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CROSS TRANSMISSION OF PEBRINE SPORES FROM WILD LEPIDOPTERAN CATERPILLAR, *CRICULA TRIFENESTRATA* HELFER TO MUGA SILKWORM, *ANTHRAEA ASSAMENSIS* HELFER

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ABSTRACT

Muga silkworm is a very important bioresource and it is found mainly in Assam and its neighbouring states of North east India. It has high aesthetic value and economic importance among the Assamese people. Muga silk production generates additional rural employment in the North eastern region of India. Muga silkworm is reared outdoor on its primary host plants, Som (*Persea bombycina*) and Soalu (*Litsea monopetala*). Due to its nature of outdoor rearing, Muga silkworm is vulnerable to attacks from different pests and pathogens. Among the pathogen attacks, infection caused by microsporidian spores, called pebrine disease is very serious concern. In Muga silkworm rearing, pebrine disease incidence reached up to 50-60% and it caused crop loss up to 30% or more. In Muga rearing fields, many wild caterpillars, specially lepidopteran caterpillars are inflicting Som and Soalu food plants, thereby sharing the same food plants with Muga silkworm. Such coexistence, sharing of food plants facilitates cross transmission of pebrine spore from wild lepidopteran caterpillars to Muga silkworm. From the study, it has been confirmed the occurrence of cross infection/transmission of pebrine disease from *Cricula trifenestrata* to *Antheraea assamensis*.

Keywords: *Antheraea assamensis*, bioresource, coexistence, *Cricula trifenestrata*, Microsporidian, outdoor rearing, pebrine disease.

Introduction

Seri-biodiversity represents the variability of sericigenous insects and their host plants. In general, sericulture sector is divided broadly into mulberry and vanya sectors. Mulberry silk is produced by *Bombyx mori* and vanya silk is produced by a group of other insects mostly present in the wild. The vanya silkworms belong to the family Saturniidae, one of the largest groups of Lepidoptera comprising more than 1500 species spread over the world, and a few belong to Lasiocampidae. Among them, about 80 species are known to produce silk which has some economic value (Nässig *et al.*, 1996). The saturniids, which include some of the largest and most spectacular species of all lepidopterans, are distributed in temperate and tropical conditions and are univoltine to multivoltine, depending on the climatic conditions (Regier *et al.*,

2008). Jolly *et al.* (1975) reported that many species are present in Asia and Africa that produce wild silk of economic value. Arora and Gupta (1979) estimated as many as 40 species from India, and among them nine species are present in Northeastern India (Thangavelu 1991, Thangavelu *et al.*, 1987). Out of these nine species, the genus *Cricula* Walker (Helfer) is present in the North-eastern part of India and is comprised of three species viz., *Cricula trifenestrata* Helfer (Lepidoptera: saturniidae), *C. Andrei* (Jordan 1909) and *C. andamanica* (Veenakumari *et al.* 1996). Of these, *C. trifenestrata* is widely distributed in the Indian Sub-continent. This silk producing insect, *C. trifenestrata* feeds on mango (*Mangifera indica* Lin.), Som (*Persea bombycina*) and Cashew plant (*Anacardium occidentale* Lin.) and produces an open ended 'net-like' cocoon of beautiful golden yellow

colour. The silk is rich in luster and is used for making spun silk. The habitat of *Cricula trifenestrata* ranges from the low land to highland at an altitude of over 2000 m in Meghalaya, Assam, Tripura and West Bengal. Availability of large numbers of alternative host plants facilitates migration of this insect from place to place (Tikader 2011). The population of *Cricula* occasionally increases to a level of causing significant economic injury to the muga silkworm (Sarmah *et al.*, 2010).

In muga ecosystem, many insect visitors, lepidopteran caterpillars are infesting Som and Soalu food plants in the field. Such coexistence may facilitate cross infection of pebrine spore from lepidopteran caterpillars to muga silkworm. Regarding to it, this study had been conducted to reveal the cross transmission of pebrine disease from other lepidopteran insect to muga silkworm sharing the same food plants. The present study was undertaken with the objectives to confirm the cross infectivity of pebrine disease from wild lepidopteran caterpillars to Muga silkworm coexisting in the rearing farms.

Pebrine is an insidious and chronic disease in muga silkworm, *Antheraea assamensis*, Helfer, caused by highly virulent microsporidian parasite *Nosema assamensis*. Pebrine disease is being transmitted to offspring by transovarial/ transovum means from mother moths or it is transmitted through contamination of environment. It is also reported that the perpetual incidence of microsporidian infection in silkworm may be due to various sources of secondary contamination or crossed infection from the alternate hosts. The periodic occurrence of pebrine disease in the rearing field indicates the possibility of cross infection of pebrine spore from the other alternate host.

When healthy larvae get infected through contamination during rearing, it is called secondary infection. Per oral infection occurs when silkworms eat contaminated leaf with spores. Primary infection refers to that occurring in the first and second instars and excretion of spores through faeces by these infected ones in third or fourth instars. Ingestion of these spores by other healthy worms constitutes secondary infection. Secondarily infected silkworms are able to feed normally and become moths but they lay eggs with infected embryos. The infected larvae show significant changes in the cocoon weight, shell weight, denier, reelability etc. (Bhat and Nataraju, 2005). Transovarian transmission reveals the rate of infection to increase with each successive hatching date resulting in 100 per cent infection among larvae hatching on last day (Talukdar, 1980).

Materials and Methods

Survey for wild caterpillars, *C. trifenestrata* in Muga rearing fields was conducted at different places of Upper Assam, Middle Assam and Lower Assam in different crop seasons. The wild caterpillars of *C. trifenestrata* were collected from the different Muga rearing fields and procured for pebrine spore testing in the laboratory. Location and date of larva collection of wild caterpillars was also recorded for revealing their seasonal occurrence thereof in Muga ecosystem. The occurrence of *C. trifenestrata* is very common on Som (*Persea bombycina*) food plants, which is primary food plant of Muga silkworm. Considering its feeding nature on same food plant, biology, silk producing insect belong to same family saturniidae, *C. trifenestrata* became an interested area of study. Following the Fujiwara method, the collected *C. trifenestrata* caterpillars were tested for pebrine spores.



Fig. 1 : *C. trifenestrata* caterpillar feeding on Som muga food plant, its cocoon and moth

Pebrine spore isolation from *C. trifenestrata*

Collected wild caterpillars of *C. trifenestrata* were tested for pebrine spores following Fujiwara method. Procedure for Pebrine spore detection and isolation is given below-

1. The collected *C. trifenestrata* caterpillars were homogenously crushed by using mortar & pestle with 6-8 ml of 0.6% K_2CO_3 solution.
2. The homogenate was transferred to a test tube and allowed for settling.

3. The bottom liquid of the test tube was filtered carefully through 2-3 layers of clean absorbent cotton.
4. The filtrate was then centrifuged at 4000 rpm for 4 minutes.
5. After centrifugation, the supernatant solution was decanted off.
6. The sediment was then dispersed by using a glass rod with few drops of 0.6% K_2CO_3 solution.
7. After then, the solution was smeared on a dried and cleaned slide from each sample and five fields per smear was subjected to examined/observed at 600x magnification under a microscope.
8. Examination for detection of pebrine spores were performed under a compound microscope at 15X eyepiece lens and 45X objective lens.
9. Pebrine spores were observed with oval-cylindrical in shape and in moving condition viz., Brownian motion. It also looks like rice grains in shape.



Fig. 2: Detection of pebrine spores from *C. trifenestrata* following Fujiwara Method.

After microscopic examination of the samples, it was found that *C. trifenestrata* infected by pebrine spores. The spores were isolated and purified by centrifugation at 3000 rpm for 10 minutes using percoll following standard protocol of Sato and Watanabe 1980. After centrifugation, the spores were suspended in 0.85% NaCl and stored at 4°C. To check further multiplication of spores and to obtain fresh spores, third instar caterpillars of *C. trifenestrata* were orally inoculated with same spores isolated from same species at the concentration of 1×10^6 spore ml^{-1} . The caterpillars of *C. trifenestrata* were reared till sixth

instar in control condition at a separate place feeding on som leaves. During rearing, some of the caterpillars were observed dead before attaining cocoon stage. Dead caterpillars of *C. trifenestrata* were tested for pebrine spores. However, the matured caterpillars were allowed to form cocoons in som leaves. After seven days of cocoon formation, the cocoons were harvested and preserved for moth emergence in a separate control room. The emerged moths of *C. trifenestrata* were tested for pebrine spores. The spores were isolated following same procedure as mentioned above and counted using haemocytometer.

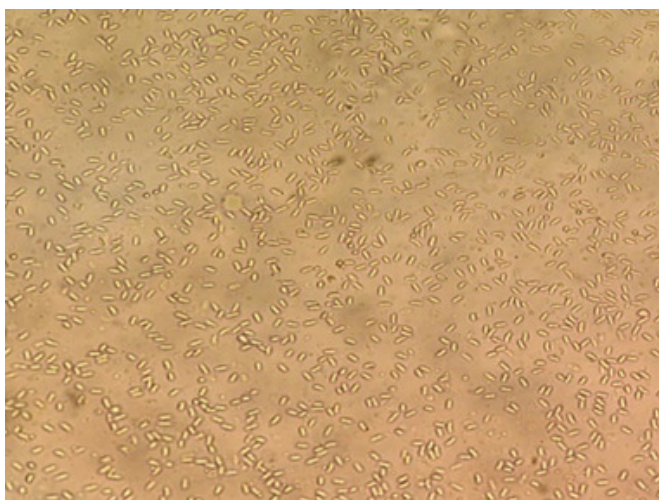


Fig. 3: Neubauer slide used in Pebrine spore counting**Purification process of isolated pebrine spores**

1. The homogenate was filtered through two layers of cheese cloth and collected the filtrate.
2. The filtrate was centrifuged at 3000 rpm for 10 minutes.
3. After centrifugation, the supernatant was discarded and the pellet was washed (six times), suspended in 0.85% NaCl or water.
4. Density gradient centrifugation was performed employing Percoll or sucrose (50-60%) to purify the Nosema spores; the suspension of spores was layered over the gradient and centrifuged at 10000rpm for 30 min. in an RC5C high speed refrigerated centrifuge with swing-out-rotor (SH-MT12) to obtain purified spores (Bhattacharya *et al.*, 1993).
5. Collected the visible spore layer (viable spores) at the bottom of centrifuge tube and re-suspended in 1ml distilled water.
6. Spores were washed by centrifugation at 3000rpm for 10 min thrice with distilled water.
7. Finally re-suspended the pelleted spores in phosphate buffer solution (pH 7.2) or distilled water and observed the smear by light microscopy for presence of any debris/aggregates.
8. After then the purified spores were preserved in 0.8% NaCl and stored at 4°C for further inoculation process and study cross infectivity.

Cross-infectivity test of isolated pebrine spores

Cross-infectivity test of microsporidia isolated from *C. trifenestrata* to Muga silkworm, *A. assamensis*

was conducted. Microsporidia isolated from *C. trifenestrata* were prepared at a concentration of 1×10^6 spore ml^{-1} . Before inoculation, spores were counted using haemocytometer (Neubauer slide). After then, the spores were inoculated per orally, by smearing the aqueous solution of pebrine spores at the edges of cleaned fresh leaves to the thirty 3rd instar worms of *A. assamensis*. Continuous observation was taking place for any behavioural changes and mortality of the larvae. The treated worms were compared control which fed some leaves without smearing aqueous suspension of pebrine spores.

Results and Discussion

Pathogenicity test was conducted on the host *C. trifenestrata* using spores isolated from same species and positive result was obtained. Before inoculation, spores were counted using haemocytometer (Neubauer slide). After then, the spores were inoculated to 3rd instar larval stage of *C. trifenestrata*. Continuous observation was taking place for any changes in the behaviour and its mortality of the larvae. Mortality of *C. trifenestrata* larvae was observed after third and fourth moult. Percent mortality of *C. trifenestrata* was upto 67 till sixth instar of the larvae. Dead *C. trifenestrata* worms were collected carefully and observed for pebrine spores. When we observed the sample, presence of pebrine spores was confirmed and density of spores was found increased.

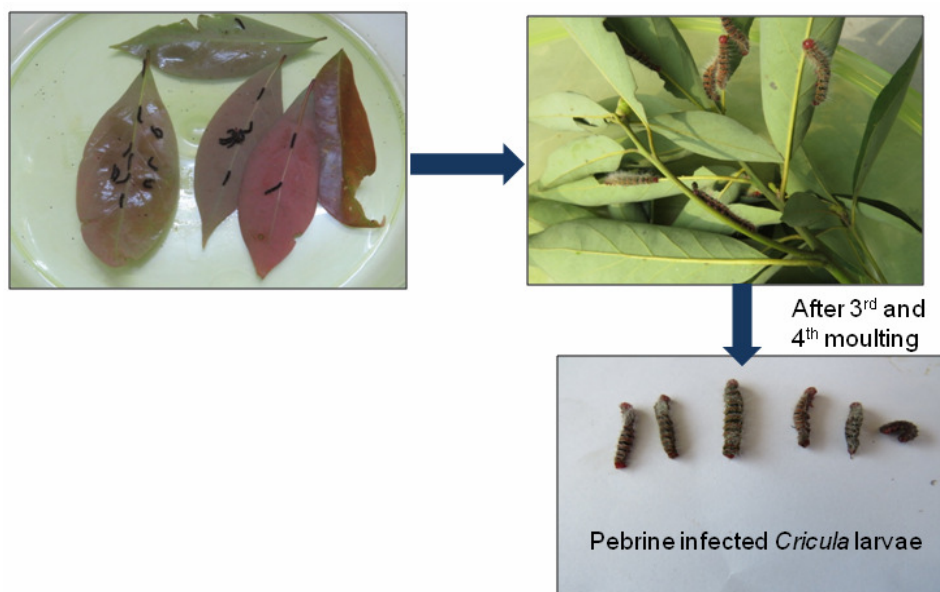
**Fig. 4:** Pathogenicity test of pebrine spores isolated from *C. trifenestrata*

Table 1: Percent mortality of *C. trifenestrata* infected with pebrine spores isolated from same species.

<i>C. trifenestrata</i> instars	No. of larva alive (nx)	No. of larva dead (dx)	Age specific survival (lx) (in %)	Age specific mortality (qx) (in %)
3 rd instar	30	2	100	6.70
4 th instar	28	3	93.35	10.70
5 th instar	25	16	83.33	64.00
6 th instar	9	6	30.00	67.00
Late 6 th instar	3	-	10.00	-

Cross infectivity test

Cross infectivity test was also conducted from *C. trifenestrata* to healthy muga silkworm with the spores isolated from *C. trifenestrata* and positive result was found. After 9 days of inoculation, muga worms were found died. We collected the dead muga worms

carefully and observed for pebrine spores. In the observation, pebrine spores with higher density was detected, which confirms the cross transmission of pebrine spores.

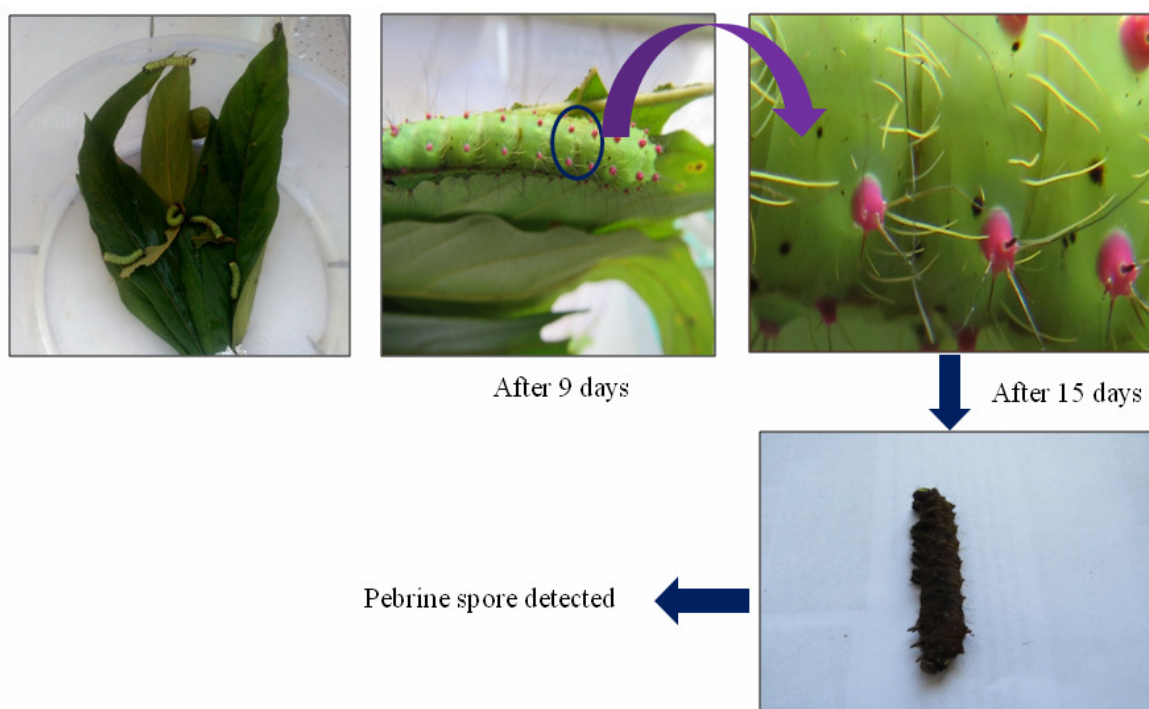


Fig. 5: Cross infectivity test of pebrine spores isolated from *C. trifenestrata* to *A. assamensis*

Thirty numbers of healthy *A. assamensis* larvae of third instar were selected for the cross infectivity/transmission test.

Table 2: Percent mortality of *A. assamensis* infected with pebrine spores isolated from *C. trifenestrata*

<i>A. assamensis</i> instars	No. of larva alive (nx)	No. of larva dead (dx)	Age specific survival (lx) (in %)	Age specific mortality (qx) (in %)
3 rd instar	30	0	100	0
4 th instar	30	11	100	36.67
5 th instar	19	18	63.33	94.73
Late 5 th instar	1	-	3.33	-

The muga larvae were inoculated with pebrine spores with the concentration of 1×10^6 spore ml^{-1} isolated from *C. trifenestrata*. Observations of larval mortality and behavioural changes were recorded daily after the treatment. It was recorded that 94.73% larval mortality on the 15th day after the treatment, showing cent percent larval mortality in the late 5th instar of *A. assamensis*, which confirms cross infectivity of pebrine spores between two saturniid species coexisting on the same host plant, Som (*P. bombycina*).

Conclusion

From the study, it is confirmed that cross transmission of pebrine spores occurred between two saturniid insect species, *A. assamensis* and *C. trifenestrata*, which are living together in an ecosystem sharing same host plant. It was found 94.73% larval mortality of *A. assamensis* on the 15th day inoculation. Eventually, after fifteen days, 100 % larval mortality was found in the late 5th instar of *A. assamensis*, which confirms cross infectivity of pebrine spores between two saturniid species. Mortality due to pebrine was confirmed in dead larvae by microscopic examination. From this study, it has also confirmed that *C. trifenestrata* is a vector of microsporidian, which needs to be given special attention to control this insect in Muga ecosystem to prevent cross transmission. The population of *C. trifenestrata* can be controlled mechanically as egg masses are generally laid in the on the edges of host plant leaves, which can be seen easily. Biocontrol agents such as web weaving and hunting spiders, mantids and several other insect predators which are highly effective in controlling this insect.

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