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Confinement, Purging and Portrayal of Microsporidian Spores from Insect Pests of Mulberry and Few Other Agricultural Yields

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ABSTRACT

The silkworm, scientifically known as *Bombyx mori* L., is a fragile lepidopteran insect cultivated to produce silk. Microsporidia, a type of bacteria, have been identified as the primary pathogens that infect insects in their natural habitats. Various insects discovered in mulberry gardens with a circular shape have been identified to harbor microsporidia that can infect silkworms. Two distinct microsporidia that can infect silkworms have been discovered in the mulberry pest known as "Bihar hirsute maggot, *Spilosoma oblique*". These insects can contaminate silkworm rearing by depositing the pathogen's spores on the mulberry leaves in the garden through scales and litter. Microsporidia from the genera *Nosema, Vairimorpha, Thelohania, Pleistophora*, and unclassified microsporidia have been identified as pathogens (white butterfly, *Pieris rapae*) in the cabbage. This butterfly is a pest that affects agricultural crops such as cabbage, cauliflower, broccoli, and others, excluding mulberry. At night, it is common to see cabbage white butterflies resting in mulberry gardens. As a result, the butterfly excrement and tiny pieces of their wings that contain microsporidian spores adhere to the mulberry leaves. This leads to the transmission of microsporidian infection to silkworms. This study examines the process of isolating, purifying, and characterizing microsporidian spores from insect pests that affect mulberry and other agricultural crops.

Keywords Microsporidian, Silkworm, Agriculture, Mulberry, Insect pests.

INTRODUCTION

The microsporidia, belonging to the Kingdom Protista and Phylum Microspora, are parasitic organisms that must live inside the cells of animals. They may be found in all animal phyla and are particularly prevalent in arthropods. These protozoan pathogens are the most significant agents that cause infections in both beneficial and dangerous insects. Additionally, they possess the highest potential among protozoa for the microbial regulation of insect pests in agriculture, horticulture, and forestry fields. Microsporidia are regarded as natural regulators of specific economically important insect pests. Microsporidia are distinguished by the existence of an infectious

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single-celled spore stage. This stage has one or two nuclei, known as sporoplasm, and an extrusion apparatus. The extrusion apparatus always includes a polar filament that extends to the apex of the spore and is anchored by a disc. They lack mitochondria. The life cycle phases of these organisms are characterized by their unique and unusual ultrastructure, which sets them apart from other protozoa that create spores. This distinctive ultrastructure is crucial in defining their taxonomic classification.

Silkworm *Bombyx mori* is an economical insect. The cocoon crop is at risk of failure because the plant has forfeited its natural resilience to illnesses brought about by centuries of cultivation. Pebrine, flacherie, grasserie, and muscardine are the most common silkworm diseases, and they cause a loss of cocoon crops ranging from 27 to 35%. One of the infectious disorders in this group is muscardine. In a typical year, muscardine causes a 10–40% drop in crop yield, and it tends to happen during the wet and winter months when silkworm rearing is at its best. The organism's haemolymph is a vital, ever-changing fluid that is intimately connected to every metabolic process. Studying the degree of change in many functions, haemocyte population dynamics, and biochemical contents in the haemolymph can help track the progress of the fungal infection *Beauveria bassiana.* This pathogen remains in the haemolymph until the silkworm larvae are almost dead. To improve the quality and quantity of cocoon yield parameters, it is helpful to know what changes occur in the haematological, biochemical, toxic waste product, and enzymatic systems of *Bombyx mori* larvae when the fungal pathogen infects them (Ahamad *et al.* 2016).

Lalitha *et al.* (2018) reported that the mulberry whitefly, *Dialeuropora decempuncta*, feeds on plant sap, leading to symptoms such as chlorosis, leaf dryness, leaf curls, and sooty mold disease. This infestation can significantly reduce leaf yield, ranging from 10% to 24%.

According to Kumar *et al.* (2015) the pest's condition is highly volatile due to its fluidity and is generally influenced by abiotic sources. In light of this, the current study investigated the presence of a whitefly, thrips, mealybug, and natural enemies such as coccinellids and spiders in mulberry plants. The study also examined the relationship between these organisms and abiotic conditions to understand their population dynamics.

At the end of the nineteenth century, only "*Nosema bombycis*" was recognized as a causative agent of pebrine in the "silkworm, *Bombyx mori* L. At present, several microsporidia are known to cause the disease in silkworm. Among them the most common ones are *Nosema* sp. (NIS-001, NIS-M11, NIS-M14 and NIK-2r and NIK-3h), *Vairimorpha* (NIS-M12 and NIK-4m), "*Microsporidium* sp. (NIS-M25), *Pleistophora* sp. (NIS-M27)", *Thelohania* sp. (NIS-M32) and *Leptomonas* sp." The environmental spores of these microsporidia are oval, cylindrical or ovo-cylindrical, either uninucleate or binucleate, and the size of environmental spores of these microsporidia ranges from 2.5 to 5.1 µm in length and 1.3 to 2.8 µm in girth Table 1.

The spore emergence is a fascinating and distinctive biological event. The ability of microsporidian seeds to germinate can be used to determine their viability. The spores germinate by releasing the Antarctic monofilament when exposed to potassium hydroxide (KOH) during incubation. This is then quickly neutralized in Phosphate buffered saline, along with other macromolecules present within the spore. Germinated spores are seen as black when observed under a phase contrast microscope. Also, slender polar filaments extend from the spore, with sporoplasm at their landfills.

Table 1. Morphology of Environmental spores of different microsporidia infecting silkworm.

Microsporidia	Spore size (µm)		Spore shape
	Length	Width	
Nosema bombycis, NIS-001	3.6	2.2	Oval
Nosema sp. NIS-M11	3.9	1.7	Cylindrical
Nosema sp. NIS-M14	4.2	2.4	Cylindrical
Vairimorpha sp. NIS-M12	5.1	2.1	Cylindrical
Microsporidium sp. NIS-M25	4.9	2.8	Oval
Pleistophora sp. NIS-M27	2.5	1.3	Oval
Thelohania sp. NIS-M32	3.4	1.7	Oval
Nosema bombycis NIK-1s	3.8	2.6	Oval
Nosema sp.NIK-2r	3.6	2.8	Round
Nosema sp. NIK-3h	3.8	1.8	Oval
Vairimorpha sp. NIK-4m	5.0	2.1	Ovocylindrical

Recently, the mulberry pest "Bihar hairy caterpillar, *Spilosoma obliqua*" has been found to harbour two different microsporidia cross infective to silkworm. Microsporidia belonging to genera *Nosema, Vairimorpha, Thelohania, Pleistophora* as well as unclassified microsporidia have been described as pathogens in the "cabbage white butterfly", which is a pest of agricultural crops other than mulberry viz., cabbage, cauliflower, broccoli. At night, the cabbage white butterflies are frequently observed resting in mulberry gardens and thus the butterfly excrement and tiny structures on their bodies that contain microsporidian spores adhere to mulberry leaves and pass on microsporidian infection to silkworms Sharma (2015).

From the above account of microsporidia, it's evident that there are myriad variations in the morphology, serology, pathogenicity and transmission of different microsporidia in silkworm that are encountered in insects especially those frequently visiting mulberry gardens and other agricultural crops in sericultural areas. These microsporidia are released on the mulberry leaves by the infected insect pests, which the silkworm, *Bombyx mori* L., exclusively feeds on this particular plant and multiplies in the silkworm body. Some of them show low pathogenicity in silkworms, while others exhibit high pathogenicity. It is, therefore, very important to characterize these microsporidia and study them for their specific characteristics such as morphology, ultrastructure, arrangement of polar filament coils inside the spores, serological affinity, germination response and sporulation at different temperatures.

MATERIALS AND METHODS

Collection of insect pests of mulberry and other agricultural crops

Different insect pests viz. *Sesamia inferens, Phytomyza atricornis, Pieris rapae, Eupterote mollifera, Catopsilia crocale, Terias hecabe, Catopsilia pyranthe, Spilosoma obliqua, Laphygma exigua, Colias eurytheme* and *Diphania pulverulentalis* (as presented in the following Table 1 and Fig. 1) insect

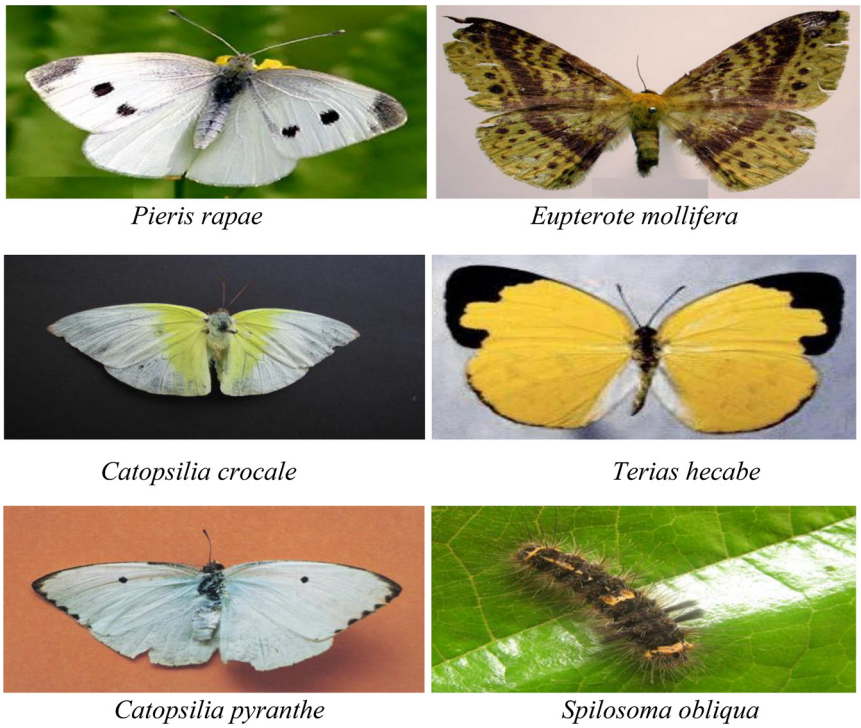

Fig. 1. Different insect pests collected from mulberry orchards and farmlands near Mysore, Karnataka.

specimens were gathered using conventional methods from mulberry orchards and farmlands near Mysore, Karnataka, India.

RESULTS AND DISCUSSION

Insect pests of mulberry and other agricultural crops

The aforementioned insect pests were collected with the help of an insect collection net (with a light and strong handle of 1-meter length, a rim of 0.3-meter diameter made of heavy wire and a nylon net bag with open mesh), forceps, a killing jar and vials. Swinging the net back and forth as one gently walked with the mulberry gardens and agricultural areas, diurnal airborne insects like butterflies were gathered. After each catch, the net was flipped by twisting its opening downwards so as to fold the net over the rim and trap the insects inside. The collected insects were then grasped through the net and transferred to the killing jar covered until the insects were stunned. The larval stages of the insects were collected by individual hand-picking with the help of forceps and transferred to vials containing alcohol. Light traps were used to collect the nocturnal insects. Crushing individual insect pests of each species in a pestle and mortar screened the collected insects for microsporidian contamination. The homogenate was microscopically examined under the microscope, and the microsporidian spore was isolated if any noticed.

Isolation and purification of microsporidian spores

Each insect pest was separately homogenized in 1 ml of a 0.6% K_2CO_3 solution. The specimen was processed and examined for any evidence of microsporidian spores using a Nikon (Type-104) phase difference microscope at a magnification of 600X. In order to cleanse the separated microsporidian spores, the mixture was left undisturbed for 5 minutes and subsequently strained using a dual-layered muslin towel to eliminate any remnants of tissue. The liquid mixture was subjected to centrifugal force at a speed of 5,000 revolutions per minute for 15 minutes to separate and settle the spores.

Finally, the sediment was suspended in water, purified through distillation and then separated by spinning it at 3,000 revolutions per minute for up to 15 minutes using a centrifuge. The silt collected was mixed with a small amount of distilled water subjected to discontinuous neutralized Percoll (Sigma) gradient centrifugation using Hitachi Ultra centrifuge CPO56G11 and Swingout rotar P56ST. The percoll gradient was constructed by sequential layering in equal volume of 100, 75, 50 and 25% percoll in distilled water. A spore suspension of 1 ml was layered on the gradient and centrifuged at 10,000 rpm for 2 hours. The band formed along the vertical length of the tube was collected separately using Pasteur pipette, diluted with distilled water by 5 times in the original volume. The suspension underwent centrifugation at a speed of 5000 revolutions per minute for a duration of 20 minutes, resulting in the removal of the supernatant. The sediment was dispersed in 1 ml of distillate water and then subjected to three rounds of washing with distilled water through repeated centrifugation. The residual debris from every microsporidian was mixed with physiological saline solution containing 0.85% NaCl and preserved at a temperature of 5ºC for subsequent experiments.

Temperature stands out among determining factors of insect susceptibility and multiplication of pathogens. The optimum temperature for infection of most of the microsporidia lies between 20 to 30ºC and no development occurs below 10ºC. In the present study, the multiplication and spore yield of microsporidia in silkworm larvae at 10 days post inoculation was significantly higher at higher temperatures (30ºC and 33ºC) than room temperature (25±2ºC) (Fig. 2). In *Vairimorpha necatrix*, high and low-temperature extremes delayed the microsporidian development,

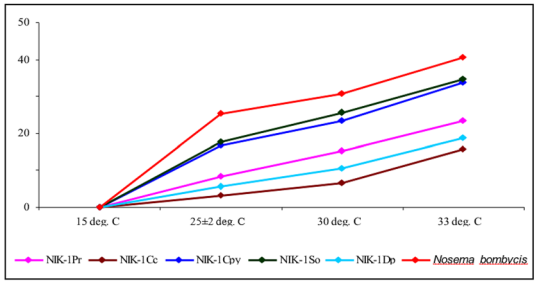

Fig. 2. Impact of different temperatures on sporulation of different microsporidia in silkworm.

producing fewer spores. In our study also, there was no spore production after 10 days post inoculation at 15ºC in all the microsporidia tested. The shifting of the experimental larvae from 15ºC to room temperature and rearing for seven more days also did not result in any spore production revealing that infectivity and development of microsporidia in insects are dependent on the temperature at which host is being reared. Although, microsporidian spores can tolerate a wide range of temperatures, the infection process is influenced greatly by temperature.

CONCLUSION

Among the different microsporidia tested, the highest spore yield in the host larvae at different temperatures (excluding 15ºC) was recorded in *Nosema bombycis,* the standard strain causing deadly pebrine disease in silkworm, followed by NIK-1So, NIK-1Cpy, NIK-1Pr, NIK-1Dp and NIK-1Cc. The variation in spore production can be ascribed to the discrepancy in virulence between the two species, as stated by Madana Mohanan (2015). A similar differential in virulence was also seen in the larvae of the Indian meal moth, *Plodia interpunctella,* between *Nosema heterosporum* and *Nosema plodia*. The variations in spore production could be attributed to the interaction between the host and parasite, potentially resulting from disparities in the virulence of the microsporidia. The analysis indicates that the expansion of microsporidia and spawn generation in the host is influenced by two factors: The aggressiveness of the microsporidia and the ambient temperature that occurs as the host is raised. Based on these studies, it is evident that the microsporidia obtained from parasitic insects of mulberry and crop cultivation have characteristics typical of microsporidia, but also display differences from the standard strain, *Nosema bombycis* in several aspects viz., spore morphology, spore ultrastructure, serological affinity, germination response and multiplication and spore production at different temperatures.

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