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Studies on the characterization and pathogenicity of microbial pathogens isolated from agricultural pests visiting mulberry fields in Kashmir

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ABSTRACT

Despite so many efforts to revive the age-old silk industry of Jammu and Kashmir we are not able to harvest the tangible results due to multifarious reasons. Among these reasons outbreak of silkworm diseases caused by various microbial pathogens poses a great threat to the survival of this economically important insect. The extent of damage to cocoon crops on account of these diseases is huge (30-40%). Despite adopting all the disease management measures, the pathogen is still making its way toward the rearing areas and infecting the worms. During the present study, various agricultural pests were screened and characterized to identify the alternate hosts of silkworm pathogens. Morphological characterization of the isolated spores revealed that Nosema spp isolated from Pierisbrassicae measured 3.5µm x 1.8 µm in size with oval shape, while Beauveria sp isolated from Oxyaindica, Glyphodespyloalis and Cicadulinambila measured 3.7 µm x 2.5 µm, 4.2 µm x 2.3 µm and 3.9 µm x 2.4 µm respectively.

Keywords: *Pathogen, Nosema, Beauveria, Cross- infectivity, Silkworm, Cocoon.*

INTRODUCTION

Among many constraints that influence the success of cocoon crop production, the menace of diseases is the primary one. The major diseases affecting mulberry silkworms are Pebrine, Muscardine, Grasserie, and Flacherie. Cocoon crop losses due to these diseases have been reported to be as high as 30 to 40 percent in India [1]. Apart from causing diseases in silkworms, these pathogens are known to infect insect pests of mulberry and other crops. Therefore, they always serve as a potential threat to gaining access to silkworm rearing through contaminated mulberry leaf and taking a heavy toll of the sericulture industry. Certain species of *Catopsilia* which are frequent visitors of mulberry gardens carry an enormous quantity of microsporidian spores capable of infecting silkworms [8]. Infection due to *Nosema bombycis* has also been recorded in the insect orders Diptera (45 species), Lepidoptera (25 species), Ephermeroptera (13 species), Hymenoptera (6 species), Trichoptera (3 species), Coleoptera (2 species), Isoptera (2 species), Plecoptera (2 species) and one species in each of the following orders: Anoplura, Hemiptera, Odonata, Siphonoptera and Thysanura [14].
Recently, out of various insects collected from mulberry gardens in district Baramulla (J&K), five were found to be infected with *Nosema* and *Beauveria* species [9]. These and many other insects are, therefore, a potential source of contamination in silkworm rearing as they drop the spores of the pathogen on the mulberry leaves in the garden through scales and litter [12]. However, a lot of confusion is prevailing regarding their proper identification which may lead to wrong diagnosis and ultimately wrong decision making. Therefore the current study was undertaken to characterize the isolated pathogens which will pave the way for their effective management.

MATERIALS AND METHODS

The collected insect pests were homogenized individually in 1 ml of 0.2% KOH and 0.6% K_2CO_3 solution. The smear was prepared and observed for the presence of microsporidian and fungal spores at 600X magnification under the microscope. To purify the isolated spores, the homogenate was allowed to stand for 5 minutes and then filtered through a double-layered muslin cloth to remove the tissue debris. The filtrate was centrifuged at 5,000 rpm for 15 min to sediment the spores. Finally, the sediment was suspended in distilled water and centrifuged at 3,000 rpm for 15 min. The supernatant was discarded and sediment was suspended in a minimal volume of distilled water and subjected to microscopic analysis.

1. Morphological characterization of the isolated spores: The isolated spores from insect pests of mulberry and crops were subjected to morphological characterization following the standard method as described by [4] and compared with the spores of the standard strain *Nosema* and *Beauveria* sp. Observations were recorded on spore shape and size.

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To determine the spore shape, the spores were immobilized on a micro slide coated with mineral oil droplets and observed at 600 X magnification.

1.1 Micrometry: To determine the spore size, the spores were first immobilized using a drop of mineral oil. A drop of mineral oil was placed on a slide and a cover slip with a small drop (< 5 µl) of spore suspension of each pathogen was applied on top of the oil. Water, having a better affinity for glass, spreads out on the surface of the cover-slip, leaving spores individually trapped in "holes" in the oil for measurement. Ten spores of each pathogen were measured using ocular and stage micrometers for their length and width following the standard micrometry method [4].

1.2 Confirmation of the infectivity of the isolated pathogens: To confirm that the spores isolated from insect pests of mulberry and crops cause the disease in the silkworm, *Bombyx mori* L., the isolated spores were subjected to tests for their infectivity following Koch's postulates. To conduct the test, an inoculum of concentration 1×10^6 spores/ml of *Nosema* sp was prepared from the stock inoculums by following the standard protocol using a haemocytometer [2]. One ml of inoculum was smeared on mulberry leaves and fed to 100 third instar silkworm larvae of the CSR_4 breed immediately after 2nd moult. Likewise, another batch of silkworms was surface inoculated with aqueous conidial suspension (1×10^6 conidia/ml) by uniform spraying with the help of an atomizer. The larvae were allowed to feed on the treated leaves for 12 hours to ensure complete consumption of the treated leaves. After 12 hrs, the larvae were fed on untreated mulberry leaf and reared till cocooning. The dead larvae and pupae were examined for the presence of infection. The emerged moths were homogenized individually and the smears were observed under the microscope to assess the percentage of infection at the moth stage.

RESULTS

Morphological characterization of the isolated spores

The results of the morphological characterization of identified pathogens (Nosema and *Beauveria spp*) from the insect pests of mulberry and other crops are presented in Table- 1. The *Nosema* spores isolated from *Pierisbrassicae* were oval measuring 3.5µm in length and 1.8 µm in width. The length- width ratio of the spore was 1.94:1 (Plate-1).Micrometric analysis of *Beauveria spp.* isolated from *Oxyaindica* revealed that its conidia were ovoid to globular in shape, one-celled, born singly on small lateral sterigmate, 3.7 µm in length and 2.5 µm in width. The length: width ratio of the conidia was recorded as 1.48:1.The conidia of *Beauveria* sp. isolated from *Glyphodespyloalis* measured 4.2 µm in length and 2.3 µm in width with a length: width ratio of 1.82:1. The shape of the conidia was recorded as ovoid. The *Beauveria* conidia isolated from *Cicadulinambila* were globular in shape measuring 3.9 µm in length and 2.4 µm in width with a length: width ratio of 1.62:1.The conidiophores of *Beauveria* sp. isolated from the insect pests were single or irregularly grouped, erect, zigzag in appearance (Plate-2).

Table- 1: Morphological characterization of identified pathogens

S.NO.	Name of the pathogen	Spore/conidia size(µm)		Spore Shape	Length width ratio
		Length	Width		
1.	*Pierisbrassicae (Nosema* sp.*)*	3.5	1.8	Oval	1.94:1
2.	*Oxyaindica (Beauveria* sp.*)*	3.7	2.5	Ovoid/Globular	1.48:1
3.	*Glyphodespyloalis (Beauveria* sp.*)*	4.2	2.3	Ovoid	1.82:1
4.	*Cicadulinambila (Beauveria* sp.*)*	3.9	2.4	Globular	1.62:1

Confirmation of Infectivity of isolated pathogens

To confirm that the isolated pathogens from mulberry and other crops cause disease in the silkworm, *Bombyx mori* L. the isolated pathogens were inoculated into the healthy silkworm batches by following Koch's postulates. Separate batches of the silkworm larvae of the CSR_4 breed were maintained for *Nosema* and *Beauveria* inoculation respectively. The larvae inoculated with the spore suspension of $1x\ 10^6$ were observed regularly for the appearance of disease symptoms. *Nosema* inoculated batch of healthy silkworm larvae developed characteristic symptoms viz., loss of appetite, sluggishness, retarded growth, and irregular development (Plate-3). Under the artificial conditions of inoculation, the symptoms of muscardine were recorded which included loss of appetite, sluggishness, the appearance of oily specks on the cuticle, general paralysis, hardening, and mummification of cadavers (Plate-4). The larvae inoculated with *Nosema* sp. Isolated from *Pierisbrassicae* showed larval mortality of 73.1 percent and at pupal stage mortality was recorded to the extent of 14.0 percent. The total infection rate in the said batch was recorded to the extent of 100 percent as all the moths that emerged were found to be infected with *Nosema* sp. During the present study, *Nosema* sp. isolated from *Apismellifera* was also subjected to infectivity test, however, the isolated sp. of *Nosema* did not cause any mortality to the silkworm at any stage of its life cycle. (Table - 2)**.** Larval mortality of 55.7 percent was recorded when silkworm larvae were inoculated with *Beauveria* sp. isolated from *Oxyaindica*. At the pupal stage, 16.9 percent mortality was recorded while 9.6 percent of moths were found to be infected with the same pathogen. Results of the present studies showed that *Glyphodespyloalis* also acts as an alternate host of *Beauveria* sp. The same pathogen was found to record mortality of 31.0 percent, 15.5 percent and 19.0 percent at larval, pupal, and moth stages respectively.

The present studies also revealed that *Beauveria* sp. isolated from *Cicadulinambila* is also pathogenic to silkworms, while confirming the pathogenicity of this species to silkworms, it was found that the pathogen recorded mortality of 37.0 percent, 19.0 percent, and 16.0 percent at larval, pupal and moth stage respectively (Table-2).

Table- 2: Infectivity of pathogens isolated from insect pests to CSR4 breed of silkworm

Name of the pathogen	Mortality %		% moth emerged	% moth infected	Infection rate (%)	Virulence
	Larva	Pupa				
Pierisbrassicae (Nosema sp.)	73.10	14.0	11.7	11.7	100	High
Apismellifera (Nosema sp.)	Nil	Nil	Nil	Nil	Nil	Non virulent
Oxyaindica (Beauveria sp.)	55.71	16.90	13.90	9.60	69.06	High
Glyphodespyloalis (Beauveria sp.)	31.00	15.50	42.00	19.00	45.23	Medium
Cicadulinambila (Beauveria sp.)	37.00	19.00	32.00	16.00	50.00	Medium

Discussion

During the present study, the identified pathogens were subjected to morphological characterization through micrometric measurement using ocular and stage micrometers. Light microscopy revealed that *Nosema* spores isolated from *Pierisbrassicae* were oval with 3.5μm in length and 1.8 μm in width. The micrometric measurements of *Beauveria* species isolated from *Oxyaindica*, *Glyphodespyloalis,* and *Cicadulinambila* include 3.7 μm x 2.5 μm, 4.2 μm x 2.3 μm and 3.9 μm x 2.4 μm respectively. The shape of the conidia was found ovoid-- globular in all the cases. [7] Carried out the morphological characterization of microsporidian spores isolated from *Catopsiliacrocale* and *Catopsiliapyranthe*. The spores were found to be ovo-cylindrical in shape with a size of 4.5 μm x 1.7 μm and 4.2 μm x 2.1 μm respectively. Though in our study the measurements of the spore vary slightly compared to the ones reported by [7], [13] stated that most of the microsporidian spores fall in the size range of 3-6 μm x 2-4 μm , therefore, our findings are very much close to the findings of [13]. [3] Carried out the characterization of microsporidian isolated from *Pierisrapae* and reported that the mature spore of this microsporidium is long, oval and 3.8 μm x 2.0 μm in size. These findings are, therefore, very close to the present findings. While studying the morphological characteristics of fungus isolated from different insect pests, it was found ovoid to globular in shape, conidiophores single as well as irregularly grouped, erect and zigzag. Conidia were one-celled, born singly on short stalks. Conidia size was 3.7 μm x 2.5 μm, 4.2 μm x 2.3 μm and 3.9 μm x 2.4 μm in case of *Oxyaindica*, *Glyphodespyloalis* and *Cicadulinambila* respectively. Concerning the above morphological characters, the present findings conform with those reported by [5], [11] and [1]. However, further studies are required to be under taken for identifying the standard strains of these pathogens.

In the present study *Oxyaindica*, *Glyphodespyloalis,* and *Cicadulinambila* tested positive for *Beauveria* infection. *Beauveria* species isolated from all three above agricultural pests were found to be infective to silkworm *Bombyx mori* L. at varied levels of virulence. [10] Also studied the susceptibility of *Glyphodespyloalis* (Lepidoptera: Pyralidae) to the entomopathogenic fungus, *Beauveria bassiana* and found that *Glyphodes* acts as a strong carrier of fungal conidia. [15] Reported that a new mulberry pest *Alticahimensis Shukla* (Coleoptera: Chrysomelidae) is susceptible to the entomopathogenic fungus *Beauveria bassiana*. The findings of the above authors are therefore, in agreement with the present findings.

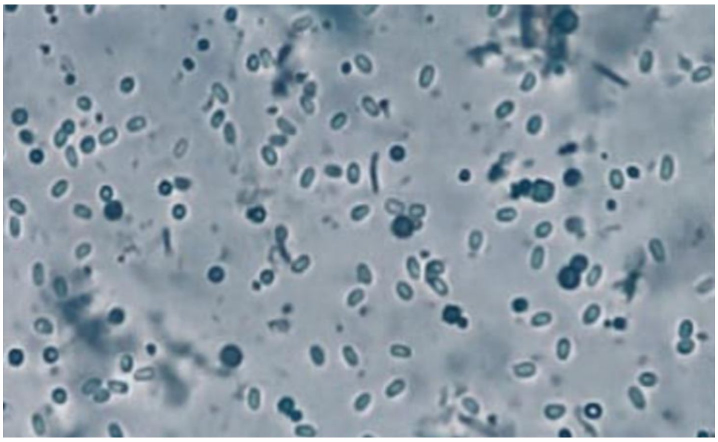

Plate-1: Nosema spores under microscopic field (600 X)

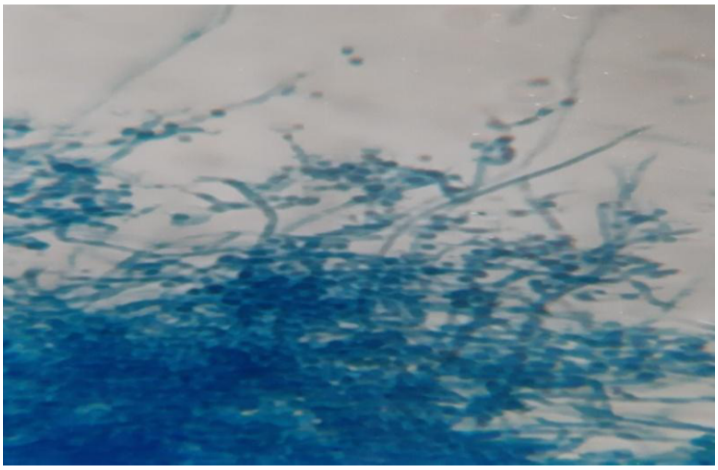

Plate-2: Conidiophores with conidia on host integument

Plate-3: Silkworm larvae infected with Nosema sp

Plate-4: Silkworm larvae infected with Beauveria sp.

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