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Intrinsically Coloured Silk Produced with Dye Containing Diet by Domesticated Silkworm, Bombyx mori (PM x CSR2)

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ABSTRACT

Silkworm silk is among the most widely used natural fibers for textile and biomedical applications due to its extraordinary mechanical properties and superior biocompatibility. There is a growing interest to introduce more new functionalities into silk while maintaining its advantageous intrinsic properties. Various methods with their merits are assessed to produce functional silk, specifically those with colour and luminescence. There is a highlight on intrinsically coloured and luminescent silk produced directly from silkworms for a wide range of applications and a discussion on the suitable molecular properties for being incorporated effectively into silk while it is being produced in the silk gland. This paper elicits an in vivo uptake of neutral red, a weak cationic azine dye by *Bombyx mori*, which results the production of vibrantly coloured cocoons. It is a green process without any chemical pollution, which offers the development of an insect system to produce coloured silk. It has significant potential applications in sericulture research. The colour change observed in haemolymph and silk gland is evident for the efficacy of the selected dye to incorporate into the silkworm body. The results of larval and cocoon traits shows that there is no significant difference between the control and dye added diet fed groups. The findings suggest that neutral red is a promising candidate dye for the production of environmental friendly colour silk production, which eliminates the generation of dye waste.

Introduction

The textile industry is one of the fastest growing industries of the world. It requires a sequence of chemical processes those are hazardous to the environment. The current inefficient and harmful dyeing processes, which utilize enormous water to rinse excess dye from the coloured fabrics, pollute a large number of water bodies and thus affect the aquatic population (Shahid et al., 2016). Most of these dyes are fade resistant due to their complex chemical structure and gives the water a turbid appearance and foul smell (Kant, 2012; Samanta and Agarwal, 2009; Robinson et al., 2001). Apart from this, the textile effluent comprises various chemicals, heavy metals, carcinogenic amines, etc. Carcinogenic by products formed during the chlorination of most of the dyes (Dias and Petit, 2015). On this background recently researchers are in urge to develop eco-friendly techniques for the production of coloured silk.

The generation of coloured silk using dye mixed diet is a novel area of research to reduce dye waste pollution. It is a promising technology that requires only a small amount of dye as silkworm feed. Besides that, it avoids the excess use of water that is inevitable in conventional dyeing methods. The staining of insect larvae using dye mixed diet has been reported by scientists from the first half of 20th century (Koyler, 1965; Lombardi, 1920; Edwards, 1921). They made trials with vital dyes, anilinic dyes, etc., (Jucci and Ponsevernoi, 1930; Goris and Muehlemann, 1936) and observed that certain dyes pass through the midgut of insects (Roeder, 1953; Zacharuk, 1963). Tansil et al. (2011) introduced an in vivo uptake of rhodamine dyes by *B. mori*. It was a successful attempt, which leads to the production of intrinsically coloured silk, and the characterization studies revealed that the dye incorporated into the fibroin – the core of the silk thread. Successful production of pink cocoons using rhodamine B and formation of differently coloured cocoons using azo dyes were reported by researchers recently (Trivedy et al., 2016; Nisal et al., 2014). The success of this effort relies on the effective incorporation of dye into the silk gland.

There has been a wealth of new information on the pathway of colour giving carotenoid pigments through the silkworm body, in the recent years. Mulberry leaves contain two kinds of pigments such as yellowish carotenoids and green coloured flavonoids (Sakudo et al., 2007). These pigments from the midgut of silkworm reach the silk gland through a biochemical pathway via the peritoneal membrane of midgut and the haemolymph. This is the reason for the yellow coloured haemolymph, silk gland and cocoon of wild silkworm *B. mori* that fed on mulberry leaves (Sakudo et al., 2013). The recent investigations (Suehida et al., 1998; Jouni and Wells, 1996; Doira, 1992) related to this emphasize the biochemical pathway through which the pigments reach the silk gland.

In this study, our concern is the production of coloured silk using the Neutral red dye and the analysis of larval and cocoon traits to find the effect of the dye on *B. mori*.

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Materials and methods

Preparation of modified diet

Neutral red, the dye used in this study had purchased from Merck Chemicals. The modified feed was prepared by spraying an appropriate concentration of dye on to the mulberry leaves. Initially the dye concentration in food was of 0.05 wt % and it was increased then to 0.1 wt %, 0.2 wt % and 0.4 wt% to find whether there is a better colour incorporation in the silkworm body. After these trials, a concentration of 0.2 wt % was fixed.

Rearing of silkworm

PM X CSR2, a multivoltine X bivoltine hybrid is used for the experiment. The eggs had obtained from the Grainage centre, Sericulture Department in the Regional Deputy Directors Office, Trichy, Tamil Nadu and Rearing was done under standard conditions recommended by Krishnaswami et al. (1973). The silkworms were fed with fresh mulberry (*Morus alba*) leaves of V1 variety. On the first day of fifth instar, silkworms were divided into control and experimental groups each having 50 worms. The experimental group was fed with 0.2 wt % neutral red sprayed mulberry leaves from the fifth instar and larvae from each group dissected on the seventh day, before spinning. Both the haemolymph and silk gland were observed to confirm the colour accumulation.

Larva and cocoon traits

The physical parameters such as duration of life cycle, length and weight of larvae, cocoon weight, pupa weight and shell weight of control and neutral red mixed feed administered groups were measured.

Statistical analysis

Data on larval and cocoon traits has statistically analyzed by One Way Analysis of Variance (ANOVA) by using SPSS version 20. All the data were presented as mean ± standard deviation of mean. The significance was calculated at 5% level (values are significant when $P< 0.05$).

Results

The experimental group silkworm fed with neutral red dye sprayed mulberry leaves were observed for feeding and morphological changes. The worms fed mulberry leaves sprayed with 0.2 wt % neutral red incorporate the red colour within one hour. The weight and length of the larval forms in the control and the experimental groups, the colour of the body, silk gland, haemolymph, pupa and cocoon were the parameters studied. The larvae, pupae, cocoon and the emerging moth were maintaining the imparted colour throughout. To the extend the adult silk moth laid red colour eggs. A comparison between control and neutral red fed group has shown in Figure. 1. The duration of the larval instars was same for both the groups and spinning started on the eighth day of V instar. The experimental group fed with the dye had spun intrinsically colored silk with red colour. In the initial trial, the experimental group fed with 0.05 wt % of dye sprayed mulberry leaves spun cocoons with mild red colour. In order to analyze the effect of high concentration on colour incorporation, increased concentrations of dye were evaluated. The concentration of neutral red used and the effects are tabulated in Tables 1&2.

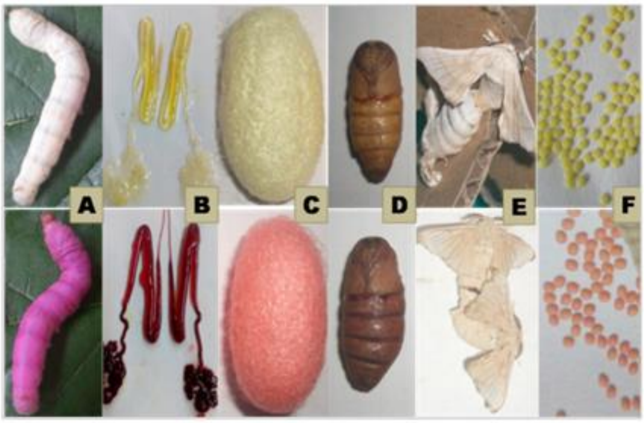

Fig 1. A) V instar larva, B) silk gland, C) cocoon, D) Pupa, E)moth and F) eggs of control and Neutral red added diet group.

Table 1. Concentration and molecular weight of the neutral red used and its impact on the colour of haemolyph, silk gland and cocoons.

Experimental condition	Dye con. (wt %)	Molecular weight (g/mol)	Haemo lymph	Silk gland	Cocoons
Control	–	–			
Neutral red	0.2	288.78			

The results observed demonstrate that the colour intensity of cocoons can reduce by lowering the dye concentration (Fig. 2). However, there is no significant increase in cocoon colour with an elevation in concentration of dye above 0.2 wt %. It is consistent with the studies of Natalia Tansil et al. (2011) using rhodamine B.

Table2. Cocoon colour imparted by different concentrations of dye.

Dye con. (wt %)	Cocoon colour
0.05	Orange
0.1	Light pink
0.2	Pink
0.4	Pink

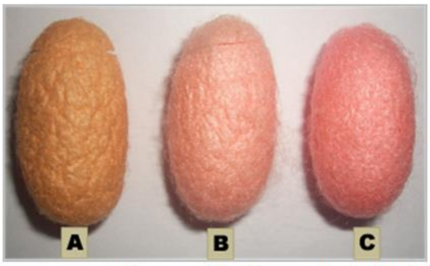

Fig 2. Cocoons of silkworms fed with neutral red added diet in different concentrations. A) 0.05 wt %, B) 0.1 wt %, C) 0.2 wt %.

The statistical analysis of the larval and cocoon traits of control and the experimental group reveals that there is no significant difference between the groups (Table. 3, 4 & 5). From this, it is evident that Neutral red has no harmful effect on the physical and economical parameters of *B. mori*. This is similar to the reports by Kanika Trivedi et al. (2016) which showed no significant difference of cocoon characters between control and rhodamine B added diet fed group of silkworms. Among the two groups neutral red fed group has an average weight of 3.994 ± 0.173 gm on the seventh day of V instar and the average weight of control group silkworms was 3.958 ± 0.134 gm. Lengths of these groups on the same day were 6.99 ± 0.160 cm and 6.97 ± 0.125 cm respectively. Though the maximum cocoon (1.609 ± 0.282 gm) , pupa (1.310 ± 0.243 gm) and shell weight (0.289 ± 0.047 gm) were recorded for control group, highest shell ratio was observed for neutral red fed group (18.204 ± 1.833). As the dye has no significant impact on silkworms other than changing colour, the selected dye in appropriate concentration is suggested for the production of coloured silk**.**

Table 3. Effect of Neutral red on the weight of V instar larva.

Exp. condition	**Day of V instar**						
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control	0.734 ± 0.005	0.848 ± 0.031	1.384 ± 0.147	2.278 ± 0.135	3.314 ± 0.195	3.577 ± 0.171	3.958 ± 0.134
Neutral red	0.734 ± 0.009	0.856 ± 0.055	1.491 ± 0.368	2.533 ± 0.309	3.319 ± 0.236	3.759 ± 0.284	3.994 ± 0.173

Each value is mean ± standard deviation of ten observations
Values are non-significant (P> 0.05)

Table 4. Length of control and Neutral red fed V instar larva.

Exp. condition	**Day of V instar**						
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control	3.830 ± 0.200	4.360 ± 0.227	4.720 ± 0.270	5.740 ± 0.324	6.320 ± 0.388	6.750 ± 0.190	6.970 ± 0.125
Neutral red	3.850 ± 0.184	4.370 ± 0.177	4.800 ± 0.211	5.820 ± 0.199	6.380 ± 0.339	6.750 ± 0.425	6.990 ± 0.160

Each value is mean ± standard deviation of ten observations
Values are non-significant (P> 0.05)

Table 5. Economical parameters of cocoon in the control and Neutral red fed group.

Exp. condition	**Cocoon weight**	**Pupa weight**	**Shell weight**	**Shell ratio**
Control	1.609 ± 0.282	1.310 ± 0.243	0.289 ± 0.047	18.081 ± 2.037
Neutral red	1.561 ± 0.176	1.270 ± 0.163	0.282 ± 0.024	18.204 ± 1.833

Each value is mean± standard deviation of ten observations
Values are non-significant (P> 0.05)

Discussion

On dissecting the larvae of experimental group on the seventh day of V instar before spinning, coloured haemolymph and silk gland had observed. Interestingly, the peritoneal membrane of alimentary canal of neutral red fed group also possessed a noticeable pink colour. It is in accordance with the earlier studies by Campbell (1932) who reported a colour change of silkworm body on feeding neutral red added diet. The colour change of gut epithelia indicates the diffusion of dye through the alimentary canal to the haemolymph and from there to silk gland. This is par with the findings of Tansil et al. (2012) which states that initially the gut epithelia absorbs carotenoids pigments from mulberry leaves, then haemolymph takes its turn to transfer these pigments into the silk gland resulting yellow haemolymph and yellow silk gland.

Hamamoto et al. (2005) reported that midgut of silkworm larva has a permeability barrier against compounds with a molecular weight greater than 400 g/mol and compounds with smaller molecular weight have a faster transport rate through the midgut membrane. As the molecular weight is an important factor for the passage of molecules, the required range of molecular weight of neutral red (288.78 g /mol) might be aided the better colour accumulation in silk gland of this group and thus the production of bright coloured cocoons. However, further investigations are required to confirm the factors, which facilitated the passage through the biochemical pathways and accumulation of dyes in the silk gland.

This paper confirms two issues in the present scenario, viz. the reduction of textile dye pollution and in vivo production of coloured silk by *B. mori* fed by a dye added diet. As the title denotes this technique has no harmful effect for the insect and the environment. It provides a cost effective method for dyeing silk, which avoids hazardous chemicals. The process has no unfavourable effects on the silkworm, as there is no significant difference in the observed traits of normal and neutral red fed group silkworms. It was observed that neutral red modified the cocoon colour noticeably. This proves it is a promising technology for producing various vital coloured silk in the near future. Thus, it can be recommended for large-scale production of coloured cocoons. Further characterization studies are required to confirm whether the colour incorporated into the fibroin of silk.

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