2, respectively.

The SLIA scheme produced a better egg counting accuracy for both Image Set-1 and Image Set-2 compared to ULIA scheme. The ULIA scheme is based on k-means clustering. (Jain *et al.*, 1999). This corroborates the observations in related literature (Lempitsky and Zisserman, 2010) that unsupervised learning methods have not been successful in providing high object counting accuracy compared to supervised learning methods.

We plan to undertake further experimentation for silkworm egg counting using larger data sets and on improvising scheme 2, to produce better fecundity accuracy. Also, the schemes can be applied for multi-class classification with parameters such as colour variation, texture *etc.*, for studies on hatching percentage, embryonic

development and other economic parameters relevant to sericulture industry.

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Article scientifique

APPLICATION DES METHODES D'ANALYSE D'IMAGES A LA QUANTIFICATION DE LA FECONDITE CHEZ LE VER A SOIE *BOMBYX MORI* L.

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RESUME

La fécondité et le taux d'éclosion sont des paramètres économiques importants qui ont une incidence directe sur les caractères de rendement en cocons en sériciculture. La fécondité varie largement dans une race géographique donnée et entre races de vers à soie en fonction des caractères génétiques, de la région, de la saison, de la nutrition *etc.* Aujourd'hui, le comptage manuel d'un échantillon et l'extrapolation des résultats au lot entier de pontes est la règle. Ceci demande beaucoup de temps, est fastidieux et se fait sans contrôle des comptages. L'automatisation de cette activité avec une fiabilité acceptable réduirait considérablement le temps de comptage, améliorerait la fiabilité de l'organisation des aménagements de l'espace d'élevage pour le succès d'une récolte de cocons et la validation des paramètres de rendement. Cet article décrit deux méthodes, la « Unsupervised Learning Image Analysis » et la « Supervised Learning Image Analysis » pour estimer la fécondité à travers le comptage d'images d'œufs chez *Bombyx mori*. Les résultats initiaux sont prometteurs et donnent une fiabilité de l'estimation de 91-95% par ponte saine. La portée pour améliorer la fiabilité ainsi que l'application de la méthode à des classifications de paramètres multiples tels que la variation de couleur, la texture *etc.*, pour des études concernant les paramètres en ériculture sont discutées.

Mots-clés: *Bombyx mori*, fécondité, taux d'éclosion, analyse d'image.



POSSIBILITIES FOR BREEDING NEW HIGHLY PRODUCTIVE SILKWORM, *BOMBYX MORI* L. POPULATIONS GENETICALLY SEX – LIMITED FOR COCOON COLOR

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ABSTRACT

The present study aimed at creation of two comparatively highly productive silkworm populations sex – limited for cocoon color which may be used as initial material in the further breeding programmes. Three silkworm breeds namely, the Japanese breed, D 50, sex limited for cocoon color and the Bulgarian breeds, Super 1 and Hesa 2 were used. The breeding target was to create two new populations possessing morphological characters and productivity levels similar to that of the original Bulgarian breeds, Super 1 and Hesa 2, but sex limited for the cocoon color. It was detected that the breed, D 50 is heterozygous as regards the allele for translucent skin which is recessive. By using the methods of selection as described, two new silkworm populations sex limited for cocoon color were created. The new populations are characterized with the same qualitative parameters as the parental breeds, Super 1 and Hesa 2 except for their female cocoons being golden yellow and the male cocoons white in color. The productivity of the newly bred populations was found lower than that of the original breeds Super 1 and Hesa 2, but much higher than that of the Japanese breed, D 50. The new sex limited populations may be used as initial material for cross breeding with other white cocoon silkworm strains, in studies aimed at creation of new, highly productive silkworm breeds, sex limited for cocoon color or may be further improved following the same selection scheme of backcrossing with the original breeds, Super 1 and Hesa 2.

Key words: *Bombyx mori* L., breeds, cocoon, color, sex limited, silkworm.

INTRODUCTION

The present day's world sericultural industry is based exclusively on the rearing of F₁ silkworm hybrids displaying higher survivability and productivity compared with the pure breeds. Therefore, in order to produce F₁ hybrids, the parental breeds should be separated by sex which is however a laborious process and not sufficiently précised. Usually, the sex separation is made in the pupal or moth stages based on the sexual marking of the insects. The method is not only labour intensive but also causes considerable physical damage to

a number of pupae while cutting the cocoons. Moreover, both at the pupal and moth stages, the workers can commit mistakes in the sex separation leading to inbreeding instead of a cross between the two parental breeds and subsequently, the eggs produced are not 100 % hybrid, but mixed with pure breed eggs as well. All these aspects make the traditional technology for hybrid silkworm egg production, less efficient. A safe sex separation is possible by using sex limited silkworm parental breeds. Sex chromosome constitution in silkworm is ZZ in males and ZW in females. Hasimoto (1948) unequivocally demonstrated that the W chromosome nearly monopolizes

the power of determination of femaleness through the findings that even triploids with ZZW and tetraploids with ZZZW chromosome constitution become normal females.

Even though the theoretical principles for developing the sex limited silkworm were proposed by the Russian geneticists *viz.*, Tichomirov (1891), Astaurov (1933) and Serebrovskiy in 1930s (Strunnikov, 1983), the first sex – limited silkworm strain was created in Japan by Tazima in 1941 by treating newly laid silkworm eggs with X – rays and translocation of the P' allele and its connection with the sex W chromosome. Later, using the same method, Hasimoto (1948) managed to develop a new sex-limited breed having zebra female and plain male larvae. Strunnikov and Gulamova (1969) using the radiation method, created two sex – limited breeds in which the female eggs have gray color and the male eggs are light yellow. Many other breeders then used the already created strains sex – limited for larval markings and egg color as initial material by crossing them with other races, having plain larvae or yellow eggs. Using same methods, in Bulgaria, Petkov (1995) selected two lines sex limited for egg color and 5 lines sex limited for larval marking.

However, the breeds sex – limited for egg color have lower survivability and productivity and in the case of stocks sex – limited for larval markings, though the sex separation in the larval stage is easier, it is still a too laborious activity. Hence during 1970s, more studies were initiated and directed towards the concept of sex separation based on cocoon color. Kimura *et al.* (1971) succeeded in breeding a new silkworm strain with yellow female cocoons and white male cocoons. They translocated the yellow blood gene (y) from the 2nd chromosome through gamma irradiation to the female pupae of yellow cocoon race. Subsequently, a few newly developed strains sex limited for cocoon color were selected in Japan (Yamamoto, 1989), South Korea (Lim *et al.*, 1995), India (Datta *et al.*, 2001) and China (Chen, 2002). In Bulgaria, so far, no silkworm strains sex limited for cocoon color are available. The present research work was an exhaustive attempt aimed to develop two comparatively highly productive silkworm populations

sex – limited for cocoon color which may be used as initial material in the further breeding process.

MATERIALS AND METHODS

The study was carried out during the period 2007 – 2009 at the Sericulture Experiment Station, Vratza, Bulgaria. Three silkworm breeds namely, the Japanese breed, D 50 and the Bulgarian breeds, Super 1 and Hesa 2 were used. The Japanese breed, D 50 was obtained from the Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, Fukuoka, Japan. The breed, D 50 is genetically sex limited for cocoon color – the female cocoons are yellow and the male cocoons are white. The breed has larvae with ordinary markings and elongated cocoons with constriction. On the other hand, the breed is a very lean performer regarding productivity and the population consist of “translucent skin larvae” characterised with the recessive allele. It's known that this character is controlled by over 23 alleles (Lim *et al.*, 1995), located at different chromosome loci. The ‘translucent skin larva’ gene is also lethal or it determines sterility in the female moth. In the case of the breed, D 50, the females with translucent skin lay eggs wherein the embryos die later on. In this breed, about 50 % of the larval population are with translucent skin and the rest have normal skin. The breed, Super 1 is of the Japanese type with marked larvae and white, elongated cocoons. The breed, Hesa 2 is of the Chinese type, with plain larvae and white, oval cocoons. Both the breeds are highly productive and used as parental lines of several Bulgarian F₁ commercial silkworm hybrids.

The following were the studies conducted under the breeding scheme.

1) Investigations on the Japanese breed, D 50: As regards its general productivity, the difference between the productivity of individuals having normal skin and those with translucent skin and the inheritance of the “translucent skin larva” character for its possible removal from the population were investigated. In order to investigate the inheritance of the “translucent skin larva” character, the following crosses were produced and reared:

females with normal skin x males with translucent skin; females with normal skin x males with normal skin, but heterozygous for the translucent skin allele; females with normal skin x males with normal skin, but homozygous for the dominant allele responsible for normal skin (these were males of the breeds, Super 1 and Hesa 2); F_2 of the cross, females with normal skin x males with normal skin, but homozygous for the dominant allele responsible for normal skin.

2) Creation of two new silkworm populations: The Japanese breed, D 50 was crossed with the Bulgarian breeds, Super 1 and Hesa 2, respectively and implemented a purposive selection. The breeding target was to create two new breeds possessing morphological characters and productivity levels similar to that of the original Bulgarian breeds, Super 1 and Hesa 2, but sex limited for the cocoon color. For the purpose, female individuals of D 50 strain were crossed with male individuals of Super 1 and Hesa 2. The breeding scheme is as given in Figure 1. The first generation (G_1) was mass reared as mixture of 60 layings separated in 10 replications. In G_1 , all the female individuals spun only yellow cocoons and all the males, white cocoons which proved the stable inheritance of the “y” allele linked with the W chromosome, responsible for yellow haemolymph and yellow cocoon color. From the cocoons obtained,

females with golden yellow cocoon color were selected and then measured individually to detect their fresh cocoon and silk shell weights and the shell percentage. The individuals conforming to the breed parameters and with the best performance as well were selected and then mated with selected male individuals of the breeds, Super 1 and Hesa 2. A random cocoon sample was utilized to check the silk reeling parameters of the population. The second generation (G_2) obtained was reared and maintained by the same method and then crossed again with selected males of Super 1 and Hesa 2. During the fifth instar, the larvae of the population D 50 x Hesa 2 (G_2) were separated into plain and with markings while only females from the plain larvae group were used for further breeding. Then in the 3rd generation (G_3), a separate batch rearing was performed where the eggs of each silkworm moth were grown separately. Each population was reared in a volume of 32 layings each one having 200 larvae counted after the 2nd molt and then a selection of the 10 best layings regarding the pupation rate, cocoon, silk shell weights and shell percentage was implemented. The cocoons of the chosen 10 layings were measured individually and then only 20 female and 20 male cocoons from each laying, having the best parameters were selected. The selected cocoons from each laying were sib - mated in order to obtain the 4th generation (G_4) which was inbred. The purposes of this inbreeding were first to check the population uniformity reached at this stage through detecting the segregation after the inbreeding and secondly to reject the layings which manifest the “translucent skin larva” character. The fourth generation was reared in a volume of 32 separate batches, each batch of 4 originated from one laying of G_3 , after inbreeding. After rejection of several 4 – layings batches, manifesting the “translucent skin larva” character, the rest of the layings/individuals were the object of the same selection procedure like in G_3 . The selected female individuals of G_4 were again crossed with males of the breeds, Super 1 and Hesa 2, respectively in order to obtain the 5th generation (G_5). Then the 5th generation was further reared and all the morphological and economically important parameters were detected.

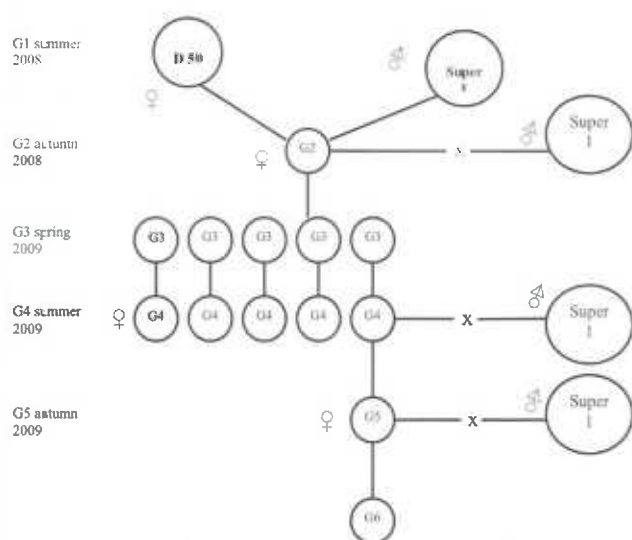


Figure 1: Breeding scheme followed for developing silkworm population sex-limited for cocoon color

During the larval rearing of each generation, separate batches of the breeds, Super 1 and Hesa 2 were also reared

in parallel in order to make comparison between the productivity of the newly selected populations and the original breeds. The data obtained were processed statistically using the well recognized methods (Dispersion analyses). The newly bred populations were named as Lim 1 (D 50 x Super 1) and Lea 2 (D 50 x Hesa 2).

RESULTS AND DISCUSSION

The progeny of the cross between females with normal skin and males with translucent skin manifested a segregation of around 50 % of the larvae of each sex with normal skin and 50 %, with translucent skin. It proves that the gene for translucent skin is recessive. Actually this is the method of maintenance of the breed D 50 in order to preserve its original characteristics. Under the cross, females with normal skin x males with normal skin, but heterozygous for the translucent skin allele, the progeny consisted of about three fourth of the larval population with normal skin and one fourth, with translucent skin. This revealed that the population of the breed D 50 is heterozygous as regards the gene for translucent skin. The

progeny of the cross, females with normal skin x males with normal skin, but homozygous for the dominant allele responsible for normal skin manifested only larvae with normal skin.

In F_2 of the cross, females with normal skin x males with normal skin, but homozygous for the dominant allele responsible for normal skin, a segregation of 96.70 % larvae with normal skin and 3.30 % larvae with translucent skin was detected. This segregation is close to what was theoretically expected. Theoretically, when we make a series of backcrosses with males homozygous for the dominant allele responsible for normal skin, generation by generation, the frequency of the translucent skin character allele in the population decreases. We also may conclude that sib – crossing (inbreeding) would reveal the progenies having the recessive translucent skin allele in their genotype.

The data on the quantitative characters are shown in Tables 1 – 6. It is evident from Tables 1 and 2 that the larvae with translucent skin have significantly lower values of the characters, fresh cocoon weight, cocoon shell weight, filament length and filament weight. Therefore, the translucent skin character is considered undesirable because the appearance of such individuals would decrease the productivity of the strain.

In Table 3, the data concerning the biological traits in the different generations of the cross, D 50 x Super 1 are manifested. In G_3 , the hybrid population already exceeded the breed Super 1 as for the cocoon weight and the difference in the cocoon shell weight between them was

Table 1: Biological characters of the breed D 50

Strain	Fresh cocoon weight (mg)	Cocoon shell weight (mg)	Silk shell percentage
D 50 larvae with normal skin	1351	174	12.88
D 50 larvae with translucent skin	1218	153	12.56
td	3.05**	2.20*	0.96

The data are average from the spring rearing seasons of 2008 and 2009.

Table 2: Silk filament technological characters of the breed D 50

Strain	Filament length (m)			Filament weight (mg)			Raw silk percentage			Reelability (%)			Filament thickness (denier)		
	♀	♂	Average	♀	♂	Average	♀	♂	Average	♀	♂	Average	♀	♂	Average
D 50 larvae with normal skin	445	497	471	130	140	135	25.49	36.84	31.17	96.29	95.24	95.77	2.63	2.54	2.59
D 50 larvae with translucent skin	368	497	433	110	120	115	29.73	32.43	31.08	96.49	93.02	94.76	2.69	2.17	2.43
td			2.15*			2.26*			0.85			0.93			0.16

The data are average from the spring rearing seasons of 2008 and 2009.

Table 3: Biological characters in the different generations of the crosses between the breeds, D 50 and Super 1 and in the breed Super 1

Strain/generation	Fresh cocoon weight (mg)	Cocoon shell weight (mg)	Silk shell percentage
D 50 x Super 1 G1 (summer season)	1811	346	19.11
Super 1 (summer season)	1911	416	21.77
td	4.52**	5.15***	0.97
D 50 x Super 1 G2 (autumn season)	1297	251	19.35
Super 1 (autumn season)	1439	266	18.49
td	8.55***	1.70	0.87
D 50 x Super 1 G3 (spring season)	2477	511	20.63
Super 1 (spring season)	2278	518	22.74
td	3.56**	1.13	2.75*
D 50 x Super 1 G4 (summer season)	1680	365	21.73
Super 1 (summer season)	1779	413	23.22
td	2.45*	5.12**	3.22**
D 50 x Super 1 G5 (autumn season)	1219	221	18.13
Super 1 (autumn season)	1321	242	18.32
td	3.13**	0.87	0.44

Table 4: Biological characters in the different generations of D 50 x Hesa 2 and Hesa 2

Strain/generation	Fresh cocoon weight (mg)	Cocoon shell weight (mg)	Silk shell percentage
D 50 x Hesa 2 G1 (summer season)	1920	368	19.17
Hesa 2 (summer season)	1848	415	22.46
td	1.76	1.88	3.88**
D 50 x Hesa 2 G2 (autumn season)	1392	253	18.18
Hesa 2 (autumn season)	1318	254	19.27
td	0.98	0.76	0.92
D 50 x Hesa 2 G3 (spring season)	2105	467	22.19
Hesa 2 (spring season)	2217	504	22.73
td	1.49	1.87	0.76
D 50 x Hesa 2 G4 (summer season)	1640	362	22.07
Hesa 2 (summer season)	1725	395	22.90
td	3.30*	2.30*	0.43
D 50 x Hesa 2 G5 (autumn season)	1222	225	18.41
Hesa 2 (autumn season)	1288	249	19.33
td	3.15*	1.67	0.91

**Figure 2: Cocoons of the original Japanese breed, D 50 and newly developed breeds (Lim 1 and Lea 2) sex limited for cocoon color**

negligible. The G_4 generation which is inbred manifested lower values of the characters studied than the breed Super 1. Even though the generation G_5 was obtained by backcrossing with Super 1, its productivity was lower than those of Super 1 which may be attributed to the unfavorable silkworm rearing conditions during the

autumn season. In the cross, D 50 x Hesa 2 in G_2 and G_3 generations, the differences with Hesa 2 breed were not significant (Table 4). In G_5 inbred generation, the cocoon and shell weights were significantly lower than that in Hesa 2 breed.

The data relating to the silk filament technological characters are presented in Tables 5 and 6 wherein significant differences between the generations of D 50 x Super 1 and D 50 x Hesa 2 crosses and the pure breeds Super 1 and Hesa 2, respectively were obvious. It may be concluded that the improvement of the silk filament technological characters needs more generations of selection when compared to biological characters. The too low silk reeling parameters detected in the breed Hesa 2 in the summer rearing season of 2009 may be explained as due to the unfavorable conditions such as very high temperature during the 5th larval instar which affected the breed performance. According to Chen (2002), two

Table 5: Silk filament technological characters in the different generations of D 50 x Super 1 and Super 1

Strain	Filament length (m)			Filament weight (mg)			Raw silk percentage			Reelability (%)			Filament thickness (denier)		
	♀	♂	Average	♀	♂	Average	♀	♂	Average	♀	♂	Average	♀	♂	Average
D 50 x Super 1 G1 (summer season)	691	850	771	258	278	268	36.18	42.00	39.09	87.16	91.45	89.31	3.36	2.94	3.15
Super 1 (summer season)			1142			360			42.75			90.91			2.84
td			5.23***			5.15***			2.68**			0.87			2.87**
D 50 x Super 1 G2 (autumn season)	707	618	663	199	183	191	38.71	37.81	38.26	90.45	89.70	90.08	2.53	2.66	2.60
Super 1 (autumn season)			1010			221			37.65			91.00			1.98
td			8.76***			1.13			0.90			0.97			3.03***
D 50 x Super 1 G3 (spring season)	1044	1092	1068	400	420	410	39.60	42.68	41.14	94.11	92.92	93.52	3.45	3.46	3.45
Super 1 (spring season)			1014			500			42.73			92.59			4.43
td			1.23			2.34*			1.05			1.26			2.16*
D 50 x Super 1 G4 (summer season)	772	965	869	260	320	290	35.62	45.71	40.67	87.24	92.48	89.86	3.03	2.98	3.01
Super 1 (summer season)			1002			530			41.25			87.07			2.96
td			3.32**			1.11			1.15			2.18*			0.54

Table 6: Silk filament technological characters in the different generations of D 50 x Hesa 2 and Hesa 2

Strain	Filament length (m)			Filament weight (mg)			Raw silk percentage (%)			Reelability (%)			Filament thickness (denier)		
	♀	♂	Average	♀	♂	Average	♀	♂	Average	♀	♂	Average	♀	♂	Average
D 50 x Hesa 2 G1 (summer season)	826	963	895	273	289	281	35.13	40.25	37.69	38.35	91.75	90.05	2.97	2.70	2.84
Hesa 2 (summer season)			1179			347			38.56			89.00			2.61
td			5.67***			6.16***			0.78			0.23			0.34
D 50 x Hesa 2 G2 (autumn season)	739	818	779	193	202	198	34.77	38.40	36.58	37.70	87.10	87.40	2.35	2.22	2.29
Hesa 2 (autumn season)			889			230			36.68			88.00			2.34
td			2.14			0.93			0.22			0.54			0.16
D 50 x Hesa 2 G3 (spring season)	1011	1213	1112	380	380	380	40.00	42.04	41.02	91.78	91.34	91.56	3.38	2.82	3.10
Hesa 2 (spring season)			1226			410			40.19			93.18			3.01
td			0.86			1.13			1.03			2.34*			0.23
D 50 x Hesa 2 G4 (summer season)	981	1046	1014	260	310	285	38.24	45.58	41.91	88.14	91.17	89.66	2.39	2.66	2.53
Hesa 2 (summer season)			889			260			34.21			74.71			2.63
td			3.56***			0.88			3.97***			4.56***			0.43

silkworm strains CY_{2c} (Chinese race) and JY₆₂₈ sex limited for cocoon color were created in China. They manifested 1.543 and 1.694 g cocoon weight, 0.388 and 0.419 g shell weight values, respectively. In our newly bred populations, we could manifest corresponding values for these traits. Figure 2 depicts cocoons of the Japanese breed D 50 and the two newly selected populations sex limited for cocoon color. It is evident that the cocoon size in the new populations is much bigger than that of the breed D 50. Moreover, the cocoon shape in the population Lea 2 (D 50 x Hesa 2) is oval and completely different from that of D 50 which has elongated cocoon with constriction.

CONCLUSION

From the results obtained, we could make the following conclusions that population of the breed, D 50 is heterozygous as regards the allele for translucent skin which is recessive. The translucent skin character is a negative parameter as regarding productivity of a strain. The fixing of normal skin character in the selection process over generations may be possible by continuous back crossing with male individuals, who are homozygous for the dominant allele responsible for normal skin, combined with sib - crossing (inbreeding). The two newly developed silkworm strains sex limited for cocoon color are characterized with qualitative characters at par with the original breeds, Super 1 and Hesa 2. Though the productivity of the newly bred populations was lower than that of the original breeds, Super 1 and Hesa 2, it was much higher than that of the Japanese breed, D 50. The new sex limited populations may be utilized as initial material in other directional breeding programmes for creation of a pool of new, highly productive silkworm breeds, sex limited for cocoon color or may be further improved following the same selection scheme of backcrossing with the original breeds, Super 1 and Hesa 2.

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Article scientifique

**POSSIBILITES DE LIGNAGE DE NOUVELLES POPULATIONS
HAUTEMENT PRODUCTIVES DE VER A SOIE, *BOMBYX MORI* L.,
AYANT UNE COULEUR DE COCON GENETQUEMENT LIMITEE AU SEXE.**

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R E S U M E

La présente étude a pour but de créer deux populations de vers à soie comparativement hautement productives présentant la couleur du cocon limitée au sexe qui puissent être utilisées comme matériels de base dans des programmes de lignage. Nous avons utilisé trois souches de vers à soie, la japonaise, D 50 limitée au sexe pour la couleur du cocon et les bulgares, Super 1 et Hesa 2. La cible était d'obtenir deux nouvelles populations possédant des caractères morphologiques et de productivité similaires aux lignées bulgares originelles Super 1 et Hesa 2 mais ayant la couleur du cocon limitée au sexe. D 50 est hétérozygote pour le caractère tégument translucide qui est un allèle récessif. Après sélections, nous avons obtenu deux nouvelles populations avec la couleur du cocon limitée au sexe. Ces populations sont caractérisées par les paramètres qualitatifs des lignées parentales Super 1 et Hesa 2 sauf que les cocons femelles sont jaune d'or et les cocons mâles blancs. La productivité de ces nouvelles lignées est plus faible que celle des lignées Super 1 et Hesa 2, mais beaucoup plus élevée que celle de la lignée japonaise D 50. Ces nouvelles lignées limitées au sexe peuvent être utilisées pour des croisements avec d'autres souches à cocon blanc pour obtenir la création de nouvelles lignées hautement productives limitées au sexe pour la couleur du cocon, ou peuvent être améliorées ultérieurement en suivant le même schéma de sélection par croisement de retour avec les lignées Super 1 et Hesa 2.

Mots-clés: *Bombyx mori* L., lignées, cocon, couleur, limité au sexe, ver à soie.



PEBRINE SPORE DETECTION IN TASAR SILK MOTHS USING MICROGRAPHIC IMAGE ANALYSIS

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ABSTRACT

Tasar silk is popular in India because of its traditional value as silk sarees. In spite of the availability of natural resources and demand of tasar silk, tasar sericulture in India is challenged by the frequent outbreak of pebrine disease which is caused by a microsporidian parasite, *Nosema* sp. The disease is highly infectious and can result in complete loss of crop. Currently, the disease is detected by manual microscopic inspection of tissue smear of each and every egg laying moth, a procedure which is prone to human error and subjective in nature. This paper proposes automation of the pebrine spore detection process by capturing photo micrographic images of the smear and classifying pebrine spores using digital image processing technique to improve productivity and accuracy of the process. Captured images are subjected to gamma enhancement. Local segmentation is applied to segment the foreground objects. The segmented foreground objects are labelled individually by particle labeling technique. The specific features are then extracted to identify pebrine objects. More than 1000 moth samples were analyzed using the developed solution and the results validated with human experts. System accuracy was found to the tune of 90 %.

Key words: Digital image processing, Gamma enhancement, local segmentation, particle labeling, pebrine disease, photomicrographic image, region-growing.

INTRODUCTION

Pebrine disease (Govindan *et al.*, 1998) is caused due to trans-ovarial (through egg) transmission (Baig *et al.*, 1988) of microsporidia from mother to the progeny and also through secondary infection from diseased silkworm (Subramanya, 2005), contaminated faecal matter, rearing area and infected appliances. If silkworms ingest microsporidia in their larval stage, there will not be any visible disease symptoms in the particular generation (Shankar Rao *et al.*, 2002). However, mother moths subsequently pass on the microsporidium onto the eggs, and 100 % of worms hatching from the infected eggs will die in their early larval stage. Presently, during grainage season, every female moth after completion of egg laying is examined under the microscope. For this, a smear

sample of the middle portion of the lower abdomen from between the 8th and 9th segments (Awasthi *et al.*, 1988) is taken and examined under the microscope (675 X resolution). If pebrine disease is detected in a moth, all eggs corresponding to that infected moth are destroyed immediately. But the above procedure has got its own limitations that, it is time consuming, require expert manpower and manual examination of each slide under the microscope, which is highly subjective and prone to human errors because of individual biasness, non-repeatability nature and fatigue induced. So, there is an utmost requirement to develop a low cost, fast, high precision automated pebrine detection technology which is simple to operate and can be used even by inexperienced users.

METHODOLOGY

System overview

A low cost, student microscope with 675X resolution (15X eyepiece and 45X objective) was used which is currently being used by field level moth testers. A low cost webcam is fitted on the eyepiece with proper mount and connected to the computer through USB. An LED lighting arrangement is attached with the microscope to get constant illumination. The pebrine detection software is installed in the attached computer. As soon as the image is captured by the user through the software, the image is analyzed automatically within the software and the presence or absence of pebrine in the captured image is shown to the user.

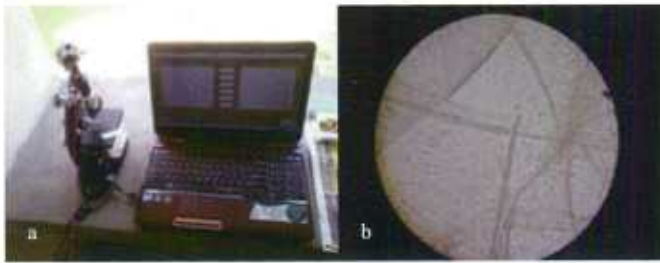


Figure 1: (a) Pebrine-O-Scope Setup (b) Photo-micrographic image

A. Image analysis techniques

Once the user places the prepared slide under the microscope, the live view of the slide smear appears on the Pebrine-O-Scope (Figure 1). User has to click the Load button to capture the image for analysis. As soon as the user captures the image, software shows the infection status of the slide. Details of image analysis steps are described below:

1) **Image capturing:** Image capturing consists of simple capturing of color photo-micrographic image of the sample and then cropping of dark and out of focus portions of the captured image. Captured image has 1600X1200 image resolution before cropping and pixel depth is of 24 bits. Cropping reduces the image resolution by dropping the unwanted portions and thereby reducing the overall processing time.

2) **Gamma correction:** Pebrine spores generally look brighter than the other non pebrine objects. The cropped RGB image is enhanced through standard gamma

correction procedure which makes the bright pebrine spores brighter but the dark regions darker, making further image processing procedures easier. Gamma correction (Gonzalez and Woods, 1992) is a non linear image enhancement procedure which simply replaces the original pixel by some exponential value of that pixel. (1) is the gamma correction equation used here.

for each pixel p of the image

$$p = p^\gamma \quad (1)$$

Where, γ is a pre-determined value to enhance the gamma

3) **RGB to gray conversion:** Till this point, all the pre-processing was done on the RGB pixels to retain all the pixel information. Image segmentation can be done on gray scale image without losing much of the image information. So the RGB pixels are converted into gray scale values after image enhancement. One added advantage of conversion from RGB to grayscale is that it also reduces the time complexity down to $1/3^{\text{rd}}$. RGB to grayscale conversion follows the algorithm (2).

for each pixel p of the image

$$\text{gray} = (R + G + B) / 3 \quad (2)$$

R, G and B are the three color components of each RGB pixel.

4) **Image segmentation:** Modified local thresholding based Region Growing (Gonzalez and Woods, 1992) technique is applied here to segment the image into foreground and background objects. Region-growing is a two step process which consists of finding the seed points and recursively growing the regions starting from those seed points. Here, seed points are selected by a harsh thresholding within a fixed size local sliding window. If average of the pixels within that window is within some threshold, then the center of that window is taken as seed point. (3) shows the seed point selection algorithm. Then within that window, every seed point is grown in region growing step. (4) shows the region growing technique in pseudo code form. This same process is repeated by sliding that window over the image array.

for each sub – image

$$\text{pixelavg} = \left(\sum_{i=1}^h \sum_{j=1}^w \text{pixel}(i,j) \right) / (h * w)$$

if (low threshold $\leq$ pixelavg $\leq$ high threshold)

add the center pixel to seed point array (3)

for each seed point k

regiongrow(k)

function : regiongrow(seed point k)

mark k as foreground

n = one of k's neighbor

if low threshold $\leq$ n $\leq$ high threshold

call function regiongrow(n)

end function

(4)

5) Particle labelling: Particle labeling gives one unique identification number to every particle which makes the feature detection easier. Particle labeling is done using standard two pass labeling algorithm (Gonzalez and Woods, 1992). In first pass, the whole pixel array is scanned. If any unlabelled foreground pixel is found, then the top and left neighborhoods of that pixel are checked. If none of them is labeled then the pixel is labeled by some number. In this way, every pixel is checked with its two neighborhoods and labeled accordingly. After first pass, the labeled pixel array has some unique labels and some equivalent classes of same label. In second pass, those equivalent labels are given new labels which ultimately make the pixel array consisting of each object with different labels.

6) Feature extraction: The feature parameters calculated here are given below:

- Particle area is the number of pixels within that particle.
- Particle perimeter is the number boundary pixel of that particle.
- Particle length has been calculated by measuring the total number of pixels after doing advanced binary morphological thinning operation and length adjustment technique of each particle.

- Particle average width was determined by calculating the average vertical width at different parts of the particle along the thinning length.
- Particle maximum width is the maximum value of all the widths calculated at different parts of the particle along the thinning length.
- L/B ratio is the value of particle length divided by the particle maximum width.
- Shape factor was obtained by dividing the R.M.S. value of particle width by particle average width.
- Compact ratio is the measure of 4* particle area divided by particle perimeter.
- Edge area ratio is the particle perimeter divided by particle area.

All these feature parameters are then used to filter out the pebrine objects from the other objects and finally count the number of pebrine spores within the analyzed area of the sample and show it on the output window of the pebrine detection software along with an infection grade indicator to display whether the sample slide is characterized with high, medium or low population of pebrine spores or the sample is completely safe with no pebrine infection.

RESULTS AND DISCUSSION

Testing of the proposed system was carried out by 1000 slides prepared by moth testers at the grainages. Slides were prepared by taking body fluid randomly from different moths either infected or not infected with microsporidia. The tasar moths were taken from various conditions such as regular samples, dead samples, deformed samples and random samples. The sample slides, which were tested in the proposed system, have been tested at the same time by the moth testers and compared with software results. To cover the maximum regions of the sample slides, the images are captured from 5 different locations of the same slide. If any one of the 5 images contains infection, the sample was declared as infected.

Some of the field testing results are shown in Table 1. It

includes the software as well as manual examination output by human expert and an indication of false identification by software. Based on this, the overall accuracy of the system was calculated.

Table 1: Manual and software examination of silkmoth tissue smears for pebrine detection

Sl. No.	Pebrine		False detection (Y/N)	Remarks
	Manual (Y/N)	Software (Y/N)		
1	N	N	N	
2	Y	Y	N	
3	Y	Y	N	
4	N	N	N	
5	N	Y	Y	
6	N	N	N	
7	N	N	N	
8	Y	Y	N	
9	Y	Y	N	
10	N	N	N	
11	Y	Y	N	
12	N	N	N	
13	N	N	N	
14	N	Y	N	Doubtful
15	N	N	N	

CONCLUSION

The above result is quite encouraging and establishes that the system can be used successfully in automating the whole pebrine spore detection process. If the slide quality is good, the overall accuracy of the system can be above 90 %, though there is a slight chance of false detection. As of now, no such system is available to automate the spore detection process in sericulture industry which is presently being carried out fully by human expert, which is many a time error prone. The proposed system offers a vast range of possibilities and it would be more realistic and more automatic if the slides bearing samples can be placed under the microscope objective automatically

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Article scientifique

DETECTION DES SPORES DE PEBRINE CHEZ LES PAPILLONS TASAR PAR ANALYSE D'IMAGES MICROGRAPHIQUES

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RESUME

La soie Tasar est populaire en Inde en raison de la tradition des saris de soie. En dépit de la disponibilité naturelle de cette ressource et de la demande de soie tasar, la sériciculture tasar est contrariée par de fréquentes poussées de pébrine causée par un parasite microsporidien du genre *Nosema*. La maladie est très contagieuse et peut entraîner la perte totale de récolte. En général, la maladie est détectée par une inspection microscopique des œufs de chaque ponte, un procédé de nature subjective et soumis à l'erreur humaine. Cet article propose une méthode automatique de détection des spores de pébrine en capturant des images microphotographiques et en classifiant les spores de pébrine par une technique de digitalisation des images, afin d'améliorer la productivité et la sécurité du procédé. Les images sont soumises à une augmentation gamma. La segmentation locale est appliquée aux objets du premier plan. Les objets du premier plan segmentés sont marqués par la technique du marquage particulière. Les éléments spécifiques sont extraits pour identifier les objets pébrine. Plus de 1000 papillons sont analysés en utilisant cette méthode et les résultats validés par des experts. La fiabilité du système est estimée à 90%.

Mots-clés: Procédé d'images digitales, Augmentation Gamma, segmentation locale, marquage particulière, maladie de la pébrine, image microphotographique, croissance régionale.



QUALITY CHARACTERISTICS OF RAW SILK PRODUCED FROM AUTOMATIC SILK REELING FILATURES USING CSR RACE BIVOLTINE HYBRID COCOONS

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ABSTRACT

The present study was conducted to analyze the reeling performance and quality characteristics of raw silk produced from Automatic silk reeling filatures, during the period 2011-12, 2012-13 and 2013-14. Results indicate that renditta of the cocoon lots achieved in the filature were in the range of 6.58 to 6.92. The quality characteristics of majority of the lots produced on Automatic silk reeling filatures were of 2A to 3A international grade. Further from the individual quality characteristics of raw silk, it was observed that Evenness variations I, II & III, neatness, low neatness, cleanness, tenacity, elongation and cohesion characteristics of raw silk were excellent and consistently of 3A to 4A international grade, whereas, size deviation, winding breaks and maximum deviation characteristics of raw silk were of 2A to 4A grade. Better quality raw silk consistency is attributed to parameters such as better quality cocoons, optimum degree of drying of cocoons, cooking of cocoons uniformly to the required level using conveyor cooking machine and reeling with denier indicating and mechanical denier correction mechanism in the case of Automatic silk reeling machine. Results indicate that if good quality CSR race bivoltine hybrid cocoons are reeled by adopting Automatic silk reeling machinery (technology) package with appropriate process parameters, better reeling performance is assured resulting in consistent production of superior grade raw silk of 3A to 4A grade.

Key words: Cleanness, international grade, neatness, size deviation, tenacity and elongation.

INTRODUCTION

There is a combination of factors such as cocoon quality, reeling process parameters, reeling machineries, skill of the workers and water quality which play a decisive role on raw silk quality, raw silk yield and productivity (Shimazaki, 1964; Subhas *et al.*, 2000, 2003). Reeling involves a series of intricate processes converting the cocoons into raw silk. In order to improve the quality of raw silk, scientists of Central Silk Board in association with Japanese experts have evolved new bivoltine hybrid (CSR) races, which possess a number of promising features (Somashekar and Kawakami, 2003).

The majority of the silk reeling in India is managed by

a small scale sector which employs a variety of reeling devices viz., charka, cottage basin, domestic basin and multi-end reeling machinery. Presently, silk industry is facing the very serious issue of labour shortage. In order to address this problem and to increase the production capacity of the unit, it is essential to establish bigger raw silk reeling filatures with Automatic raw silk reeling machineries for the consistent production of international / superior grade raw silk from bivoltine hybrid cocoons. Accordingly, Central Silk Technological Research Institute (CSTRI), Central Silk Board in association with Departments of sericulture of state govt. has established a few Automatic silk reeling filatures in southern states of India under Catalytic Development Programme of 10th and

11th plan, by importing Automatic silk reeling machineries from China. Since inception, this Automatic silk reeling filatures have been working successfully. Technical guidance is provided to these filatures by CSTRI, Bangalore and Silk Conditioning and Testing House (SCTH), CSB, Dharmavaram for achieving better performance and results. The present study was conducted to analyze the reeling performance and quality characteristics of raw silk produced by the Automatic silk reeling technology by selecting two Automatic silk reeling filatures, one at Andhra Pradesh and one at Karnataka, for a period of 3 years viz., 2011-12, 2012-13 and 2013-14.

MATERIALS AND METHODS

Raw material: Bivoltine hybrid cocoons of CSR race procured from farmers in the Govt. cocoon markets were reeled on Automatic Silk Reeling Machineries under the following sequence viz., conveyor cocoon drying machine – deflossing machine – cocoon sorting machine – cocoon vacuum permeation chamber – conveyor cocoon cooking machine – mechanical brushing and Automatic silk reeling machine – small reel permeation chamber – re-reeling machine – lacing, skeining, bundling and packing.

Cocoon drying: Cocoons were hot air dried to the optimum level with 4-band conveyor type cocoon drying machine. This drying machine can dry 2 tonnes of cocoons in 24 hours. The following temperature profile was used to dry the cocoons. The cocoons were dried for 5 hours per batch.

Band	Temperature (°C)
I	- 110 ~ 115
II	- 95
III	- 90
IV	- 70 ~ 85

Cocoon deflossing: Hot air dried cocoons were deflossed using deflossing machine before passing on to cocoon sorting machine. Deflossing machine can easily handle about one tonne of cocoons per 12 hours shift.

Cocoon sorting: Defective cocoons were sorted out from

reeling cocoons using cocoon sorting machine. The output of the cocoon-sorting machine is one tonne per 12 hours.

Cocoon cooking: Cocoons were cooked using vacuum permeation chamber as a pre-treatment followed by conveyor cocoon cooking machine. Initially, the cocoons were treated in the vacuum permeation chamber at 0.09 MPa vacuum pressure and then water was released into the chamber so that cocoons are completely filled with water (at room temperature). Total time required for this treatment is about 5-7 minutes. Vacuum permeation chamber can permeate 1.5 tonnes of cocoons in shift of 8 hours. The water-permeated cocoons were then cooked in conveyor cocoon cooking machine under the following temperature profile for 8 ~ 9 min.

Cooking zone	Temperature (°C)
1 st	50 - 60
2 nd	98 - 100
3 rd	98 - 100
4 th	60
5 th	45 - 50
6 th	40 - 45

The cooked cocoons were then collected and fed to Automatic silk reeling machine.

Automatic raw silk reeling: Cooked cocoons were brushed mechanically using mechanical brushing unit and reeled on Automatic silk reeling machine. The Automatic silk reeling machine mainly consists of mechanical brushing, groping and end picking, cocoon carrier system, denier indicator and mechanical casting of cocoons and dropped cocoons/pelade carrier and separation system. The cocoons were reeled on Automatic silk reeling machine of 400 ends capacity with the following process parameters.

Reeling speed:	120 - 130 m/min
Croissance length:	8 to 10 cm

Small reel permeation: Small reels were treated in reel permeation chamber for better water absorption by silk before re-reeling. Small reels were subjected to vacuum permeation pressure of 0.6 MPa and this process was repeated for 8 cycles.

Re-reeling: Re-reeling of raw silk was carried out on closed type of re-reeling machine. Double-sided closed type of Re-reeling machine consists of 40 windows. Re-

reeling was carried out adopting 170 – 180 rpm and by maintaining 35 – 40 °C temperature and 45 % RH inside the re-reeling machine.

The reeling performance data *viz.*, quantity of cocoons reeled and raw silk produced (based on one shift in a day) were collected month-wise from the filature for three years *viz.*, 2011-12, 2012-13 and 2013-14. Further, two sample lots (25 silk skeins from each lot) of raw silk were selected from the silk lots produced in the filature per month during the above three years and tested at CSTRI, SCTH, CSB, Bangalore and at SCTH, CSB, Dharmavaram following ISA test procedures during the three years. In total, 72 lots (25 silk skeins from each lot) of raw silk samples were tested.

RESULTS AND DISCUSSION

The details of cocoon consumption, raw silk production and renditta achieved in the filatures during the period 2011-12, 2012-13 and 2013-14 are presented in Table 1. The grades of raw silk produced in the filatures during the above three years are indicated in Table 2. Analyses of individual quality characteristics of raw silk produced in the filatures in the 3 years are presented in

Table 1: Details of consumption of cocoons and production of raw silk from Automatic silk reeling filature for the period of 3 years (2011-12, 2012-13 & 2013-14)

Year	Consumption of bivoltine hybrid cocoons (kg)	Raw silk production (kg)	Renditta
2011-12	127893.09	18487.05	6.92
2012-13	234077.93	34779.14	6.73
2013-14	230024.25	34941.70	6.58
Total	591995.27	88207.89	6.71

Raw silk production is based on day shift production

Table 2: Distribution of over all grades of raw silk samples produced from Automatic silk reeling filature for the period of 3 years (2011-12, 2012-13 & 2013-14)

Year	No. of lots	4A Grade	3A Grade	2A Grade	A Grade	B Grade
2011-12	24	2	8	11	3	0
2012-13	24	2	8	9	5	0
2013-14	24	2	12	5	5	0
Total	72 (100 %)	6 (8 %)	28 (39 %)	25 (35 %)	13 (18 %)	0

Values shown in parentheses are percentage of lots; No. of lots tested: 72.

Table 3. Further, individual quality characteristics of raw silk (minimum and maximum) and respective grade of raw silk with reference to 20/22 denier raw silk samples tested during the above period are presented in Table 4.

From Table 1, it is observed that renditta of the lots reeled during the period was in the range of 6.58 to 6.92. Results indicate that there is further scope to reduce the renditta by improving the raw silk recovery.

It could be observed (Table 2) that majority of lots produced on Automatic silk reeling filatures were of 2A to 3A international grade. Out of 72 lots tested during the 3 years, 8 % of the lots were of 4A grade, 39 %, of 3A grade and 35 %, of 2A grade, thus indicating that majority of the lots (82 %) produced in the filatures are of 2A and above grade of raw silk.

Further, from the individual quality characteristics of raw silk (Table 3), it is observed that evenness variations I, II & III, cleanness, neatness, low neatness, tenacity, elongation and cohesion characteristics of raw silk were consistently of 3A to 4A international grade, whereas, size deviation, winding breaks and maximum deviation characteristics of raw silk were of 2A to 4A grade.

It could be observed from Table 4 that the quality characteristics of raw silk of 45 lots of 20/22 denier reeled from the filatures belong to 2A to 3A international grade. The results of raw silk quality characteristics indicate that if certain precautions / steps are taken to further improve the grade of size deviation and winding performance of silk, superior quality raw silk of 3A to 4A international grade can be produced consistently from the Automatic silk reeling filatures.

The production of quality raw silk consistently in Automatic silk reeling filatures is attributed to a combination of factors such as better quality cocoons, optimum degree of drying of cocoons, sorting of cocoons for defective cocoons, cooking of cocoons uniformly to the required level and reeling with denier indicating and mechanical denier correction mechanism. It is to be noted that in Automatic reeling machine, majority of the important reeling operations are carried out mechanically and automatic reeling machineries facilitate adopting essential process parameters and hence superior grade raw

Table 3: Distribution of lots based on individual quality characteristics of raw silk samples produced from Automatic silk reeling filature for the period of 3 years (2011-12, 2012-13 & 2013-14)

Major quality characteristics						
Grade of silk	Size deviation	Evenness I	Evenness II	Cleanness	Neatness	Low neatness
4A	20 (28 %)	72 (100 %)	60 (83 %)	29 (40 %)	39 (54 %)	42 (58 %)
3A	26 (36 %)	0	12 (17 %)	35 (50 %)	31 (43 %)	29 (40 %)
2A	21 (29 %)	0	0	6 (8 %)	2 (3 %)	1 (2 %)
A	5 (7 %)	0	0	1 (2 %)	0	0
Auxiliary quality characteristics						
Class of silk	Max. size deviation	Evenness III	Winding breaks	Tenacity	Elongation	Cohesion
1	32 (44 %)	63 (88 %)	6 (8 %)	72 (100 %)	72 (100 %)	72 (100 %)
2	18 (25 %)	9 (12 %)	65 (90 %)	0	0	0
3	13 (18 %)	0	1 (2 %)	0	0	0
4	9 (13 %)	0	0	0	0	0

Values shown in parentheses are percentage of lots; No. of lots tested: 72.

Table 4: Quality characteristics of 20/22 denier raw silk samples produced from Automatic silk reeling filature for the period of 3 years (2011-12, 2012-13 & 2013-14)

I T E M					
I	Average Size (d)	20/22 Denier (No. of lots - 45)			
II	Major tests	Min.	Grade	Max.	Grade
1	Standard size deviation (d)	0.70	4A	1.55	2A
2	Evenness variation- I (Stripes)	20	4A	139	4A
3	Evenness variation- II (Stripes)	0	4A	16	3A
4	Cleanness (%)	93	2A	99	4A
5	Neatness (%)	90	2A	99	4A
6	Low neatness (%)	85	2A	95	4A
III	Auxiliary Tests	Class		Class	
1	Maximum Deviation (d)	1.3	1	4.3	3
2	Evenness variation- III (Stripes)	0	1	1	2
3	Winding Breaks / 40 skeins / hour	4	1	10	2
4	Tenacity (g/d)	3.7	1	4.4	1
5	Elongation (%)	18	1	23	1
6	Cohesion (Strokes)	64	1	142	1
Overall grade of the lots:		2A to 3A			

silk can be produced consistently.

CONCLUSION

In Automatic silk reeling technology, many of the manual operations at each stage of silk reeling are replaced by automation to handle bulk quantity of cocoons and is the best suitable for large scale production of superior grade raw silk consistently, with less labour requirement.

Results indicate that if the good quality CSR race bivoltine hybrid cocoons are reeled by adopting Automatic silk reeling machinery (technology) package with appropriate process parameters, better reeling performance and superior grade raw silk of 3A to 4A grade in large quantity are assured consistently.

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Article scientifique

CARACTERISTIQUES QUALITATIVES DE LA SOIE GREGE OBTENUE PAR DEVIDAGE AVEC UNE BASSINE DE FILATURE AUTOMATIQUE DE COCONS DE L'HYBRIDE BIVOLTIN CSR

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R E S U M E

Cette étude a été entreprise pour analyser la performance de dévidage et les qualités de la soie grège produite par une bassine automatique pendant les années 2011-12, 2012-13, et 2013-14. Les résultats indiquent que les rendements à la bassine des cocons atteints à la filature sont entre 6,58 et 6,92. La qualité de la majorité des lots produits correspond aux grades internationaux 2A et 3A. On observe également que les variations de régularité I, II, et III, la netteté, la netteté basse, la propreté, la ténacité, l'élongation et la cohésion de la soie grège sont excellentes et correspondent aux grades 2A et 3A, alors que la régularité du titre, les ruptures et la déviation maximale correspondent aux grades 2A à 4A. La meilleure qualité de soie grège dépend de la qualité des cocons, du degré optimal d'étouffage des cocons, d'une cuisson uniforme des cocons au niveau souhaité à l'aide d'un convoyeur et du dévidage avec un contrôle et une correction mécaniques du titre sur une bassine de dévidage automatique. Nous montrons que si des cocons de bonne qualité de l'hybride bivoltin CSR sont dévidés en adoptant le paquet technologique du dévidage automatique avec les paramètres appropriés, la performance de dévidage permet d'obtenir de la soie grège de grade 3A à 4A.

Mots-clés: Propreté, grade international, netteté, déviation du titre, ténacité et élongation.



AN ECONOMIC ANALYSIS OF ADOPTION INTENSITY OF BIVOLTINE HYBRID TECHNOLOGY PACKAGE BY FARMERS IN KARNATAKA, INDIA

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ABSTRACT

The adoption intensity of Bivoltine Hybrid Technology Package by sericulture farmers in Karnataka, India was analysed using a Poisson Count Regression model to identify the factors determining selection of technology components of the HYV package, as against the usual practice of classifying farmers into adopters and non - adopters based on cut-off percentages of inputs / technologies purchased. The results reveal some of the socio economic factors to be significant determinants of farmer's choice of adoption of individual technologies from a package of technologies.

Key words: Bivoltine hybrid, partial adoption, Poisson count model.

INTRODUCTION

In spite of its small volume in global textile production, silk has importance in developing economies primarily because of its favourable socio economic consequences. The development of sericulture had been the state's priority in all practicing countries. The Indian sericulture industry is constrained by low productivity, low quality of raw silk, price instability and import competition. With the advent of sophisticated power looms and relaxation in exim policies, large quantities of high quality silk is being imported at prices lower to local silk.

These constraints stem from the poor quantitative and qualitative performance of the prevalent, traditional low yielding silkworm varieties. To ameliorate the traditional variety silkworms, a superior hybrid namely 'Bivoltine Hybrid (BV)' and High Yield Variety (HYV) technology namely 'Bivoltine Hybrid Technology (BVHT)' were evolved as early as 1970s and systematic popularisation efforts began during 1990s. But the hybrid technology has not diffused well. The Current percentage of Bivoltine

hybrid silk production in India is around 10 % only (Rajesh, 2011) for which a number of socio-economic and technology specific factors are found to be responsible (Rajesh, 2012, 2013).

This paper attempts to study the determinants of farmer's decision on BVHT package adoption, based on the level of adoption of individual technologies (of the BVHT package); employing Poisson Count Model, as specified by Octavio *et al.*, 2000. The aim of the paper is to generate a clearer understanding of farmer level decision making on adoption of component technologies from a given package and the impact of differential adoption of component technologies on yield.

In the vast and diverse literature on agricultural technology innovation, two major research lines are discernible: research on innovations generation and research on the adoption and use of innovations (Sunding and Zilberman, 2001). Much of the agricultural adoption literature was developed to explain adoption patterns of high-yield seed varieties (HYV). Two distinct patterns of adoption of HYV technologies observed in literature are

based on land allocation (among HYVs and non-HYVs) and choice of components (of the technology package) that is extent vs intensity.

- HYV technologies are not fully adopted by farmers in the sense that farmers allocate only part of their land to HYV while continuing to allocate land to traditional technologies (Sunding and Zilberman, 2001). Feder *et al.* (1982) categorises agricultural technologies into two types, namely scale neutral and lumpy technologies. According to him HYVs are scale neutral and adopted by all farmers, devoting a larger or a smaller portion of the land to it while lumpy innovations are adopted by only larger farms.
- HYV technologies are technology packages containing a number of 'component' technologies out of which farmers pick and use technologies as they deem fit. Feder *et al.* (1985) observes that while the components of a package may complement each other, some of them can be adopted independently. The terminology used in adoption literature to refer to this is diverse. A few are: *portfolio selection* (Holland and Oakley, 2007), *multiple technology selection*, *adoption intensity*, *adoption frequency*. (Schutjer and Van der Veen, 1977)

Differentiating between divisible and non-divisible innovations, Feder *et al.* (1985) observes that while the adoption of non-divisible technology in a given period is dichotomous, adoption of the divisible technology should be expressed by the share of farm area utilising the technology or by the per hectare quantity of input used. Thus as observed by Mann, 1978, farmer may face several distinct technological options of adopting complete package or its subsets, leading to several simultaneous adoption processes with specific (and predictable) sequential patterns. As the socio economic forces driving the choice could be different, they are to be analysed separately. This paper focuses on the second set

of choices, namely choice of components within the technology package.

According to Schutjer and Van der Veen (1977), the majority of technology issues relate to the extent and intensity of use at individual farm level rather than to the initial decision to adopt a new practice or not. Therefore, a dichotomous qualitative variable is incompetent to capture adoption of such technologies. Sharma *et al.* (2009) finds from a study among UK cereal farms that only 22 % of farms adopt more than 50 % of the 18 technologies considered. Rauniyar and Goode (1992) shows that maize farmers in Switzerland adopt specific sets of technologies (out of seven) and argues that in relation to extension activities, emphasis should be on the adoption of a package of practices and not specific practices or technologies in isolation. Lohr and Park (2002) in the case of insect management portfolios by organic farmers and Isgin *et al.* (2008) in the case of precision farming technology adoption, demonstrate that adoption of isolated technologies do not preclude the adoption of other technologies; thereby defying path dependency arguments (Cowen and Gunby, 1996). Sharma *et al.* (2009) argues in their paper on pest control strategies among UK cereal farmers that there is no limit to the number of technologies adopted by a farmer except as it relates to profitability.

METHODOLOGY

Econometric modelling of partial adoption

The most commonly applied econometric methods, bivariate statistical analysis and multiple ordinary least squares (OLS) models (Current *et al.*, 1995; Gomez, 1995) do not permit effective quantification of the relationship between bio-physical, socioeconomic and institutional variables and levels of technology adoption when the dependent variables are discrete (Octavio *et al.*, 2000) as is the case with developing countries. Judge *et al.* (1985) and Octavio *et al.* (2000) report that sometimes researchers artificially lump adoption levels into two categories (1 for full adoption and 0 for no adoption) in order to use Binomial Probit or Logit models, thereby inducing statistically undesirable measurement errors. Cameron

and Trivedi (1986) recommend use of count data models in such cases. Octavio *et al.* (2000) in a study addressing adoption of agricultural and natural resource management technologies by small farmers in Central American countries claim to have introduced the use of Poisson Count Regression model in technology adoption studies. According to them, this tool allows for a statistically efficient and sound evaluation of adoption when the dependent variable is an integer-valued gradient and the explanatory variables being farmer's socio economic characteristics and bio-physical characteristics of their farm. Another parametric specification employed in the existing count data literature on technology adoption is the Negative Binomial. A criticism on these two models is about their narrow theoretical base. As an alternative, non-parametric methods as specified in Racine and Li (2004) are used by some researchers who concurrently admit these techniques to be computationally burdensome (Octavio *et al.*, 2000.)

Source of data: Both primary and secondary data were used for the study. The study was based on the micro data generated from a sample survey conducted in Sreeerangapattanam taluk, Mandya district, Karnataka state. The study location was selected after carefully evaluating the status of sericulture production. Out of the five sericulture ranges in the taluk, two ranges (comprising of 22 villages and 665 farmers) were selected by random sampling method. From the entire list of farmers of these ranges, 71 farmers were selected at random. The data were collected through direct interview method by using a pre-tested schedule.

Variable definition

Dependent variables: Bivoltine Hybrid Technology (BVHT) is a HYV package consisting of a number of component technologies including agronomic practices for both high yielding mulberry varieties and Bivoltine Hybrid silkworms (Dandin *et al.*, 2005). At the centre of the technology package is the BV hybrid silkworm which has the potential to produce higher quantities of high quality silk in comparison with traditional varieties. This qualitative and quantitative edge is realised at the cost of 'robustness', the ability of the insect to withstand high temperature, humidity, sub-optimal quality food and pathogens. Hence a large number of technologies form

part of the package, designed to provide optimum, disease free micro-environment and quality feed. Even though there is adequate information available on free of cost to farmers, few, in practice adopt all these technologies. A list of twenty two technologies was drawn considering their degree of impact on crop success (Dandin *et al.*, 2005). These technologies were further classified into three sets as follows.

- I. **Crucial technologies (10 no.):** These technologies are crucial for ensuring a successful crop. They are: possession of separate silkworm rearing house, proper ventilation & exhaust in the rearing house, twice disinfection per crop- one before and one after the crop, maintaining airtightness after disinfection for effectiveness of the disinfectant action on pathogens, use of recommended quantity of Calcium Oxide disinfectant, scientific care during moulting of the worms, recommended number of bed cleanings, ensuring hygiene & sanitation within the rearing premises, maintenance of recommended temperature & humidity with the help of thermometer and provision of adequate rearing bed area.
- ii. **Quality enhancing technologies (7 no.):** These technologies are recommended for realising optimum quality of cocoon, the hybrid variety is capable of producing. They are: adoption of HYV mulberry for nutritious feed supply, meshed doors & windows against parasitic uzi fly, use of chawki reared worms for healthy brood, separate spinning hall for provision of optimum spinning conditions, use of improved mountage, timely harvest and disinfection of mountage to prevent mortality due to post spinning infection.
- iii. **Labour saving and advanced technologies (5 no.):** These technologies help the farmer cut expenses on labour and reduce drudgery and enhance crop production and protection. They are: adoption of shoot rearing, use of phytoecdysteroid, use of chemical weedicides, use of plant growth regulators (GR) for better quality leaf yield and use of uzi-trap/ uzicide against parasitic uzi fly.

Explanatory variables: Fifteen socio economic variables including four dummy variables (with values 1 for yes and 0 for no) were used as explanatory variables. These variables are defined as follows

1. Family labour availability (number): Labour availability to the farmer is captured in the variable as the family labour available in the household. The farmers were asked how many members of the family actively participate in the farm operations, namely mulberry cultivation and silkworm rearing. Only the number of hands with considerable participation is counted and recorded.
2. Farmers' experience in sericulture (no. of years): The total number of years of experience of the farmer in sericulture.
3. Age (years): Age in years as reported by the farmer.
4. Education (no. of years of schooling): The number of years of formal education received by the farmer.
5. Ratio of area under mulberry to total land: Ratio of mulberry acreage to total land holding is used as a proxy for the relative importance attached by the farmer to sericulture. When the relative importance of sericulture is more, it is expected that the farmer's dependency on it for his livelihood is more and it is more probable that the farmer adopt the new technology. Here the total landholding is inclusive of own and leased land.
6. Crop failure ratio: The ratio of number of eggs failed to the total number of silkworm eggs purchased in a year, expressed in percentage is a strong measure of profitability, independent of prices.
7. Number of days of training received: The farmers were asked whether they received any technology related training and if so, how many days of training did they undergo.
8. Average selling price per kg of BV cocoon (₹): The farmers were asked to recall price per kg of BV cocoons they received in every crop for the previous year.
9. Total amount of subsidies received (₹): Farmers were asked whether they received any subsidy as cash from government in their entire history of sericulture and if so, how much?
10. Average yield of BV hybrid (kg/Yr.): Average yield is a common measure used in sericulture literature for profitability. It is the quantity of cocoon realised (for BV hybrid here) per 40,000 eggs (also called 100 layings) in kg.
11. Visits of govt. extension agent per crop (number): Farmers were asked how many times the government extension worker visited their crop for every crop during previous year. The average is worked out per crop.
12. Participation in mass contact programs (1=Yes, 0=No): The farmers were asked whether they participate in any mass contact programmes related to sericulture.
13. Access to credit (1=Yes, 0=No): Farmers were asked whether they borrowed any amount from bank any time in their sericulture history.
14. Possession of separate rearing house (1=Yes, 0=No): Whether the farmer possesses a separate building constructed exclusively for the purpose of silkworm rearing.
15. Status as BV hybrid starter (1=Yes, 0=No): The farmer was asked whether he/ she started out as a BV hybrid rearer or not. This variable is expected to capture pure BV rearing experience and save the farmer from exposure to un-scientific practices by previous experience with traditional varieties.

The Poisson Count Model

The Poisson Count Model is an Event Count Duration Regression (ECDR) model (King, 1989) useful in analysing adoption data from developing countries where farmers seldom adopt all the practices in a HVY package, but modify them according to their means and perceived needs (Nelson, 1994), which render their measurements the form of categorically ordered variables undertaking values such as "none, low, average, high and total" (Octavio *et al.*, 2000). These models assume that the

dependent variable results from a counting of events using positive integer numbers. This process implies an ordering scheme like that observed when measuring adoption. The model predicts expected level of adoption by a farmer, given the type of extension program in which the farmer participated and his/ her socio economic profile. Quantifying the impact of each independent variable on the level of adoption is also straight forward. In the Poisson Even Count Model (King, 1989):

$$(1) \quad E[Y_i] = \exp\{\beta X_i\} \quad (i = 1, \dots, n)$$

Where $E[Y_i]$ is the expected value of the dependent variable for the i^{th} observation, $\exp$ - the exponential function, β - is a 1 by k vector of parameters, X_i is a k by 1 vector with the values of the k independent variables in the i^{th} observation and n is the number of observations. Equation (1) can be used to predict the expected level of adoption given the value taken by the vector of independent variables X_i

Equation (1) can also be expressed as

$$(2) \quad E[Y_i] = \exp\{\beta_1 X_{i1}\} \exp\{\beta_2 X_{i2}\} \dots \exp\{\beta_k X_{ik}\}$$

$$E[Y_i] = \exp\{\beta_j X_{ij}\} C_j \quad (i = 1, \dots, n)$$

Where j can take any one value from 1 to k and identifies a specific explanatory variable and C_j is a constant representing the product of the remaining exponential terms in (2). For dichotomous explanatory variables, if $X_{ji} = 0$, $E[Y_i] = C_j$ and when $X_{ji} = 1$, $E[Y_i] = \exp\{\beta_j\} C_j$. Therefore:

$$(3) \quad 100 \times (\exp^{\beta_j} - 1)$$

calculates the percentage change on $E[Y]$ when X_j goes from zero to one, for all observations (i). In general, for independent variables those take several integer- values, the percentage change in the expected level of adoption when X_j goes from X_{j1} to X_{j2} can be calculated as:

$$(4) \quad 100 \times (\exp\{\beta_j X_{j2}\} - \exp\{\beta_j X_{j1}\}) / \exp\{\beta_j X_{j1}\}$$

RESULTS AND DISCUSSION

Table 1 presents the 22 technologies under three sets and the percentage of farmers adopted them. Table 2 presents the 15 variables used as explanatory variables and their average values in the sample. The four technology sets (including one with all 22 technologies put together) were used as independent variables against 15 independent explanatory variables for estimating four separate Poisson Count Regression models. The results are summarised in Table 3. Standard Error estimates and significance levels

Table 1: Per cent adoption levels of 22 technologies, categorised into three technology sets

Sl. No.	Technology	Percentage adopted
SET I: Crucial technologies (10)		
1	Possession of Separate Rearing House	45
2	Proper ventilation & exhaust	52
3	Twice disinfection per crop	59
4	Airtightness after disinfection	33
5	Use of recommended CaO 6 kg/100 dfl	63
6	Scientific moulting care	72
7	Recommended bed cleaning	21
8	Hygiene & sanitation	42
9	Temperature & Humidity control in rearing house	7
10	Adequate rearing bed area	64
SET II: Quality enhancing technologies (7)		
11	HYV Mulberry adoption	94
12	Meshed doors & windows against uzifly	77
13	Use of chawki reared worms	96
14	Separate spinning hall	7
15	Use of improved mountage	7
16	Timely harvest	40
17	Disinfection of mountage	16
SET III: Labour saving & advanced technologies (5)		
18	Shoot rearing adoption	97
19	Use of phytoecdysteroid	14
20	Use of chemical weedicides	0
21	Use of plant GRs	29
22	Use of uzi-trap/ uzicide	38

for the parameters of the Poisson Count Regression Models of four technology sets are furnished as Tables 4 to 7.

Table 2: Explanatory variables used in the study

Sl. No.	Variables and units	Average values
1	Family labour available (no.)	4
2	Experience in sericulture (no. of years)	15
3	Farmer's age (years)	43
4	Education level (no. of years of schooling)	5
5	Ratio: Area under mulberry to total land	0.56
6	Crop failure ratio	0.17
7	Training received by farmer (no. of days)	4
8	Av. selling price per kg of BV cocoon (₹)	140
9	Total subsidies received (₹)	7604
10	Average yield of BV hybrid (kg/Yr.)	51.5
11	Visits of govt. extension agent per crop (no.)	2
12	Participation in mass contact programs (1=Yes, 0=No) Yes: 31 %	
13	Access to credit (1=Yes, 0=No) Yes: 8 %	
14	Possession of separate rearing house (1=Yes, 0=No) Yes: 45 %	
15	Status as BV hybrid starter (1=Yes, 0=No) Yes: 10 %	

Table 3: Significance level and probable relative impacts in %- Summary

Explanatory variables	Dependent variables from 4 models			
	22 TECH	10 TECH	7 TECH	5 TECH
Family labour available (no.)			19.1**	-19.5**
Experience in sericulture (no. of years)	8.0**	20.3**		
Farmer's age (years)	-3.8*	-21.2**	-19.5**	21.8**
Education level (no. of years of schooling)	-10.0**	-14.8**	-10.8**	
Ratio: Area under mulberry to total land	7.4**	31.8**	13.1**	-12.0*
Crop failure ratio				
Training received by farmer (no. of days)	-12.6**	-4.8**	-6.3**	-5.5**
Av. selling price per kg of BV cocoon (₹)		10.9*	15.0**	
Total subsidies received (₹)				
Average yield of BV hybrid (kg/Yr.)	21.1**	30.0**	33.3**	
Visits of govt. extension agent per crop (no.)				5.9*
Participation in mass contact programs (1=Yes, 0=No)	35.5**	36.1**	35.7**	34.9**
Access to credit (1=Yes, 0=No)	16.5*	29.8**		
Possession of separate rearing house (1=Yes, 0=No)	44.6**	73.3**		66.0**
Status as BV hybrid starter (1=Yes, 0=No)				

**Significance at 99 % level

*Significance at 95 % level

Out of the 15 explanatory variables, 12 were found to

have some degree of influence over adoption levels of at least one set of technologies. The 3 variables: Crop failure ratio, Subsidies and BV hybrid starter status were not found to have any causal influence on level of adoption of any technology set. Family labour availability while exhibiting a highly significant positive probability of influence over adoption rate of 'quality enhancing' technology set, showed a highly significant negative probability of influence on 'labour saving/ advanced' technology set. One unit of additional labour in both cases is indicative of nearly 20 % change in either direction (estimated using equation - 3). It is probable that families with more labour are better off in timely harvest and disinfection, the labour demanding activities in the technology set while they are not in dire necessity of labour saving devices as cheap labour availability save them from additional capital investment. Number of years of experience in sericulture imparted a strong positive probability of influence on adoption of the set of 'crucial technologies' with nearly 20 % additional adoption for every incremental year of experience. This goes very well with the theory.

Farmer's age showed a significant negative probability of influence on all sets of technologies except labour saving/ advanced technologies. The result is in conformity with the theory and are in agreement with the findings of Shetty (1966) and Subrahmanium (1982) that the middle age group show greater tendency to adopt recommended farming practices. The older farmers are too traditional and security conscious, do not take the risk of adopting the innovation (Dasgupta, 1987). Another reason for this may be that being in a traditional sericultural area; more age also means more experience. The older farmers (with the exemption of the too traditional and risk averting ones) by virtue of their long experience might be able to harvest better crops using the highly productive but more susceptible BV hybrids. Thus age appears to be a decisive factor in adoption of BV hybrids. The older farmers may tend to adopt labour saving technologies, as their daughters are likely to get married away which reduce domestic labour availability. Especially phytoecdysteroids could be of value to such farmers which could save them additional labour for fetching feed for

Table 4: Standard Error estimates and significance levels for the parameters of the Poisson Count Regression Models of adoption of 22 integral technologies in the Bivoltine Hybrid Technology Package (BVHTP) by sericulture farmers

Variable	Coef.	Relative impact ! of X on Y	S.E. Coef	Chi- Square	P- value	Significance level
<i>Constant</i>	2.917	-	-	-	-	-
Fam ly labour available (no.)	0.0036	1.5	0.0120	0.09	0.766	
Experience in sericulture (no. of years)	0.00771	8.0	0.00274	7.93	0.005	**
Farmer's age (years)	-0.00770	-3.8	0.00331	5.45	0.020	*
Education level (no. of years of schooling)	-0.02107	-10.0	0.00666	10.10	0.001	**
Ratio: Area under mulberry to total land	0.2868	7.4	0.0947	9.10	0.003	**
Crop failure ratio	-0.021	-1.5	0.127	0.03	0.866	
Training received by farmer (no. of days)	-0.01351	-12.6	0.00303	20.94	0.000	**
Av. selling price per kg of BV cocoon (₹)	0.00215	11.3	0.00118	3.31	0.069	
Total subsidies received (₹)	-0.000002	-2.0	0.000002	1.42	0.233	
Average yield of BV hybrid (kg/Yr.)	0.00765	21.1	0.00185	17.17	0.000	**
Visits of govt. extension agent per crop (no.)	0.0193	8.0	0.0130	2.18	0.139	
Participation in mass contact programs (1=Yes, 0=No)	0.3036	35.5	0.0564	29.35	0.000	**
Access to credit (1=Yes, 0=No)	0.1528	16.5	0.0660	5.33	0.021	*
Possession of separate rearing house (1=Yes, 0=No)	0.3688	44.6	0.0607	36.90	0.000	**
Status as BV hybrid starter (1=Yes, 0=No)	0.0411	4.2	0.0879	0.22	0.641	

Note: Adjusted R-Sq value: 58.69 % **Significance at 99 % level *Significance at 95 % level ! Relative impact of continuous X variables on Y are calculated between the minimum & average values of X. Unit change in categorical X variables is expected to elicit the % relative impact on Y

larvae with extended feeding periods.

Level of education, quite surprisingly showed significant negative probability of influence on all sets of technology adoption levels. The theory of diffusion and majority of empirical studies do not support this finding. However, an impartial analysis of the present sericulture scenario in India may explain this. We have two breeds, the traditional Cross Breed (CB), though low yielding is sure to yield a reasonable crop with minimal attention and inputs use. The bivoltine hybrid is a better yielder but notorious for its proneness to disease and demand for more food and extra care. Thus there is a tradeoff between expected profit and perceived risk in bivoltine sericulture, for which the 22 technologies are developed. The comparative advantage is determined by the relative profitability. Thus when market prices of Bivoltine cocoon are low, difference in net returns tends to be small. In such circumstances, educated farmers who keep

accounts of their profit and losses tend to dis-adopt bivoltine in favour of CB.

Ratio of area under mulberry to total land, which is the relative measure of importance attached to sericulture; showed significant positive probability of influence on adoption levels of all sets of technologies except that of labour saving technology (but with low degree of impact). Unit increase in the ratio is capable of producing 30 % additional probability of adoption of the set of 'crucial' technologies, which is a quite logical finding. The number of days of training received by farmer, surprisingly showed a significant negative probability of influence on all sets of technology adoption level. This is against all perceived notions and information available in literature. However, a plausible explanation for this finding may be found. From Table 2, the average number of days of training received by a farmer is just four days. This is quite insufficient to grasp any idea about a very complex

Table 5: Standard Error estimates and significance levels for the parameters of the Poisson Count Regression Models of adoption of 10 crucial technologies in the Bivoltine Hybrid Technology Package (BVHTP) by sericulture farmers

Variable	Coef.	Relative impact ! of X on Y	S.E. Coef	Chi- Square	P- value	Significance level
Constant	2.689	-	-	-	-	-
Family labour available (no.)	-0.0026	-1.0	0.0116	0.05	0.822	
Experience in sericulture (no. of years)	0.01321	20.3	0.00272	23.92	0.000	**
Farmer's age (years)	-0.01254	-21.2	0.00335	14.16	0.000	**
Education level (no. of years of schooling)	-0.03196	-14.8	0.00653	24.25	0.000	**
Ratio: Area under mulberry to total land	0.5520	31.8	0.0935	34.59	0.000	**
Crop failure ratio	0.075	1.3	0.124	0.37	0.544	
Training received by farmer (no. of days)	-0.01232	-4.8	0.00292	18.64	0.000	**
Av. selling price per kg of BV cocoon (₹)	0.00259	10.9	0.00118	4.79	0.029	*
Total subsidies received (₹)	-0.000003	-2.3	0.000002	3.77	0.052	
Average yield of BV hybrid (kg/Yr.)	0.00874	30.0	0.00184	22.43	0.000	**
Visits of govt. extension agent per crop (no.)	0.0140	2.8	0.0129	1.17	0.279	
Participation in mass contact programs (1=Yes, 0=No)	0.3084	36.1	0.0559	30.92	0.000	**
Access to credit (1=Yes, 0=No)	0.2610	29.8	0.0653	15.79	0.000	**
Possession of separate rearing house (1=Yes, 0=No)	0.5499	73.3	0.0610	81.64	0.000	**
Status as BV hybrid starter (1=Yes, 0=No)	0.1713	18.7	0.0866	3.83	0.050	

Note: Adjusted R-Sq value: 52.75 % **Significance at 99 % level *Significance at 95 % level ! Relative impact of continuous X variables on Y are calculated between the minimum & average values of X. Unit change in categorical X variables is expected to elicit the % relative impact on Y

technology package which demands investment and hands-on learning (a silkworm crop is of 28 days duration). In such a situation, a brief exposure to high technology could have negative impact. The farmer training programmes on Bivoltine Hybrid technology for which the 22 technologies are developed, are conducted by State and National level training institutes, where high end technologies are demonstrated. Some of these technologies are installed at high cost and with the technical and financial support of externally aided projects. An ordinary farmer who receives short-term induction training in such high technology environment could easily think that atleast some of these technologies are beyond his means.

The variables: average selling price per kg of BV cocoon, average yield of BV hybrid, participation in mass contact programs, access to credit and possession of separate rearing house showed significant positive

probability of influence on adoption levels of various sets of technologies. These results underline the causal effect of income and crop success (the first two variables) on levels of technology adoption. Out of these, average yield demonstrates a stronger probable influence, which is quite logical. Farmers receiving higher average yields are more probable to adopt 'crucial' and 'quality enhancing' technology sets. The results do not indicate any strong probability of influence of extension visits by government extension agents on adoption of technology sets except labour saving/ advanced technology. This finding finds parallels in the adoption literature (Octavio *et al.*, 2000). At the same time, participation in mass contact programmes by farmers showed significant positive probability of influence on adoption levels of all sets of technologies, with around 35 % of impact for every additional number of participation. Access to credit elicited significant positive probability of influence on adoption levels of 'all 22 technologies' and 'crucial'

Table 6: Standard Error estimates and significance levels for the parameters of the Poisson Count Regression Models of adoption of 7 quality enhancing technologies in the Bivoltine Hybrid Technology Package (BVHTP) by sericulture farmers

Variable	Coef.	Relative impact ! of X on Y	S.E. Coef	Chi- Square	P- value	Significance level
<i>Constant</i>	3.013	-	-	-	-	-
Family labour available (no.)	0.0438	19.1	0.0115	14.15	0.000	**
Experience in sericulture (no. of years)	0.00350	5.0	0.00267	1.73	0.189	
Farmer's age (years)	-0.01142	-19.5	0.00314	13.35	0.000	**
Education level (no. of years of schooling)	-0.02277	-10.8	0.00663	11.91	0.001	**
Ratio Area under mulberry to total land	0.2462	13.1	0.0911	7.25	0.007	**
Crop failure ratio	-0.169	-2.8	0.121	1.95	0.162	
Training received by farmer (no. of days)	-0.01638	-6.3	0.00301	31.51	0.000	**
Av. selling price per kg of BV cocoon (₹)	0.00350	15.0	0.00112	9.82	0.002	**
Total subsidies received (₹)	0.000000	0.0	0.000002	0.04	0.840	
Average yield of BV hybrid (kg/Yr.)	0.00959	33.3	0.00177	29.19	0.000	**
Visits of govt. extension agent per crop (no.)	0.0238	4.9	0.0126	3.56	0.059	
Participation in mass contact programs (1=Yes, 0=No)	0.3052	35.7	0.0551	31.10	0.000	**
Access to credit (1=Yes, 0=No)	0.0882	9.2	0.0646	1.86	0.173	
Possession of separate rearing house (1=Yes, 0=No)	0.0683	7.1	0.0583	1.37	0.242	
Status as BV hybrid starter (1=Yes, 0=No)	-0.0250	-2.5	0.0857	0.09	0.770	

*Note: Adjusted R-Sq value: 44.72 % **Significance at 99 % level *Significance at 95 % level ! Relative impact of continuous X variables on Y are calculated between the minimum & average values of X. Unit change in categorical X variables is expected to elicit the % relative impact on Y*

technology sets with 16 % and 30 % marginal impact.

Out of all the explanatory variables, 'possession of separate rearing house' had a high degree of significant positive probability of influence on adoption levels with probable impact up to 73 % on adoption decision. This is indicative of the fact that a basic requirement for improved technology adoption is possession of a separate rearing house. Only 45 % of the sample farmers have separate rearing houses. This constraints adoption of other technologies such as proper disinfection, provision of adequate rearing space, proper ventilation, maintenance of hygienic practices, exclusion of pests etc.

CONCLUSION

The Poisson Count Regression model, allows for a statistically efficient and sound evaluation of the various complex factors affecting technology adoption by sericulture farmers in the study area. The general

conclusions that may be drawn from the study are as follows. Family labour availability is positively related to quality enhancing technology adoption and better experienced farmers tend to adopt crucial technologies better. Older farmers do not tend to adopt technologies well. Therefore, incentives to bring in more young entrepreneurs into sericulture could improve levels of technology adoption in future. This could be a finding with policy implications. For example, suitable policies could be evolved to make sericulture profession attractive to rural youth by reserving seats in agricultural universities for rural youths from sericultural families. Education level is found to be negatively related to adoption of technologies, an explanation for which could be found by more detailed investigations, in relation with general performance of the bivoltine hybrid silkworm and crop losses associated with it. It is found that farmers who devote considerable portion of their land for sericulture tend to adopt more number of technologies. The

Table 7: Standard Error estimates and significance levels for the parameters of the Poisson Count Regression Models of adoption of 5 labour saving & advanced technologies in the Bivoltine Hybrid Technology Package (BVHTP) by sericulture farmers

Variable	Coef.	Relative impact ! of X on Y	S.E. Coef	Chi- Square	P- value	Significance level
<i>Constant</i>	3.208	-	0.252	-	-	-
Family labour available (no.)	-0.0542	-19.5	0.0142	15.36	0.000	**
Experience in sericulture (no. of years)	0.00159	2.3	0.00298	0.28	0.594	
Farmer's age (years)	0.01038	21.8	0.00356	8.46	0.004	**
Education level (no. of years of schooling)	0.00429	2.2	0.00717	0.36	0.550	
Ratio: Area under mulberry to total land	-0.255	-12.0	0.108	5.64	0.018	*
Crop failure ratio	0.056	1.0	0.146	0.15	0.701	
Training received by farmer (no. of days)	-0.01407	-5.5	0.00346	17.56	0.000	**
Av. selling price per kg of BV cocoon (₹)	-0.00138	-5.4	0.00131	1.10	0.295	
Total subsidies received (₹)	-0.000002	-1.5	0.000002	1.76	0.185	
Average yield of BV hybrid (kg/Yr.)	0.00230	7.1	0.00202	1.3	0.254	
Visits of govt. extension agent per crop (no.)	0.0287	5.9	0.0140	4.18	0.041	*
Participation in mass contact programs (1=Yes, 0=No)	0.2991	34.9	0.0611	24.21	0.000	**
Access to credit (1=Yes, 0=No)	-0.0148	-1.5	0.0708	0.04	0.834	
Possession of separate rearing house (1=Yes, 0=No)	0.5069	66.0	0.0656	59.82	0.000	**
Status as BV hybrid starter (1=Yes, 0=No)	-0.1409	-13.1	0.0962	2.19	0.139	

Note: Adjusted R-Sq value: 45.45 % **Significance at 99 % level *Significance at 95 % level ! Relative impact of continuous X variables on Y are calculated between the minimum & average values of X. Unit change in categorical X variables is expected to elicit the % relative impact on Y

sericulture extension system may take a cue from this finding and encourage mulberry cultivation as mono crop for bivoltine rearing. As the farmer training programmes are found to be negatively influencing farmer decision on technology adoption, the training institutes may study the issue in detail to find out deficiencies in their training programmes and make necessary amendments. The average yield realised by the farmer and average selling prices are strong motivators for technology adoption. The extension visits by government extension workers is found to be not very effective. The extension department may devote attention to this. The effectiveness of the extension services is an important and frequently debated issue in developing countries. However, farmer participation in mass contact programmes shows a very significant impact on adoption levels. This information could be utilised to exploit the potential of mass contact programmes, which could be excellent opportunity for

facilitating peer learning among farmers. Access to credit is found to impact technology adoption favourably and hence, this deserves attention of policy makers in utilising its un-tapped potential. One of the key findings of this study is the positive impact of possessing a separate silkworm rearing house by farmers on their technology adoption. Assistance for construction of rearing houses could improve the general level of technology adoption to a considerable extent.

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Article scientifique

ANALYSE DE L'INTENSITE DE L'ADOPTION PAR LES FERMIERS DU PAQUET TECHNOLOGIQUE D'HYBRIDES BIVOLTINS AU KARNATAKA EN INDE

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R E S U M E

Nous avons analysé l'intensité de l'adoption du paquet technologique d'hybride bivoltins avec le modèle de régression de Poisson afin d'identifier les facteurs déterminant la sélection des composants de la technologie HYV contrairement à la pratique habituelle de classification des fermiers en adoptants et non adoptants basée sur le pourcentage apport/technologies utilisées. Les résultats montrent que quelques facteurs socio-économiques sont des déterminants significatifs du choix des fermiers dans l'adoption de technologies individuelles prises parmi un paquet technologique.

Mots-clés: Adoption partielle, hybride bivoltin, modèle de Poisson.



INTONATION OF PHYSIO-BIOMOLECULAR INDICES IN TASAR SILKWORM *ANTHRAEA MYLITTA* CHALLENGED WITH *NOSEMA* SP.

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ABSTRACT

Biochemical indices were measured in haemolymph and fat body of *Antheraea mylitta* challenged with *Nosema* sp. In addition to the changes in lipid peroxidation and total hydroperoxides, the activities of enzymes such as phenol oxidase, acid phosphatase and catalase were investigated in order to understand their variation with respect to *Nosema* infection. We observed a significant increase in lipid peroxidation level (0.077 and 0.139 nmol /mg protein of fat body) and total hydroperoxides (1.88 and 2.45 nmol/mg protein) of the fat body during infection period, suggesting that the animals are under stress. Similarly, higher level of phenol oxidase (11.55 and 18.11 nmol dopachrome/ min/ mg protein) and acid phosphatase (1.41 and 2.39 nmol of p-nitrophenol released/h/mg protein), observed during infection indicate that the animals may use these enzymes to counteract stress in the tissues. This study points towards the changes in lipid peroxidation, total hydroperoxides, phenol oxidase, acid phosphatase and catalase in tissues of *A. mylitta* as physio-bio molecular biomarkers of infection.

Key words: *Antheraea mylitta*, enzymes, *Nosema*, oxidative stress.

INTRODUCTION

Tropical tasar silkworm, *Antheraea mylitta* D. is an economically important polyphagous sericigenous insect. It is an important cog of Indian non-mulberry silk industry. Since these tasar silkworms are reared outdoors, are more vulnerable to various diseases, viz., pebrine, virosis, bacteriosis and mycosis. Among these diseases, pebrine caused by *Nosema* sp. is considered a major threat causing production loss up to 20-25 % (Sahay *et al.*, 2000). In this regard, substantial amount of work has been done on the impact of *Nosema* sp. on *A. mylitta* such as, larvae infected with *Nosema* sp. show declined food consumption and relative growth rate (Rath *et al.*, 2003), deterioration of cocoon characters (Velide *et al.*, 2013), enhanced level of total haemocyte count (Madhusudhan *et al.*, 2011) and lower level of excretory substances (Renuka and Shamitha, 2012). However, the detailed physiological mechanism behind deterioration of larval and cocoon characters, fecundity and hatching, which is

sure to have a negative impact on the next generation is still unclear.

As ingestion is the main entry route of many pathogens, the intestinal epithelium is the first line of defence against invasion and dissemination of pathogenic microorganisms. In this view, insect innate immune system plays a vital role in the defence against microorganisms (Medzhitov and Janeway, 1997). One of the most immediate epithelial responses in mammals to combat the pathogen is the generation of antimicrobial reactive oxygen species (Cohn *et al.*, 1994; Geiszt *et al.*, 2003). It is to be noted that ROS can affect both entomopathogens and the host tissues, and may lead to damages of cellular biomolecules such as lipids, proteins and nucleic acids.

Further, the main source of cell damage under intestinal pathogens of vertebrates is lipid peroxidation (LPX) and enhanced production of ROS. However, there is a scarcity

of reports on exploring the oxidative stress to which tissues are subjected to and the relative enzyme activities when insects are infected with pebrine. Therefore, the purpose of the study was to assess whether *Nosema* sp. infection can lead to change in the balance between ROS and antioxidants in tasar silkworm *A. mylitta* as well as change in enzyme activity. To test the hypothesis, we examined the effect of *Nosema* infection on oxidative stress and stress enzyme in the fat body of *A. mylitta*.

MATERIALS AND METHODS

Isolation of spores and insect inoculation

To isolate *Nosema* spores, diseased larvae of Dabacoraces were homogenized in 0.6 % K_2CO_3 , filtered and the filtrate was centrifuged at 3000 rpm for 15 min. Spores were purified on discontinuous sucrose gradient (25, 50 and 75 %) by centrifugation at 4000 rpm for 10 min. The spores were collected from the sediment and washed in distilled water thrice and stored as stock at 4 °C in 0.85 % NaCl until use. The spores were suspended in distilled water and spore count was enumerated by haemocytometer. The stock solution was diluted to obtain an inoculum dosage of 10^6 spores ml^{-1} . Healthy fifth instar (after fourth moult) larvae of Daba TV race were starved for 3–4 h to induce hunger and then fed on the *Terminalia arjuna* leaves smeared with inoculum dosage. Healthy larvae were maintained to establish control values for physio-biomolecular indices.

Sample preparation

The fat bodies were dissected out from seven day old Vth instar larvae and thoroughly washed in ice-chilled phosphate buffer (50 mM, pH 7.0) to remove traces of haemolymph. Tissue samples (fat body) were homogenized in ice-cold buffer (50 mM sodium phosphate buffer, pH 7.0). Homogenization was carried out in an ice-chilled motor driven Teflon Potter-Elvehjem homogenizer and centrifuged at 8000 x g for 15 min at 4°C. The supernatant was used for biochemical analysis.

Estimation of lipid peroxidation

Lipid peroxidation (LPX) level was assayed by

measurement of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids hydro peroxides. MDA was determined by the TBA reaction as described by Baror *et al.* (2001) with slight modification. Briefly, the reaction mixture contains 0.1 ml of sample and 0.9 ml of 0.8 % aqueous solution of TBA (in 20 % TCA). The mixture was heated at 95 °C for 60 min and cooled under room temperature. The supernatant was read at 532 nm after removal of any interfering substances by centrifuging at 4000 x g for 10 min. The amount of MDA formed was expressed as nmol MDA/mg protein.

Estimation of total hydroperoxides

Total hydroperoxides were determined spectrophotometrically according to the method of ferrous oxidation with xylene orange (FOX1) (Wolff, 1994). Hydroperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined by using ferric sensitive dyes as an indirect measure of hydroperoxide concentration. Xylene orange binds ferric ions with high selectivity to produce a coloured (blue-purple) complex. The absorbance was read at 560 nm after removal of any flocculated material by centrifugation at 4000 x g for 10 min. The signal was read against an H_2O_2 standard curve and levels were expressed as nmol/mg protein.

Catalase

Catalase activity was determined according to Aebi (1974). The method is based on the decomposition rate of H_2O_2 by the enzyme. The assay mixture contained 2.9 ml of 12 mM H_2O_2 and 0.1 ml of sample (100 µg protein). Absorbance was measured at 240 nm and CAT activity is expressed as nkat/mg protein (1 katal = 1 mol sec^{-1}).

Phenol oxidase

The phenol oxidase (PO) activity was recorded in the form of dopachrome production, a product of the oxidation of L-dopa by the enzyme PO. The reaction mixture contains 850 µl phosphate buffer (100 mM, pH 7.4), 100 µl L-dopa (10 mM) and 50 µl sample. The change in absorbance was recorded at 490 nm and enzyme activity was expressed as nmol dopachrome/ min/ mg protein

using a molar extinction coefficient of $3.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Acid phosphatase

Different samples of haemolymph and fat body of control and pebrinised insects were subjected to ACP activity assay as per protocol used by Arif *et al.* (2004) with slight modifications. The p-nitrophenol was used for the preparation of a standard curve. The activity of the enzyme was articulated as nmol of p-nitrophenol released/h/mg protein.

Protein assay

The protein content was estimated by the Bradford (1976) method using bovine serum albumin as standard.

Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). Difference between control and treatment was analyzed by Student's t-test. Differences were considered statistically significant when $P < 0.05$. Further inter-relationship was analyzed by determining the correlation effects.

RESULTS AND DISCUSSION

Significant variations in LPX levels were observed among the control and treated lots. LPX mean levels in control and infected larvae were 0.077 and 0.139 nmol/mg protein, respectively (Figure 1A). LPX serves as an indicator of oxidative damage in cells and tissues (Pampanin *et al.*, 2005). Earlier studies suggested that enhanced level of LPX was detected during microbial infection in insects (Wang *et al.*, 2001; Dubovskiy *et al.*, 2008). It has been reported that one of the most immediate immune response of the tissues involves the production of ROS to fight against microbial infection in insects (Ha *et al.*, 2005 a, b; Ryu *et al.*, 2010). The increased immune response during infection could be the possible reason for the increase in the rate of ROS formation, resulting in oxidative stress. Further, higher level of total hydroperoxide (H_2O_2 and other water soluble hydroperoxides) as observed in infected larvae (Figure 1B) when compared to healthy ones may indicate

increased formation of free radicals, thereby, enhancing the LPX level in fat body of larvae which corroborates the earlier report (Wolff, 1994). This is also evident from a significant positive correlation between total hydroperoxide and LPX in fat body samples (Figure 2A, $P < 0.05$). In *A. mylitta*, reduction of larval (Madhusudan *et al.*, 2012) and cocoon weight (Velide *et al.*, 2013) was also observed during *Nosema* infection. This may be due to an increase in the production of ROS and oxidative damage in response to diseases.

In the present study, higher level of PO activity was recorded in pebrine infected larvae (Figure 1 C, D). Change in PO activity may be taken as immediate response of insect during infection. Cell organelles are surrounded with lipid membranes that get damaged due to lipid peroxidation. Damaged membrane loses its fluidity and integrity. In the present study, ACP activity was significantly enhanced in diseased larvae when compared to healthy ones (Figure 1 E, F, $P < 0.05$). Earlier studies reported that, injured lysosomes release hydrolytic enzymes in cytoplasm leading to auto degradation of cellular proteins and interferes with protein synthesis and intracellular transport of vital compounds. Lipid peroxidation in membrane lipids plays an important role in cell physiology and pathology as there are a number of membrane bound enzymes whose activity is altered by lipid peroxidation (Gaikwad *et al.*, 2010).

Determination of antioxidant status of larva exposed to *Nosema* is important to understand the toxic mechanism and predict the damage potential in the organism. An increased oxidative stress suggests elevated activity of antioxidant enzymes, thereby protecting animals from oxidative stress (Halliwell and Gutteridge, 2001). The principal H_2O_2 scavenging enzymes CAT showed increased levels in fat body of *A. mylitta* in response to *Nosema* infection (Figure 1 G). This suggests that during infection period, cell might elevate the rate of H_2O_2 production in tissues, and as a consequence, an elevation of CAT may be observed. In this regard, upregulation of CAT activity was observed in honey bee, *Apis mellifera* infected with *Nosema ceranae* (Dussaubat *et al.*, 2012).

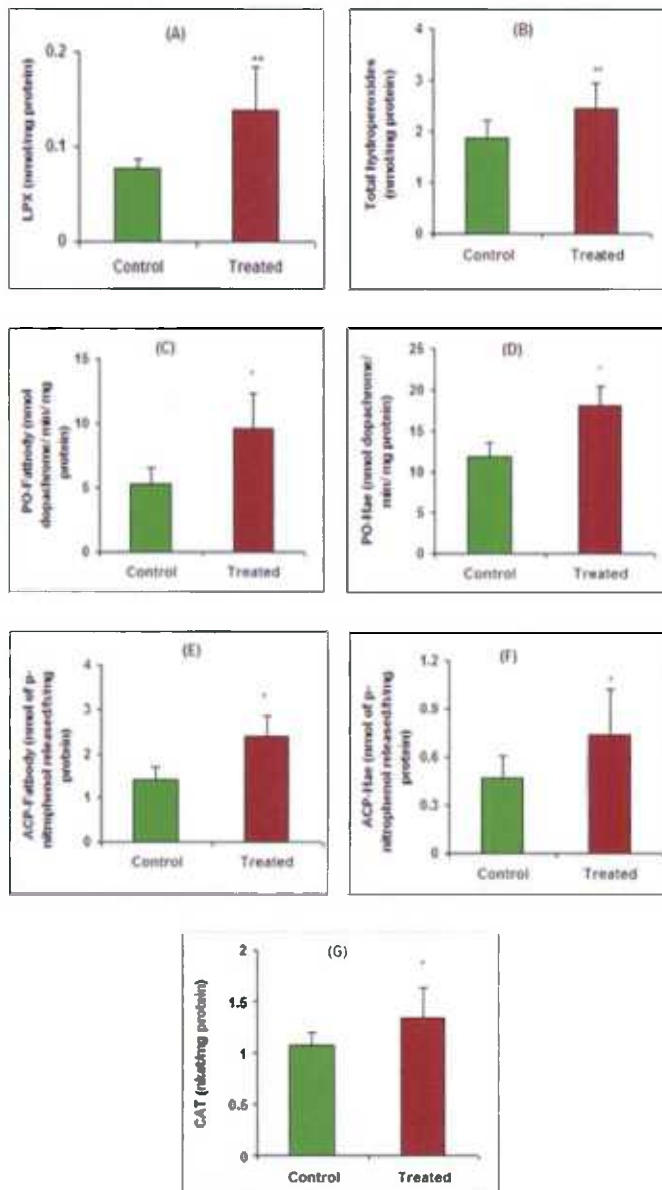


Figure 1 A-G: Intonation of physio-biomolecular indices in tassar silkworm *A. mylitta* challenged with *Nosema* spores (A) Lipid peroxidation, (B) Total hydroperoxide, (C, D) Phenol oxidase activity of fatbody and haemolymph, (E, F) Acid phosphatase activity of fatbody and haemolymph and (G) Catalase activity of fat body of control and treated tassar silkworm, *A. mylitta*. Data are expressed as mean $\pm$ SD (n = 9). Symbols indicate the significant difference between control and treated silkworm at $P < 0.05$ and $P < 0.01$.

However, in early stages (newly hatched larvae), a biphasic response of CAT activity was observed in whole body mass of pebrinised larvae (Madhusudhan *et al.*,

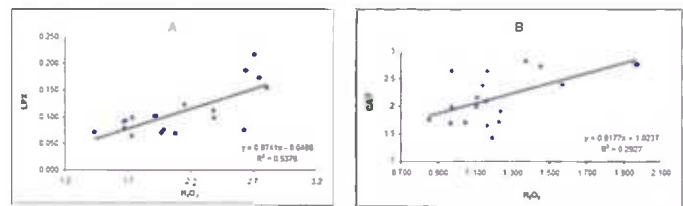


Figure 2: Correlation of physio-biomolecular indices in tassar silkworm, *A. mylitta* challenged with *Nosema* spores (A) Correlation of LPX vs H_2O_2 and (B) Correlation of CAT vs H_2O_2 .

2012).

The present study demonstrates that *Nosema* can significantly modulate LPX and ROS production in the fat body of tassar silkworm, which is a disorder of pebrine disease. To protect against oxidative stress, CAT activities get activated in fat body of *Nosema* infected larvae. This is indicated by a significant positive correlation observed between oxidative stress indices and antioxidant enzymes (Figure 2 B). Despite this protective response, *Nosema* infected larvae have reduction of growth and development compared to healthy ones (Rath *et al.*, 2003), suggesting that they are unable to cope with the physiological stress due to *Nosema* infection over a long period.

CONCLUSION

A significant increase in lipid peroxidation and total hydroperoxides of the fat body as determined during infection period is an indication that the insects are under stress. Similarly, higher level of enzymes manifested during infection may be used by the animals to counteract stress in the tissues. The changes in lipid peroxidation, total hydroperoxides, phenol oxidase, acid phosphatase and catalase in tissues of *A. mylitta* are identified as physio-biomolecular biomarkers.

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Article scientifique

INDEXES PHYSIO-BIOMOLECULAIRES CHEZ LE VER A SOIE TASAR *ANTHRAEA MYLITTA* SOUMIS A DES SP. DE *NOSEMA*

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RESUME

Nous avons mesuré des indexes biochimiques dans l'hémolymph et le corps adipeux d'*Antheraea mylitta* soumis à *Nosema* sp. En plus des changements dans la peroxydation des lipides et les hydroperoxydes totaux, nous avons mesuré les activités enzymatiques telles que la phénol oxydase, les phosphatases acides et la catalase afin de comprendre leurs variations lors d'une infection par *Nosema*. Nous observons une augmentation significative du niveau de peroxydation des lipides (0,077 et 0,139 nmoles / mg de poids frais) et des hydroperoxydes totaux (1,88 et 2,45 nmoles/mg de protéine) dans le corps adipeux lors de l'infection, ce qui indique un stress. De même, de hauts niveaux de phénol-oxydase (11,55 et 18,11 nmoles de dopachrome/min/mg de protéine) et de phosphatase acide (1,41 et 2,39 nmoles de p-nitrophénol relarguées /h/mg de protéine) observés pendant l'infection indiquent que les animaux utilisent ces enzymes pour contrecarrer l'effet du stress dans les tissus. Cette étude souligne que les variations de la peroxydation des lipides, des hydroperoxydes totaux, de la phénol-oxydase, des phosphatases acides et de la catalase dans les tissus de *A. mylitta* sont des marqueurs moléculaires de l'infection.

Mots-clés: *Antheraea mylitta*, enzymes, *Nosema*, stress oxydatif.



SILKWORM SEED QUALITY REGULATION IN INDIA THROUGH LEGISLATIVE ENACTMENT

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ABSTRACT

India made a giant leap by aligning itself with the countries promoting free trade of sericulture produce in line with other commodities with minimal intervention from the local and central governments, by introducing vital amendments in the Acts, Rules and Regulations governing production, possession and distribution of silkworm seed. Through this, setting of benchmarks in various aspects of quality silkworm seed production is aimed leading to achieving the basic minimum standards which are essential to foster production of quality cocoons and thereby the raw silk production to gradually meet the global standards. In the erstwhile regime wherever regulatory intervention was in force, no person was permitted to produce, possess, distribute or transact any of the sericulture produce unless he was the holder of a valid license issued by the authority. But in the wake of liberalization, privatization and globalization, the market was opened up in all sectors with a hope that fierce competition without any jurisdictional restriction would pave way for quality improvement and at the same time would protect the consumers' interest. Realizing this, close on the heels of the recommendations of diverse committees to do away with all regulatory interventions and to establish linkages between the producers and processors, the amended silkworm Seed Act was enacted followed by passing the concerned Rules and Regulations for the whole of India. To implement the uniform rules and regulations, an apex committee *viz.*, Central Silkworm Seed Committee (CSSC) and subordinate committees such as Registration Committee (RC) and Hybrid Authorization Committee (HAC) were constituted. This was followed by appointment of 63 Seed Analysts for the purpose of system certification and 295 Seed Officers for product certification across India. They were subsequently trained in all the techno-legal aspects apart from the hands-on training on disease detection. The Registration Committee met several times and after careful scrutiny, 14074 applicants were registered under the Act. These include 3033 seed producers across different sectors (Mulberry:748; Tasar:1783; Eri:78 and Muga:424), 656 chawki rearers (Mulberry 625; Tasar: 27; Eri:4) and 10385 seed cocoon producers (Mulberry:10400; Tasar:2493; Eri:371; Muga:810). The registered producers were linked with the corresponding SAs and SOs for regular inspection and monitoring. All the stakeholders are sensitized to practice self certification while producing and distributing silkworm seed. The processes involved in the implementation of the Act aiming to a massive country-wide quality consciousness and market appreciation is discussed.

Key words: Registration, Seed Act, Seed Analysts, Seed Officers, self certification.

INTRODUCTION

Silkworm seed production in India has witnessed massive change over the past few years. Silkworm seed producers both at Government and private sector have realized the importance of adopting newer and efficient methods to improve the quality and recovery and thereby improving the profitability of the enterprise as a whole.

Until recently, Karnataka, the leading silk producing state in the country had a legislative act that governed all the activities of sericulture in the state right from seed cocoon raising to production of silk yarn and its marketing. No person was permitted to produce, possess, distribute or transact any of the sericulture produce unless he was the holder of a valid license issued by the authority. But in the wake of liberalization, privatization and globalization, the market was opened up in all sectors with a hope that fierce competition without any jurisdictional restriction would pave way for quality improvement and at the same time would protect the consumers' interest.

In early 2004, the Revenue Reforms Commission set up by the Government of Karnataka in a bid to streamline the sericulture industry in the state recommended to the Government to remove all modes of licensing and control. The Commission suggested setting up a Silkworm Seed Regulatory Authority to certify the quality of silkworm seed apart from recommending to amend the existing Act to allow direct linkage between the producer and the processor. The general understanding was that all the transaction processes would be deregulated without compromising the quality of the silkworm seed.

Close on the heels of this recommendation, Central Silk Board shouldering the responsibility of developing the sericulture industry across the country, intended meaningful modifications in the existing rules and regulations governing sericulture with an intention to liberalize trade practices of sericulture produces in the country in consonance with other commodities. Accordingly, Central Silk Board (Amendment) Act, 2006 was passed by the Parliament which was followed by notification of relevant rules and regulations. The implementation of the Central Act encompassed various important modules as laid down in the Act.

Constitution of the Apex and subordinate Committees

Government of India constituted the Central Silkworm Seed Committee (CSSC) which would oversee the implementation of its various provisions and hence referred to as the Apex Committee. CSSC is a 17 member committee chaired by the Vice-chairperson of Central

Silk Board. The Member Secretary, Central Silk Board is the Secretary to the committee. Subsequently, CSSC constituted the Hybrid Authorization Committee to recommend to the Govt. of India, the silkworm hybrids for commercial exploitation after due multilocal independent trials and assessments. CSSC also constituted the Registration Committee which is chaired by the Director, National Silkworm Seed Organization (NSSO), for registering Silkworm Seed Producers, Chawki Rarers and Seed Cocoon Producers and to monitor the production and distribution as laid out in the Regulations.

Method of achieving basic premise of quality improvement in seed sector

Traditionally, Indian sericulturists enjoy third party surveillance for quality assurance and this surveillance principally vested with the various arms of the Government. It implies that neither the seller nor the buyer is responsible for assuring the quality of the produce. The provisions of the amended Act are a glaring departure from this practice. It exhorts the producer and user to ensure the quality of the produce where the interference from the govt. arm is minimal. To ensure such standards, the Act and the Regulation stipulate qualitative and quantitative benchmarks for each aspect involved.

Setting the benchmarks in various aspects of quality silkworm seed production are mainly aimed at achieving the basic minimum standards which are essential to foster production of quality cocoons and thereby the raw silk production to gradually meet the global standards. It is pertinent to mention that the Act, Rules and Regulations cover only the movements of various inputs in seed production such as seed cocoons, seed and chawki silkworm (young instar larvae). Laying down quality guidelines in seed production and distribution ensures that adequate investment (both human and infrastructure) are made at the national level without destabilizing the existing seed production system and quality consciousness inculcated both in small and large scale seed producers. The registration processes for seed producers would benefit all the stake holders involved in the downstream and upstream activities of commercial seed production.

Registration process of Producers

It is basic to concept of silkworm seed sector reforms that all the entrepreneurs involved and the producers of the raw material - the seed cocoons - are registered. The Registration Committee meets after specific intervals, examines the applications submitted and award the registration to eligible candidates which is valid for 5 years. While existing producers get their registration straight away, the new applicants need to undergo training in the concerned discipline for 3 months. Through the arrangement made to assist the new applicants, 57 seed producers and 52 chawki rearers have been trained and obtained their registrations. In total, 14074 persons have been registered under the Act as detailed in Table 1. These include Registered Seed Producers (RSPs), Registered Chawki Rearers (RCRs) and Registered Seed Cocoon Producers (RSCPs) from all the four different sectors such as Mulberry, Tasar, Eri and Muga.

Table 1: State-wise Details of registration done under Seed Act

State	RSPs	RCRs	RSCPs	Total
Andhra Pradesh	30	47	296	373
Assam	433	6	213	652
Bihar	39	5	147	191
Chhattisgarh	49	27	45	121
Jharkhand	1255	0	480	1735
Karnataka	408	336	7953	8697
Madhya Pradesh	131	49	11	191
Maharashtra	53	6	21	80
Manipur	17	20	0	37
Meghalaya	32	0	423	455
Nagaland	6	4	41	51
Orissa	252	26	0	278
Tamil Nadu	24	30	189	243
West Bengal	276	65	515	856
Other States	28	35	51	114
Total	3033	656	10385	14074

The RSPs, RCRs and RSCPs registered so far belong to different sectors such as Mulberry, Tasar, Muga and Eri. While a major share of RSPs are from Tasar (58.79 %), Eri was minimal (2.57 %). Mulberry and Muga constituted 24.65 and 13.98 %, respectively. Quite interestingly, the lion's share of registration in the case of RCRs, belonged

to Mulberry sector (95.27 %) followed by Tasar (4.12 %) and Eri (0.61 %). In the case of RSCPs, 86.92 % belongs to Mulberry, 6.58 % to Tasar, 2.78 % to Eri and 3.72 % to Muga (Table 2). This gives a clear picture that the awareness among the stake holder to get registered is fairly wide mainly because of the effort of the local extension officers.

Table 2: Sector-wise break-up of Registered Producers

Producer type	Mulberry	Tasar	Eri	Muga	Total
RSP	748	1783	78	424	3033
RCR	625	27	4	0	656
RSCP	9027	683	289	386	10385
Total	10400	2493	371	810	14074

Registration is awarded to each applicant subject to certain conditions. The registered producers are obligated to follow the conditions laid down failing which the registration can be suspended or cancelled apart from imposing penalties such as monetary fine in commensuration with the intensity of the violations. The basic responsibilities of the Seed Producers and Chawki Rearers are listed below:

Registered Seed Producers

1. Procure only certified parent seed cocoons from the seed cocoon producers and produce only the kind or variety of hybrid seeds as specified without changing the venue.
2. Adhere to the conditions specified in the Central Silk Board Regulations 2010 on production and supply of parent seed cocoons through seed cocoon producers.
3. Produce the seed adopting the methods and procedure as detailed in the regulation and maintain the quality standards by conducting seed testing and certifying the seed as specified in the regulation.
4. Maintain up to date record of seed cocoon procurement, quantity of seeds produced, moth examination details and disease incidence and disposal of seeds.

Registered Chawki Rearers

1. Procure only the certified hybrid seed from RSPs that meet the quality standards and chawki rear only the kind or variety of hybrid seeds as specified without

changing the venue for distribution among farmers.

2. Rear the chawki worms adopting methods and procedure stipulated in the Regulation and conduct testing of chawki worms as stipulated.
3. Maintain up to date record of hybrid seed procurement, larval examination details, disease incidence and disposal of chawki worms.
4. If there is an incidence of pebrine, discontinue the rearing and reject and destroy the entire batch of larvae and shall take prophylactic measures to prevent contamination after intimation to the concerned authority.
5. Certify the chawki worms as 'tested ok' if found disease free and authenticate with stamping including his name and date of transaction.

Continuous and mandatory inspection processes

All RSPs and RCRs are supposed to abide by the quality standards specified from time to time by the CSSC and those who contravene the conditions of registration are liable to be punished under the provisions of the Act. The personnel authorized by the Committee inspect the RSPs and RCRs as and when required for the purpose of verification and fulfilment of all the laid down conditions / guidelines and procedures for silkworm seed production and trade.

Inspection and Seed Certification

Since the silkworm seed and its quality determines the quality and productivity of the cocoons and silk, seed assumes greater significance and relevance in today's context of globalization and free trade. Testing of the seed for disease freeness in fact starts from seed cocoon level which leads to quality silkworm seed. Protocols for testing of larvae, pupae, mother moth and eggs are essential to make the testing process uniform and foolproof leading to silkworm seed totally disease free and hence incorporated in the guidelines for monitoring the quality aspects especially disease freeness. This is necessary to achieve the set quality standards at different levels and to determine / certify seed cocoons, seed and chawki worms before they are declared fit for marketing.

Seed certification system

As the reliability of certified seed is decided based on the findings through microscopic examination, chances of inadvertent mistakes in identification of pebrine spore cannot be ruled out. Therefore, it is desirable that rather than depending entirely on mother moth testing (product certification), additional safety measures in determination of disease freeness are added up in improving the overall quality of seed. Hence, in silkworm seed production process, a two tier certification system is advocated. This involves a) System certification by Seed Analysts not less than once a year and b) Product certification by Seed Officers not less than once in three months.

System Certification

Product certification cannot be considered failsafe for the purpose of ensuring the absolute disease freeness of silkworm eggs produced and supplied albeit the laid down system of mother moth testing is followed meticulously as disease can be inadvertently passed through, during the mother moth testing especially when the level of infection is low. But when system certification is also followed, it ensures multiple levels of testing at downstream and upstream level. This enhances the reliability of testing and the certification of seed. The advantages of system certification are that the inputs are raised from already checked and verified basic seed at the time of multiplication and also from the certified parental seed. Seed cocoons are raised by the progressive farmers ensuring the requisite quality controls and such cocoons are purchased only after ensuring that it has met the stipulated norms including disease freeness. Processing of seed cocoons are done in the seed production centres where required attention including testing is taken care by experienced and trained staff during seed production.

Further, by testing chawki reared worms, the resultant product is further ensured to be disease free. Thus, system certification has the advantages to enhance the reliability and proper usage of eggs as disease free. System certification and product certification are therefore considered as complementary to each other in meeting the quality standards of silkworm seed. In the entire

certification system, the procedure starts from generation or procurement of quality seed cocoons. To raise disease free seed cocoons, the P1 seed should be the certified one as disease free.

Seed Analysts have the responsibility to carry out the inspection of grainages and CRCs to ensure that the entire recommended and necessary infrastructure is in place and they possess adequate trained manpower. SAs also confirm that certified seed cocoons are procured by the grainages either from ASRs or from farmers belonging to seed areas and processing of seed cocoons are done as per the set guidelines and the Chawki centres procure only certified disease free layings from RSPs. The Seed Analyst looks into these aspects on an annual basis. It is also necessary to verify the available infrastructure physically and activities at various other levels of production and end usage by records.

Testing for pebrine disease is done at different levels, viz., late stage silkworm, pupa, moth, eggs and young worms. Preparation of slide for microscopic examination, however will vary with the testing material used, though the testing system, in principle, remains the same.

Product Certification

Silkworms are affected by diseases caused by viruses, bacteria, fungi and protozoa. These diseases cause considerable damage to the silkworm crops. Of these, disease caused by a highly virulent protozoan parasitic microsporidian, *Nosema bombycis* Nageli, known as pebrine is the most critical and unique by its bimodal infectivity and transovarial transmission. The infection of the pathogen occurs in two ways viz.,

1. Vertical infection, through trans-ovarian transmission.
2. Horizontal spread through oral entry.

Vertical transmission known as primary infection is passed on by infected mother moths to off-spring through egg, while the horizontal spread known as secondary infection is caused by ingestion of mulberry leaf

contaminated with pathogens by the silkworm. Silkworm larvae infected by the pathogen by any one of these two ways are to be controlled to certify the seed as they form potential source of further contamination resulting in the spread of disease.

The responsibility of product certification principally lies with the Seed Officers. They inspect or verify that the seed cocoons are procured either from seed area or generated through ASRs and regular crop inspection are carried out as per the crop supervision procedure. Before purchase, pupal examination for disease freeness of seed cocoons is also carried out by the seed producers. The Seed Officers conduct random checks to ascertain whether product certification procedures are properly followed. They ensure that the norms and quality checks followed in the process of procurement of cocoons, its processing and its product certifications are in accordance with the norms and standards set by the Committee under the Act.

Testing and certification location

- Besides conducting inspection and testing at P1 seed rearers level, seed production centres and CRCs, detailed analysis or cross checking shall be done at Central Seed Testing Laboratory or in the Accredited Laboratories. For this purpose, Silkworm Seed Technology Laboratory (SSTL), Kodathi, Bangalore, Karnataka is the Central Seed Testing Laboratory (CSTL).
- Central Sericultural Research & Training Institute (CSRTI), Mysore, Karnataka; Andhra Pradesh State Sericulture Research and Development Institute (APSSRDI), Hidupur, Andhra Pradesh; Karnataka State Sericulture Research and Development Institute (KSSRDI), Thalaghattapura, Karnataka; CSRTI, Berhampore, West Bengal; Central Tasar Research & Training Institute (CTRTI), Ranchi, Jharkand; CSRTI Pampore, Jammu & Kashmir and Central Muga Eri Research & Training Institute (CMERTI), Ladoigarh, Assam shall be the accredited laboratories under the provisions of this Act.

Timely action for curative/control measures in the case of disease incidences

The Seed Officers are the primary agents of implementation in the field and thus quickly act on receipt of complaints from farmers on defective seed, particularly, diseases and crop loss due to pebrine and verify the lots in production or sale and take action to withdraw the lot from sale. They take action to destroy the seed crop if found infected with pebrine after informing the Seed Analyst, the Central Seed Testing Laboratory and the farmer about the crop status. They physically check the actions of post disease incidence measures namely, clean up of premises, destruction of lots and corrective measures initiated by seed producers, including disinfection to maintain hygiene.

Training for Seed Analysts and Seed Officers in techno-legal aspects

It is imperative on the part of SAs and SOs to be aware of various provisions of the Act and the modus operandi of on-the-ground implementation. To achieve this, the Seed Officers and Seed Analysts were imparted a two days' training on the techno-legal aspects of the amended Act apart from offering hands on training in the procedure of prebrine detection. The training was imparted to 333 personnel in 29 batches at 9 centres across India (Table 3). Before initiating the training to the SAs and SOs, two trainers from each training centre were trained at the Registration Committee's facility.

Table 3: Details of training imparted to Seed Analysts and Seed Officers

Sl. No.	Training Centre	Number of batches	SAs/SOs trained
1	SSTL, Kodathi	7	92
2	CSRTI, Mysore	4	59
3	KSSRDI, Bangalore	3	28
4	APSSRDI, Hindupur	2	21
5	CSRTI, Berhampore	3	40
6	CSRTI, Pampore	2	12
7	CTRTI, Ranchi	3	47
8	CMERTI, Lahdoigarh	4	22
9	DoTH, Itanagar	1	12
Total		29	333

Impact on Import and Export of silkworm seed

The amended Act allows the import and export of hybrid silkworm seed by RSPs subject to certain laid down procedures. Permission is not granted to any RSPs to import pure silkworm races. The RSPs can import or export the seed for commercial purpose only on obtaining the import or export licence and under prior permission. Only those seed cleared by the quarantine authorities of exporting country can be imported to India, the rearing performance and disease freeness of which need to be submitted to the authorities in the specified formats, subsequent to the import within the stipulated time period. Similarly, it is obligatory to get necessary quarantine clearance from the designated laboratory for the seed before being exported.

Awareness creation among the stakeholders

Successful implementation of the various provisions of the amended Act solely depends of the awareness among the stake holders which not only include seed producers, Chawki rearers and seed cocoon producers but the official machinery and non-governmental organizations involved in the sericulture development. Quite a few awareness programmes have been conducted at different parts of the country which also doubles up as registration drives in which efforts are made to register new producers and seed farmers. Other important initiatives were to prepare and publish a manual on the implementation procedure of the Act to techno-legally empower the Seed Analysts and Seed Officers. This was followed by publishing of bilingual booklets in English and vernacular such as Hindi-English, Kannada-English, Telugu-English, Tamil-English and Bengali-English encompassing all possible questions on Seed Act with answers in simple language.

On the anvil

- ✓ Refresher training to all RSPs and RCRs
- ✓ A minimum of fifty awareness programmes in the coming 3 years across the country
- ✓ Establishment of 8 isolated testing laboratories for seed testing purpose to bolster the implementation of the provisions of the amended Act
- ✓ Training in quarantine and monitoring to 30 personnel

in the coming 3 years

- ✓ Nomination of new seed officers and seed analysts in place of those who superannuated
- ✓ Certificate training for prospective seed producers and Chawki rearers

IMPACT

Central Silk Board (Amendment) Act, 2006 principally envisages maintenance of superior quality of silkworm eggs in the whole of India despite the introduction of free trade regime where the interference of Government regulation in the transaction of reelable cocoons and silk yarn ceased to exist. The independent certification of silkworm seed, free movement of cocoons and silkworm seed would pave way for direct linkage

between various stake holders such as seed farmer & seed producer, seed producer & chawki rearer, farmer & reeler and reeler & weaver to ensure backward and forward linkages with a view to build inter-relationship among the stakeholders and to ensure availability of quality sericulture products in sufficient quantities. Since in a free trade regime, only quality produce sustains, the amendment of Central Silk Board Act would bring in quality consciousness among producers which would automatically lead to production of quality silkworm seed, its distribution and in turn high quality of cocoons and raw silk production in the country. The stakeholders express in various forums that they realize the empowerment and shoulder the responsibilities of quality maintenance of their produce by themselves.

Rapport technique

REGLEMENTATION SUR LA QUALITE DES GRAINES DE VER A SOIE EN INDE A TRAVERS L'ACTE LEGISLATIF

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RESUME

L'Inde a fait un bon de géant, s'alignant sur les pays prônant le libre échange pour les produits de la sériciculture comme pour d'autres produits avec une intervention minimale des gouvernements locaux et centraux, en introduisant des amendements vitaux dans les lois et les règlements qui gouvernent la production, la propriété et la distribution des graines de ver à soie. A travers cela, était visée la mise en place de références relatives à divers aspects de la production de graines de ver à soie de qualité conduisant à l'établissement de standards minimums de base essentiels pour obtenir une production de cocons de qualité et donc la production de soie grège afin d'atteindre progressivement les standards globaux. Sous les régimes d'autrefois où la réglementation était forte, personne ne pouvait produire, posséder, distribuer ou commercialiser aucun produit séricicole sans une licence délivrée par les autorités. Mais dans le sillage de la libéralisation, de la privatisation et de la globalisation, le marché s'est ouvert dans tous les secteurs avec l'espoir qu'une compétition féroce sans restrictions juridiques améliore la qualité et protège le consommateur. En réalisant cela, armé des recommandations de divers comités pour éliminer toutes les interventions réglementaires et pour établir des liens entre producteurs et transformateurs, la Loi amendée sur les graines de vers à soie a été promulguée et étendue à l'Inde entière. Afin d'uniformiser les règles et les règlements, un comité directeur a été constitué, à savoir le Central Silkworm Seed Committee (CSSC) ainsi que des comités subalternes tels que le Registration Committee

(RC) et le Hybrid Authorization Committee (HAC). Ceci a entraîné l'embauche de 63 analystes pour le système de certification et de 295 officiers pour la certification des produits à travers l'Inde. Ils ont été formés à tous les aspects techniques et légaux en dehors de la formation à la détection manuelle des maladies. Le RC s'est réuni plusieurs fois et, après un examen minutieux, 14074 demandeurs ont été enregistrés selon la loi. Cela correspond à 3033 producteurs de graines de différents secteurs (Mûrier, 748 ; Tasar, 1783 ; Eri, 78 et Muga, 424), 656 éleveurs de jeunes vers (Mûrier, 625 ; Tasar, 27 ; Eri, 4) et 10385 producteurs de cocons pour le grainage (Mûrier, 1040 ; Tasar, 2493 ; Eri, 371 ; Muga, 810). Les producteurs enregistrés furent liés avec les SAs et SOs correspondants pour une inspection régulière et la surveillance. Tous les intervenants sont sensibilisés à la pratique de l'auto-certification dès lors qu'ils produisent et distribuent des graines de ver à soie. Nous discutons en quoi le processus impliqué dans la mise en place de la Loi conduit à une conscience de la qualité dans l'ensemble du pays et comment le marché l'apprécie.

Mots-clés: Enregistrement, Loi sur le grainage, officiers de grainage, analystes de graines, auto-certification.

NEWS CORNER

TRAINING ON SERICULTURE & SILK INDUSTRY



A special training on sericulture and silk industry organised for the candidates nominated from ISC Member Countries was inaugurated by Prof. K. S. Rangappa, Vice Chancellor, University of Mysore at Central Sericultural Research & Training Institute, Mysore on 9th March. This programme is sponsored by the Ministry of External Affairs, Govt. of India under the Indian Technical Economic Cooperation (ITEC). For more details of the ITEC programme, please visit; www.itec.mea.gov.in

Under the programme, the sponsored candidate shall be provided with return air journey in economy/excursion class, living allowance, accommodation in the place of training in Hotel/Hostel, project allowance, study tour, etc. The cost of organizing the training shall also be met under the programme.

The programme will be of 45 days (6 weeks) duration and the batch strength is 14 persons from 5 countries. The training covers the following subjects.

- (a) Silkworm rearing (Young age and Late age silkworm rearing),
- (b) Mulberry cultivation technologies,
- (c) Silkworm seed production technology,
- (d) Extension management and transfer of technology, and
- (e) Post cocoon technology.

VISIT OF SECRETARY GENERAL AARDO TO ISC



H.E., Eng. Wassfi Hassan El-Sreihin, Secretary General, African Asian Rural Development Organisation (AARDO), New Delhi and Mr. A. W. Anwer, Executive Secretary, AARDO visited the sericultural areas and facilities in Bangalore and Mysore (India) during 25-28, February, 2015. The purpose of the visit was to establish collaboration with AARDO, ISC and Central Silk Board. A meeting between the delegation and the senior officers of ISC and CSB was held on 28-02-2015 at Central Silk Board HQs. The meeting resolved to collaborate between AARDO and ISC for the development of sericulture and silk industry in AARDO Member Countries. The expertise and support from Central Silk Board would be extended towards this purpose.



INTERNATIONAL

SERICULTURAL

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UNITED NATIONS REG. NO. 10416

INTERNATIONAL SERICULTURAL COMMISSION

An intergovernmental organization instituted in 1960 and registered with United Nations

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INFORMATION TO CONTRIBUTORS

SERICOLOGIA is a peer reviewed quarterly scientific journal dedicated to the science of sericulture, published by the International Sericultural Commission. Papers submitted to SERICOLOGIA should carry original contributions of scientific research (basic or applied) or reviews on any aspect of sericulture.

Submission of a paper to SERICOLOGIA implies that it has not previously been published, that it is not under consideration for publication elsewhere and that, if accepted in SERICOLOGIA, it will not be published elsewhere in the same form without the written consent of the Chief Editor.

Manuscripts and illustrations must be prepared in British English on a standard A4 size paper setting. It should be typed in 12 point Times New Roman or Arial font in double spacing and single column with well set margins on top, bottom, left and right. Page numbers should be given at the bottom centre of every page.

The typescript should contain the following features:

- **Title** of the paper should be in a 14 point font. It should be bold typed, centered and fully capitalized.
- **Authors'** full name, address, mobile/fixed line numbers, and email/alternate email address should be in 10 point font and centered underneath the title. The first address should be of the centre at which the study was conducted. In the case of multiple ownership, the authors may indicate the correspondence address.
- **Abstract** must be the gist of the paper which should explain the background, aims, methods, results & conclusion in a single paragraph not exceeding 200 words.
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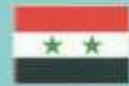
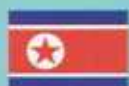
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