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SELF-DYED SILK BY FEEDING SILKWORMS WITH COLOURED MULBERRY LEAVES – APPROACHING SUSTAINABLE SILK TREATMENT TECHNOLOGY

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Abstract: Mulberry silk is one of the highest quality fibres with outstanding properties. Its traditional colouring process is dying. This study investigated a novel method called "self-dyed", where silkworms were fed with coloured mulberry leaves on the first, third, and fourth day of the fifth instar of the life cycle. The Rhodamine B dyestuff with different concentrations was applied during feeding. There was a significant difference in mortality rate, spinning, reeling processes, and colour intensity of newly fed silkworms. The silk colour was evaluated by CIE Lab colour measurement and microscope. The colour intensity also depends on dyestuffs concentrations, silkworm breed, and feeding time. This dyeing method is the premise for developing and building an environmentally friendly dyeing process for silk. It also minimizes the traditional dyeing process's water and wastewater costs. In addition, this study shares advanced knowledge and practices in the sustainable development of the textile industry.

Keywords: Sustainable, silkworms, dyeing silk, self-dyed silk, coloured silk.

Introduction

Silk is a precious material obtained from the cocoons of silkworms with many preminent aesthetic and ecological properties. Every filament comprises two long continuous fibroin fibres, accounting for about 72%, and is surrounded by a sericin layer accounting for about 28%; the sericin layer will generally be removed almost after degumming for textile garment application. The silk colour is natural ivory white, yellow, or light green, depending on the silkworm variety and the type of feeding leaves. The popular domesticated silkworm Bombyx mori is a bivoltine crossbreed that accounts for about 95% of the world's silk production; mulberry leaves feed this silkworm and produce white silk (Hallett & Johnston, 2014; Basu, 2015).

Common mulberry silkworms in Vietnam are varieties of silkworms that produce white and yellow cocoons (Figure 1). White cocoon silkworms can be imported from China or domestically bred, while yellow cocoon silkworms are "Ré yellow" or domestically bred hybrids. The "Ré yellow" or "original yellow" breeds are local varieties domesticated from a wild breed, which was believed to be the first silkworm breed to exist in Vietnam, and was raised in certain villages in Thai Binh, Nam Dinh where mulberry and mulberry silkworm farming are considered tradition. However, until recently, this silkworm breed has only been raised mainly for its pupae, which are used as food, and the waste silk is used for weaving. White cocoon silkworms are renowned for their high quality and economic value, making them a popular choice for cultivation in mulberry-growing areas of Vietnam. In order to obtain access to superior breeds more easily, researchers have conducted several studies on silkworm breeding. As part of this research, studies have been carried out on the quality of the hybrid white cocoon silkworm LD-09, which is raised in Lam Dong, Vietnam (Toan & Thuy, 2014) was conducted, which shows that all the quality parameters of silk cocoons such as silk length and average thinness are equivalent to the control group. The qualities of certain domestically-bred white cocoon silkworm varieties were also studied and compared with control samples imported from China and Japan. Results from these studies showed that the average individual silk length of the domestically-bred samples is lower than the control sample (Vu *et al.,* 2014). While yellow cocoon silkworms have an advantage in their natural colouring, white cocoon variants excel in multiple categories such as length and durability.

Various type of cocoons and silk from different species have been studied, showing obvious differences in cocoon structure morphology and characteristics of cocoon layers such as thickness, porosity, size, and colour. Cocoons of wild varieties are often much smaller and have thinner outer membranes than domesticated and hybrid varieties (Reddy & Yang, 2010; Ali *et al.,* 2016). The same has also been observed in different silkworm breeds of the same species of *Bombyx mori*. Two local Thai silkworm varieties, Nongnai and Cambogde, have been studied, and results have shown that the total length and maximum length of silk are both lower than that of the Chinese white cocoon but have a higher elastic modulus (Iizuka *et al.,* 1994). A study of silks of different Indian species such as mulberry silkworm silk, Tasar silk, Eri, and Muga silk revealed differences in colour, silk structure, shininess, and fineness; the hybrids also displayed their superiority in silk length (Chattopadhyay *et al*., 1995; Gupta *et al.,* 2000; Chattopadhyay *et al.,* 2005); varieties of silkworms give different application values. Currently, the main applications of silk are textiles garments and fashion.

Dyeing is a conventional method employed to enhance the aesthetic appeal and colour diversity of textile materials, especially silk. However, this widely practised process poses significant adverse effects on environmental aspects. The presence of certain harmful chemicals in water during the dyeing process such as formaldehyde-based dye fixatives, hydrogen-based softeners, and non-biodegradable dyeing chemicals can have potentially detrimental effects on the environment. Therefore, the dyed wastewater is often cloudy and has a foul odour, reducing the quality of water and soil resources, reducing photosynthesis, and increasing the ability of plants to mutate around the discharge environment (Sudhakar *et al*., 2001). Untreated dyeing wastewater also affects water used in daily life and is the source of some allergic diseases and cancers in humans (Kant *et al.,* 2012).

Green production is the trend and goal of many production processes to minimize the negative environmental impact. There have been many published studies on the method of colouring silkworm cocoons when changing the usual feeding with coloured mulberry leaves and achieving specific results (Tansil *et al.,* 2011; Lefèvre *et al.,* 2012; Basu, 2015). Rhodamine B dye was proposed as a suitable choice for colouring cocoons in the study of creating coloured cocoons by changing the mulberry diet (Kang *et al.,* 2011). Nisal *et al*. (2014) used some azo-based dyes during silkworm feeding to create naturally coloured cocoons. The results showed that the distribution of dyes in fibroin depended on the type of dyestuffs used to feed silkworms (Nisal *et al.,* 2014). "Green" dye-fed coloured silk was produced and made into the reported and available fabric in the Indian market (Kanika Trivedy *et al*., 2016). As for feeding silkworms with dyestuff, colouration is observed in cocoons, pupae, and eggs. The larvae hatched from these eggs are also coloured. Larvae keep colour for a few days. Then, by normal mulberry leaf feeding, become normally coloured larvae. So, the addition of dye does not affect the growth, shorten the life of silkworms, or affect future generations; further biological studies may be needed to identify genetic factors. Normally, after 3-4 days of releasing silk to create a cocoon, although the pupa inside the cocoon is still alive, the cocoons will be harvested and cooked in boiling water (reeling process) to get the silk filament, then, silkworm pupae have medicinal properties and can be used for animal nutrition (Hăbeanu *et al.,* 2023). This study proposes a solution to the aforementioned problems and wastewater issues. By reducing water usage compared to conventional dyeing processes, this study is able to make significant contributions to the field. The use of silkworms as research subjects is considered a viable alternative to other animal studies. Additionally, this study highlights the advantages of using silkworms as a model organism, as compared to other vertebrates, mammals, and invertebrates (Srivastava *et al.,* 2013). As an invertebrate model, its convenience characterizes the silkworm model, low cost, and no ethical issues (Panthee *et al.,* 2017; Matsumoto, 2019). Besides, similar studies on chemical addition have been reported in India (Nisal *et al.,* 2014; Trivedy *et al*., 2016), Korea (Kang *et al.,* 2011), Singapore (Tansil *et al.,* 2011), implying that studies on feeding silkworm with dyestuff could be silkworm is a direction of improvement for the silk dyeing process.

In addition, during the cultivation of the silkworms fed with dyed mulberry leaves, we found that the survival rate of silkworms reached about 93%, equivalent to silkworms eating regular mulberry leaves. During the first feeding, we observed that silkworms hesitated to eat the first batch but then continued to eat as usual. The life cycle of silkworms is equivalent to that of normal silkworms used for feeding. However, colour concentration and larval cultivating parameters should be discussed in detail, especially with Vietnam silkworm breeds (white and yellow cocoons). This study contributed to building a finishing process for self-dyed silk, which can minimize the wastewater of the traditional silk dyeing process. Factors such as the timing of colour leave feeding, silkworm survival rate, and colour intensity of silk were also studied and discussed. This document also serves as a reference for related studies on silk in general and Vietnamese silk in particular.

Materials and Methods

Materials

Silkworms breed: Bombyx mori white cocoons (WC) cultivated at mulberry plantations in Bao Loc-Lam Dong-Viet Nam, yellow cocoons (YC) from local breeds are provided by the Central Mulberry Silkworm Research Center, Ha Noi, Vietnam.

Dyestuff: Rhodamine B (RhB) dye stuff, 479.02 g/mol, molecular formula $C_{28}H_{31}ClN_2O_3$, melting point 210°C (483K; 410°F), from Merck-Germany.

Methods

Boiling: The Korean small silk reeling equipment M/C- WCR1 was utilized to cook dried cocoons at 90°C. YC cocoons were subjected to a three minutes cooking process while WC cocoons were heated for 5-7 minutes. The experiment involved a sample size of 10 cocoons per batch, with the silk from each cocoon combined sequentially until all cocoons were used up. Following the reeling process, raw silk was dried and utilized in subsequent experiments. To examine cocoon weight and silk length, 50 dried cocoons of each type were retained. The study was conducted at the Central Mulberry Silkworm Research Center.

Properties of cocoons: Cocoon weight, total length of cocoon and maximum length were determined according to QCVN-01-74-2011 standard by the Central Mulberry Silkworm Research Center, Vietnam.

Self-dyed silk process: The mulberry leaves were subjected to dyeing processes using spraying techniques, utilizing various dyeing solutions such as RhB at varying concentrations (100 ppm, 1,000 ppm, 1,500 ppm, 2,000 ppm). The liquor ratio was 1.5:1 (the weight in grams of mulberry leaves to the volume in millilitres of dyeing solution). These colourful leaves were line-dried until their moisture content was in equilibrium with the surrounding air.

Morphology: Observed visual of the cocoon outer layer by the microscope (Stereo Discovery-V8-Stereo-ZEISS).

Colour analysis: Based on the Kubelka-Munk theory as shown in the equation below, the colour intensity (K/S) of self-dyed silk fibres was measured and estimated using an X-Rite Color i5 Spectrophotometer and Colour Control software (X-Rite, USA) in the wavelength range of 400 nm to 700 nm.

$$K/S = \frac{(1-R)^2}{2R}$$

where K, S, and R, respectively, stand for absorbance, scattering, and reflectance.

Results and Discussion

Properties of Cocoons

Silkworm cocoons function as a protective layer for silkworms during the larval stage of development from silkworm pupa to mature moths. The cocoon has a non-woven structure, made up of a continuous silk thread released by silkworms and bound by a sericin (Mondal M. *et al.,* 2017).

Visual observation of the cocoon in Figure 1 shows that the YC cocoon is naturally gold in colour while the white WC cocoon is ivory and the length ranges from 3.5 cm-3.7 cm. The cocoon appeared elongated at both ends and porous in structure, smaller in size horizontally, and its structure was less stringent than the WC cocoon. The WC cocoon's length of approximately 3.2 cm-3.5 cm, oval in shape, and was slightly smaller in the middle of its body; it featured fine wrinkles and a dense cocoon structure with lower porosity than yellow YC. Figure 2 (a) shows that the average weight of WC was 47.13%, higher than that of YC.

The total silk length of the WC cocoons was about 70.60% higher than YC Figure 2 (b). Differences between silkworm varieties within the same species or between wild and hybrid silkworm varieties were due to genetic factors in their biological makeup. Studies show that genetic factors determine the characteristics of each species and thereby lead to differences in silk glands and silk thread secretion (Veldtman

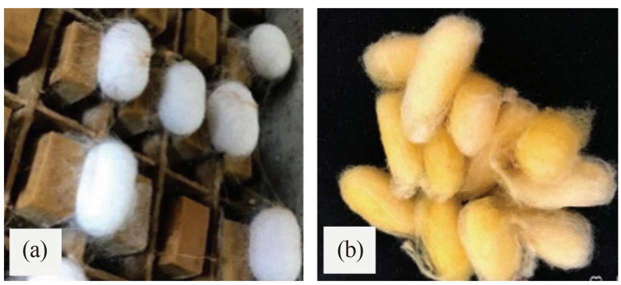
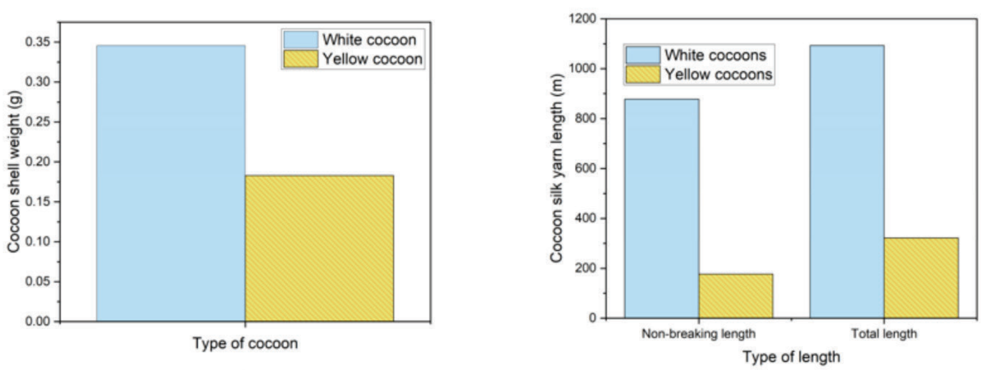


Figure 1: (a) White cocoons and (b) yellow cocoons



(a) (b)

Figure 2: (a) Cocoon weight and (b) cocoon silk yarn length

et al., 2002). The activity of vesicles in the silk glands of industrial hybrids was estimated to be 256 times higher than that of wild varieties. These vesicles released neurotransmitters and regulated the secretion of proteins in the silk glands (Ude *et al.,* 2014). The efficiency of the biosynthetic process of secreting silk protein solutions was directly related to the cocoon silk production as well as some factors such as weight and length (Vasumathi *et al.,* 2004; Chen *et al*., 2012). The non-breaking length of different type of cocoons ranged from 54.91% to 80.30% of the total silk cocoon silk filament length, of which the lowest was YC at 64%. Because the silk from the cocoon will be subjected to a tensile strength during the reeling and spinning process, the non-breaking length of the silk was also an indication of silk strength.

Colouration Effectiveness of Silk Self-dyeing Process

Experiments were conducted to study the impact of feeding dyestuff to silkworms of two different breeds, white and yellow. Results indicate that both breeds could produce a coloured cocoon, distinct from their natural colour, after being fed dyestuff. It was observed that the silkworm's body colour underwent a significant change after consuming dyestuff, and upon reaching the fifth instar, they directly initiated spinning silk filament to create a cocoon with a distinct colour (Figure 3).

The efficiency of the self-dyed process depends largely on both dyestuff properties and the body inside biochemistry of the silkworm that the dye needs to be suitable for the endogenous environment of the silkworm, thereby reacting with the liquid silk solution in the silk gland, then, spinning colour cocoons (Tansil *et al.,* 2011; Nisal *et al.,* 2014). The silkworm's silk gland is formed and fully developed at the fifth instar, containing a protein solution. The RhB dye sprayed in mulberry leaves has a molecular mass of 479.02 g/mol, making it feasible to penetrate the digestive tract wall and move to the silk gland. RhB dye has both hydrophilic and hydrophobic properties, so it is easy to bond with the protein solution in the silk gland to form a coloured protein solution, then, coloured cocoons and silks (Tansil *et al.,* 2011; Nisal *et al*., 2014). At the end of the fifth instar, silkworms are almost at a steady state, and there is a limited signal of waste excretion, which could explain why dye molecules are not excreted. The ability to grow and create coloured cocoons, the colour intensity of cocoons depends on the time moment of feeding silkworms with dyes and dye concentration.

The reaction mechanism of dyeing dyes on silk fabrics is thanks to hydrogen bonds, Van der Waals force, and ionic bonds. The -COOH and -OH groups of the dye will form an attraction to the amine and carboxylic groups (-NH and -COOH) in silk by hydrogen bonding. Rhodamine B contains carboxylic and amino groups, which helps it react effectively in silkworms. The -COOH-groups transform into -COO- negatively charged in the alkaline environment of the silkworm gut (pH 9.4-9.8). The amino groups simultaneously become positively charged when entering the circulating fluid inside the worm's body. The acid in the

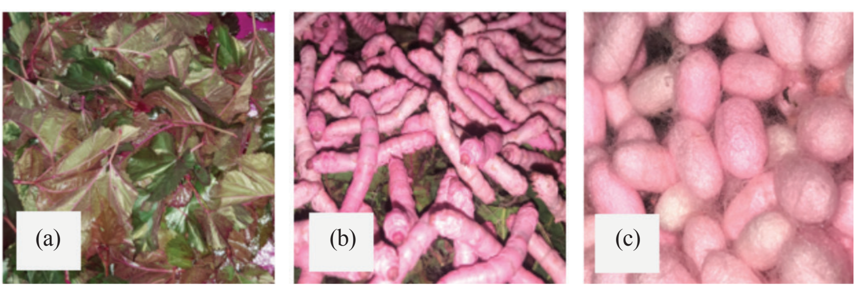


Figure 3: Self-dyed silk process: (a) Sprayed RhB mulberry leaves, (b) fed RhB silkworms, and (c) self-dyed cocoons

silkworm muscle has a pH of 6.3-6.5, which facilitates the colour binding of RhB dye in the silkworm body, penetrating various tissues, from the glandular epithelium to the silk in the glandular lumen, eventually leading to silk solution in coloured and giving colour cocoons (Tansil *et al.,* 2011).

Our observations showed that the colour of the cocoons changed with the concentration of dyes fed to the silkworms. The highest colour intensity is at the concentration of 1,500 ppm to 2,000 ppm, and the lightest one is at the concentration of 100 ppm. Figure 4 shows that the raw silk reeled from the coloured cocoon has a smooth, glossy surface, and the structure and appearance are not significantly different from the raw silk of a conventional cocoon. It is quite clear to observe the homogeneous distribution of colour in the self-dyed silk fibre at 100x magnification image (Figure 4). The fastness properties of self-dyed silk (RhB) against washing, rubbing, perspiration, and light were reported, resulting in impressive colour durability (mostly in the range of 4-5/5) which is especially superior to this silk compared to other green dyeing methods (Uyen *et al.,* 2023). After the degumming process, the colour of the dye positioned on the silk surface was partially lost. Previous studies have reported that the colour is present in both sericin and fibroin, mostly in sericin, so, the colour is reduced after degumming (Nisal *et al.,* 2014; Uyen *et al.*, 2022). However, the decrease in colour intensity depends on different degumming methods such as soap, alkali, hot water, and enzymes. Previous studies have shown that the colour of sericin and fibroin is reduced to different degrees depending on different cleaning methods such as soap, alkali, hot water, and enzymes. Accordingly, despite the degumming, the self-dyed silk (RhB) retains the characteristic colour of RhB after being treated with hot water, Na_2CO_3 or an enzyme (Uyen *et al.,* 2022).

The process of spraying dye on mulberry leaves to feed the silkworms did not affect the structure and shape of the silkworms. Morphology analysis by SEM showed that the self-dyed process had no substantial effect on silk filament because there was no significant difference between the morphology surfaces of self-dyed and white silk; the silk's diameter fluctuates from 10 µm to 12 µm, similar to conventional silk (Uyen *et al*., 2023). FT-IR spectra of self-dyed silk do not present remarkable changes

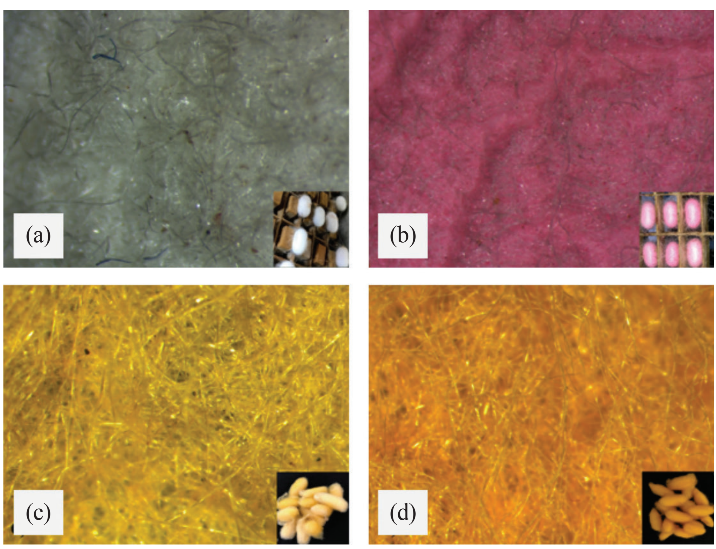


Figure 4: The change in appearance colour of silkworm cocoons (100x magnification image): (a) Natural white cocoon, (b) natural white cocoon after self-dyeing, (c) natural yellow cocoon, and (d) natural yellow cocoon after self-dyeing

in comparison with the white silk due to the superposition of identification peaks of both silk and RhB (Bawazeer & Alsoufi, 2017; Guo *et al*., 2018; Kang *et al.,* 2019; Uyen *et al.,* 2023), the significant change in crystallinity of self-dyed silk was observed. RhB is polycrystalline with identified XRD peaks located around 2θ from 10-30° (Bhat & Nadiger, 1980; Farag & Yahia, 2011; Chen *et al.,* 2016), which are overlapped by the characteristic peak of silk in self-dyed silk (Uyen *et al.,* 2023). Thermal stability of self-dyed silk by TGA analysis shows that the self-dyed process made the self-dyed silk less stable under thermal impact than the white one. Based on our findings, it can be asserted that the practice of spraying dye on mulberry leaves for feeding silkworms has no discernible impact on conventional silk structure and form. The mechanism of this technique aligns with both white and yellow silkworm breeds examined in this study, indicating that it has the potential to be implemented for a wide range of silkworm breeds. White silkworm breed and their self-dyed cocoons were used for the next experiment.

Effect of Concentration and Time Feeding on the Colour Intensity

The growth of silkworms is divided into the fifth instars (Figure 5). The first instar lasts for around 3-4 days, 2-3 days for the second instar, 3-4 days for the third instar, 5-6 days for the fourth instar, and 6-8 days for the fifth instar. In addition, the duration of the silkworm instars depends on the silkworm strain and rearing temperature.

Table 1 shows the percentage of silkworms that grow until they form cocoons. The sooner the dye is added, the lower the percentage of silkworms living until they release silk to create cocoons. Silkworms are relatively susceptible animals and susceptible to infection, especially when young (Mondal M. *et al.,* 2007).

The silkworm mortality rate is high when changing the diet at the first instar. Although the time of feeding silkworms with dyes from the first instar is more than that from the fifth instar, the colour intensity of the obtained silk shows that dye feeding time is not a decisive factor in the silk colour. At the first instar, the silk gland in the silkworm's body has not yet formed. However, at the fifth instar, especially the last days of the age of this instar, the silk glands are complete and ready for the release of silk to create cocoons. The fifth instar of silkworm life lasts for about seven days, and in the early stage of this instar, the cellular structures necessary for fibre biosynthesis are formed rapidly, while in the later stage, the fibre synthesis proceeds at full speed and uses these structures to form fibre solutions and spin silk (Tashiro *et al.,* 1968). Therefore, the addition of dye on the fourth day of the fifth instar increased the growth rate and colouration of silkworm cocoons, especially at a concentration of 1,500 ppm with high colour intensity. Although the time of feeding silkworms with dyes from the first instar is more than that from the fifth instar, the colour intensity of the obtained silk decreases; it shows that not the time feeding but the time moment is the factor that affects the colour cocoon Figure 6 (a).

The self-dyed silk's different colour intensity separated from cocoons was obtained due to the concentrations of dyestuff sprayed on mulberry leaves shown in Figure 6 (b), where the K/S colour intensity values of self-dyed silk from different feeding batches with growing dye concentrations, confirming the previous statement.

The highest colour intensity was obtained at silk from 1,500 ppm to 2,000 ppm dye concentrations batch. The results also demonstrated that the colour concentration on

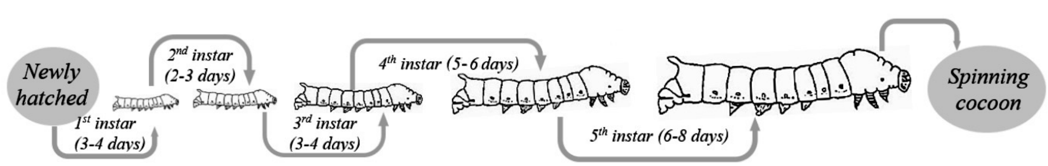
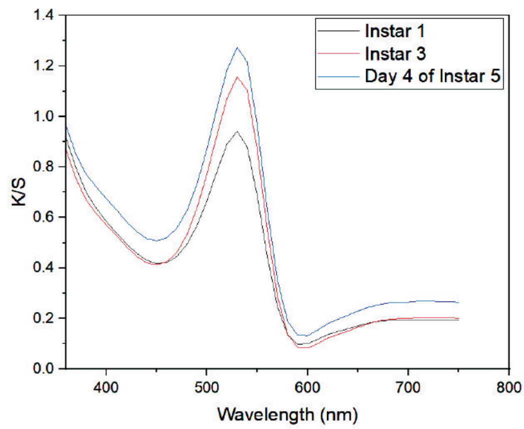
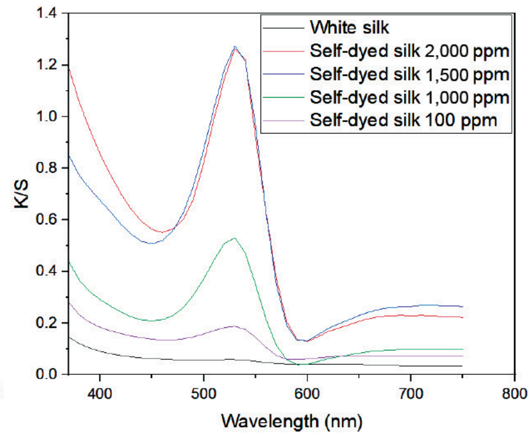


Figure 5: Five instars of silkworms' growth



(a)



(b)

Figure 6: (a) K/S colour intensity of silk from silkworm fed with 1,500 ppm at difference instar of silkworm's life cycle and (b) K/S colour intensity of silk from silkworm fed with dyestuff at 4th day of the 5th instar at difference dyes concentration

Table 1: Effect of colour feeding time on the vitality of silkworms

Dye Concentrations	1st Instar	3rd Instar	4th Day of 5th Instar
100 ppm	-	-	98%
1,000 ppm	-	-	96%
1,500 ppm	28%	35%	93%
2,000 ppm	-	-	79%

mulberry leaves is directly proportional to the colour intensity of self-dyed silk, consistent with some previous studies (Dong *et al.,* 2011; Nisal *et al.,* 2014). Nevertheless, at dye concentrations higher than 1,500 ppm such as 2,000 ppm, many silkworms refused to eat mulberry leaves or showed physiological disorders and growth retardation and did not release silk on cocoons, and the percentage of growth was decreased (Table 1).

Conclusion

The method of feeding silkworms with mulberry leaves sprayed with Rhodamine B dye resulted in silkworms changing body colour, growing and releasing silk then creating coloured cocoons. Visual observation showed that the cocoon surface has not changed in structure, is a shiny and smooth surface, and has not been far different from the morphology of conventional silk. The concentration of dye sprayed on mulberry leaves and the time moment of starting feeding silkworms with dyed mulberry leaves are the parameters affecting the survival rate of silkworms and the colour intensity of cocoons and silk filament. The colour intensity of the cocoons obtained the highest values at the dye concentration of 1,500 ppm and the lowest at the concentration of 100 ppm. The appropriate time to feed silkworms to ensure a high growth rate and intense colouration is on the fourth day of the fifth instar of the silkworm life cycle.

Even though the colour is present in both sericin and fibroin and is reduced after degumming, it still recognizes the usefulness of this "greener dyeing" technology due to the various forms of silk fabrics used in textile and fashion, usually classified by weave (habotai, satin, and jacquard) or by the amount of sericin lost after degumming as raw silk, organza, half-degumming, and lustre fabric. Different type of silk fabrics have different physical and mechanical properties and characteristics (especially gloss and drape behaviour) and are applied in fashion depending on the improvization or ideas of the designer. To limit the wet treatment processing of silk fabrics, self-dyed silk fabrics be suggested to be used in their raw form; so self-dyed could be a good alternative to conventional silk colouration processes.

In summary, colouring silkworm cocoons by feeding silkworms with mulberry leaves sprayed with Rhodamine B dye is feasible for the Vietnam popular silkworm breed. This is the premise for developing and building an environmentally friendly dyeing process for silk and silk fabrics. This development minimizes the water and wastewater costs of the traditional dyeing process. This study is also sharing of advanced knowledge and practices in the sustainable development of the textile industry.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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